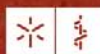


# MICRO BRAGA 01 DEC - 03 DEC BIOTEC'11

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## **Microbiotec11**

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This is the book of abstracts of the scientific presentations at Microbiotec11. The acceptance of communications was based on the submitted abstracts. The text hereby reproduced is of the responsibility of its authors.

## Preface

This book contains the abstracts presented at the 4th joint conference of the Portuguese Society for Microbiology and the Portuguese Society for Biotechnology – MicroBiotec11, held at the University of Minho, Braga on the 1st to the 3rd of December 2011. The MicroBiotec11 conference brings together young and senior researchers, as well as end users, to discuss recent developments in different areas of Biotechnology and Microbiology, including: Biocatalysis and Biotransformation; Bioinformatics; Bionanotechnology; Bioprocess Engineering; Cell culture, Stem Cell and Tissue Engineering; Clinical Microbiology; Comparative Genomics and Evolution; Downstream Processing; Drug Resistance Targets and Biotechnology; Environmental Microbiology and Biotechnology; Epidemiology; Epigenomics; Food Microbiology and Biotechnology; Functional Genomics; Health Biotechnology; Host-Pathogen Interactions; Industrial Microbiology & Biotechnology; Microbial Ecology; Microbial Pathogenesis; Microbial Physiology; Molecular Microbiology; Pharmaceutical Biotechnology; Renewables, Biofuels and Bioenergy; Synthetic Biology and Systems Biology. According to this, the conference program has been divided in 10 thematic symposia:

- S1: Industrial and Food Microbiology and Biotechnology
- S2: Environmental Microbiology and Biotechnology
- S3: Health Microbiology and Biotechnology
- S4: Next Generation Sequencing, Comparative Genomics and Evolution
- S5: Bioprocess Engineering
- S6: Molecular Microbiology and Microbial Physiology
- S7: Cell and Tissue Engineering
- S8: Functional Genomics and Systems and Synthetic Biology
- S9: Cellular Microbiology and Pathogenesis
- S10: Nanotechnology

A total of 437 abstracts are included in the book, consisting of 6 invited plenary lecturers, 21 invited keynote lectures and 39 oral presentations.

On behalf of the Scientific and Organizing Committees we wish to thank the authors who have contributed to the high scientific standard of the program. We are grateful to the sponsors who have contributed decisively to this event. We also would like to extent our gratitude to all those who, through their dedicated efforts, have assisted us in this task. Finally, we wish you a fruitful and pleasant stay in Braga and hope that you will enjoy the scientific program of this conference.

December 2011

Jorge Pedrosa & Joana Azeredo  
Organizing Committee Chairs

## Organizing Committee

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# Plenary Lectures

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*Steven M. Cramer  
John McKinney  
Merja Penttilä  
Heinz D. Osiewacz  
Hermínia de Lencastre*

*Plenary lecture 1*

## Downstream bioprocessing: recent advances and future promise

**Steven M. Cramer**

Rensselaer Polytechnic Institute, Department of Chemical and Biological Engineering, Troy, NY 12180-3590, USA,

Several recent changes have occurred that have had a profound impact on downstream bioprocessing. Major advances in upstream processes have led to dramatic improvements in cell culture titers, particularly for monoclonal antibodies (mAbs), placing increased demand on downstream bioprocessing technology. This has catalyzed many recent advances in downstream bioprocessing such as the implementation of high-throughput process development techniques, improved unit operations, and the promise of continuous bioprocessing. Recent regulatory directives such as quality by design (QbD) and process analytical technology (PAT) have resulted in a noticeable shift in the perspective of the industry towards the implementation of new downstream bioprocessing strategies. There have also been significant efforts to develop generic processes, particularly for mAbs. A powerful example of this is the two step process which includes weak partitioning ion exchange as a second step. The emergence of biosimilars has also brought entirely new bioprocessing challenges as well as a variety of product quality issues to the forefront. This presentation will highlight some of the important recent developments in downstream bioprocessing, focusing on work that has been published in the past two years. Topics covered include: recent advances in chromatography and membrane technology, monoliths, continuous bioprocessing, high-throughput process development, hybrid processes, smart biopolymers, and quality by design considerations. The emphasis of this presentation will be on purification technologies and results will include those from our lab as well as several other labs working in this area.

*Plenary lecture II*

## Individuality of microbial responses to antibiotics

**John McKinney**

Swiss Federal Institute of Technology in Lausanne (EPFL), Laboratory of Microbiology and Microsystems, 1015 Lausanne, Switzerland

Bacterial cells behave as individuals. Mutation and genetic exchange are important drivers of bacterial individuation, but these events are relatively rare. At higher frequencies, genetically identical cells display metastable variation in growth rate, response kinetics, stress resistance, and other quantitative phenotypes. These cell-to-cell differences arise from non-genetic sources, for example, stochastic fluctuations in gene expression and asymmetric partitioning of components at cell division. Temporal variation at the single-cell level generates phenotypic diversity at the population level. This diversity is critical for bacterial persistence in fluctuating environments because it ensures that some individuals will survive a potentially lethal change that would otherwise extinguish the population. A medically relevant example is the refractoriness of bacterial infections to chemotherapy, which has been attributed to spontaneous phenotypic variants ("persisters") that survive despite prolonged antibiotic exposure. The persisters are not antibiotic resistant mutants and it is unclear why they tolerate antibiotics that kill their genetically identical siblings. Our studies focus on the mechanistic basis of the reversible persister switch in bacteria. We use long-term automated timelapse fluorescence microscopy in conjunction with purpose-built microfluidic and microelectromechanical systems to study bacterial responses to antibiotics at the single-cell level. Our observations contradict the conventional idea that persistence is attributable to pre-existing subpopulations of dormant cells. Instead, we find no correlation between the growth rates of individual cells and their probability of survival during antibiotic exposure. In the specific case of isoniazid, a frontline anti-tuberculosis drug, we find that the isoniazid-activating enzyme catalase is expressed in apparently random pulses that are correlated with ensuing cell death. We also find that catalase pulsing and cell fate (death or persistence) are strongly correlated between sister cells, suggesting a role for non-genetic and metastable inheritance in these phenomena. Our observations are consistent with the idea that stochastic fluctuations in gene expression at the single-cell level can determine the fate of individual cells and cell populations in changing and stressful environments.



*Plenary lecture IV*

## Microbial factories for biorefineries. Production of enzymes, fuels and chemicals

**Merja Penttilä**

Technical Research Centre of Finland VTT, FI-02044 VTT, Helsinki, Finland

Plenary lecture V

## Unravelling a hierarchical network of molecular pathways involved in fungal senescence and lifespan control

**Heinz D. Osiewacz**

Johann Wolfgang Goethe University, Faculty of Biosciences and Cluster of Excellence Macromolecular Complexes, Max-von-Laue Str.9, 60438 Frankfurt/ Main, Germany,

*Podospora anserina* is a filamentous fungus used as a model to study the mechanistic basis of biological aging. In contrast to most filamentous fungi, this ascomycete is characterized by a limited lifespan. After a defined period of linear growth a colony turns to senescence and dies at the hyphal tips. This phenotype has been extensively studied and it is well established that the lifespan of this ascomycete is controlled by environmental and genetic factors. Already in early studies a crucial role of mitochondria became apparent. Such a role is now demonstrated for aging and degenerative processes in various systems including humans. Although essential for the oxygenic generation of adenosine triphosphate (ATP), mitochondria are at the same time the main source of reactive oxygen species (ROS) generation which, according to the 'free radical theory of ageing' (FRTA), have been suggested to cause molecular damage, cellular dysfunction and death. In the lecture I will address different molecular and cellular pathways which are crucial to keep a functional population of mitochondria for a longer period of time. Experimental modulation of the efficiency of such pathways provides good handles to intervene into the aging process. Naturally all pathways appear to be limited in their capacity. However, after one pathway is overwhelmed by molecular damage, pathways of a higher order become activated. At the basis of such a hierarchical network there are molecular pathways involved in the control of the generation and the scavenging of ROS. They are followed by repair pathways and pathways controlling the degradation of damaged cellular components and their replacement by functional ones. If the limits of these pathways are reached, cellular systems including mitochondrial fission and fusion and autophagy of mitochondria (mitophagy) become effective. Ultimately, the induction of programmed cell death leads to death of the senescent culture. From the accumulation of data raised in the past it now becomes clear that it is necessary to study complex processes like aging in a holistic approach as it is done in Systems Biology. Finally, I will discuss data of a recent study demonstrating that well controlled experiments performed under laboratory conditions may lead to unexpected and counter-intuitive results and that such experiments are prone to miss parts of the network which has evolved to deal with the constantly changing conditions (e.g. in temperature) any organism is facing in nature.

Plenary lecture VI: Nicolau Van Uden Conference

## MRSA: origin and evolution of a resistant gene and emergence and spread of resistant clones

### Hermínia de Lencastre

Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal; Laboratory of Microbiology, The Rockefeller University, New York, USA

In this lecture I will describe the origin and spread of *Staphylococcus aureus* (MRSA): First I will discuss the origin of the *mecA* gene present in all MRSA strains and responsible for  $\beta$ -lactam resistance. I will then analyze the emergence of the very first methicillin resistant MRSA, its microbiological and molecular portrait including the origin of multidrug resistance, followed by the emergence of a handful of pandemic MRSA clones, which achieved global spread. Finally, I will explain the worldwide emergence of MRSA in the community and animal husbandry. The genetic determinant of  $\beta$ -lactam resistance, the *mecA* gene, is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (*SCCmec*), and encodes a low-affinity penicillin binding protein (PBP), PBP2A. Several lines of evidence suggest that the heterologous  $\beta$ -lactam resistance gene, *mecA*, which is resident in all MRSA strains, may have its evolutionary origin in a close homologue of this gene that is ubiquitous in both  $\beta$ -lactam-susceptible and -resistant isolates of the animal commensal species *Staphylococcus sciuri*. MRSA was first isolated in the UK in 1961 and by the late 1980s and early 1990s MRSA spread globally in hospitals of the industrialized world primarily in the form of a few pandemic MRSA clones, which could be defined on the basis of their genetic background (MLST, *spa* type and PFGE) combined with the molecular type of the chromosomal cassette, *SCCmec*. By the late 1990s the epidemiology of MRSA has taken a radical turn through the appearance and rapid spread of resistant and highly virulent strains that emerged in the community and began to enter hospitals as well – blurring the traditional distinctions between risk factors of hospital acquired versus community acquired infections. It is now clear that clones of “successful” *S. aureus* lineages began to exchange not only drug resistance genes but also virulent genes leading to the emergence of new highly virulent and drug-resistant clonal types, which can cause life-threatening infections in healthy individuals. The genetic background of the most “successful” MRSA and MSSA lineages appear to be similar suggesting that critical determinants of epidemicity reside in the genetic background of the bacteria.

# Industrial and Food Microbiology and Biotechnology

*Thematic symposium 1*

**Keynote speakers:**

*Armando Venâncio*

*Teresa Crespo*

**Oral presentations:**

*Ricardo Duarte*

*Joana Guerreiro*

*Jorge Saraiva*

*Thomas Kennedy*

**Keynote speaker / S1: 5**

## **The effect of vinification on wine contamination with ochratoxin A**

**Armando Venâncio**

IBB – CEB, University of Minho, Portugal

The filamentous fungi are ubiquitous in nature, so their occurrence in natural substrates is seen as natural. When the presence of these microorganisms is associated with favourable ecological conditions (e.g., temperature and relative humidity), these may multiply and produce undesirable compounds. Classic examples of this relationship are the production of chlorinated anisoles in cork or of polyphenol oxidases in grapes. More recently, in the sixties of last century, the discovery of the production in food of toxic metabolites - mycotoxins - by filamentous fungi has revolutionized the study of food mycology and created a new field: food mycotoxicology. The occurrence of food outbreaks due to the presence of mycotoxins in food is now well recognized, and even admitted that mycotoxins are closely related to some pests described in the Middle Ages. As in any other row of the agri-food sector, in vitiviniculture field is also possible to find reports on the possible presence of mycotoxins in the vineyard, the grape or its derivatives. However, it is necessary to distinguish between the mere detection of these metabolites and their occurrence at levels that cause harm to health. In this communication we present a historical survey on the detection of mycotoxins in grapes and their derivatives, indicating the case where the presence of the mycotoxin is regarded as a hazard: ochratoxin A. From a perspective of implementing a food safety model, the carry-over of ochratoxin A from grapes to wine will be presented and discussed.

**Keynote speaker / S1: 6**

## **Emerging pathogens and their impact in research and global market**

**Maria Teresa Barreto Crespo**

IBET, Oeiras, Portugal

One of the 21st century challenges are foodborne diseases, because they not only significantly affect people's health and well-being, but they also have economic consequences for individuals, families, communities, businesses and countries. These diseases impose a substantial burden on health-care systems and markedly reduce economic productivity. Globalization of food and feed products trade are changing the patterns of food production and distribution. Therefore, both known and new foodborne diseases can become a hazard. Food and feed are distributed over far greater distances than before, creating the conditions necessary for widespread outbreaks of foodborne illness. Other factors can be added to this equation namely changes in urban lifestyle, in terms of eating habits (such as the increase in consumption of raw or lightly cooked food and the demand for exotic products) or an increasingly transient human population. The introduction of new food processing technologies, including irradiation, new packaging materials and packaging technologies, nanotechnologies, in this climate of concern about food safety, are special challenges. According to the European Food safety Authority (EFSA) "an emerging risk to human, animal and/or plant health is understood as a risk resulting from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard". Microbial pathogens, although some of them know pathogens, keep reappearing with new strains that have new combinations of acquired genes and as such are more aggressive to humans and cause more mortality. The recent case of *E. coli* O104:H4 outbreak is a clear example of how globalization and bacterial genomic plasticity can cause high mortality rates in a small period of time. Enterococci, that are present in so many traditional foods, due to the risk of dissemination of virulent and/or antibiotic resistances in the community, can be considered as an emerging pathogen. Therefore it will be considered as a case study in this talk. Virulence factors, antibiotic resistances, geographical distribution will be approached. In this context all actors in the scene of public health have to continue to be alert: microbiologists, hospital personnel, food producers and handlers or animal husbandry responsible staff.

S1: 1

## Microevolutionary changes of commercial *Saccharomyces cerevisiae* strains recovered from vineyard environments identified by comparative genome hybridization on array

**R Duarte<sup>1</sup>, L Carreto<sup>2</sup>, B Cambon<sup>3</sup>, S Dequin<sup>3</sup>, MAS Santos<sup>2</sup>, M Casal<sup>1</sup>, D Schuller<sup>1</sup>**

<sup>1</sup>CBMA (Centre of Molecular and Environmental Biology) / Department of Biology / University of Minho, Portugal; <sup>2</sup>RNA Biology Laboratory, CESAM, Biology Department, Aveiro University, Campus Universitário de Santiago, Aveiro; <sup>3</sup>UMR Sciences pour l'Oenologie, Microbiologie, INRA, Montpellier, France

The use of commercial *Saccharomyces cerevisiae* wine yeast strains as fermentation starters has been extensively generalised over the past two decades. These strains are used by wineries every harvest time in large quantities. Within our previous work we showed that such strains are disseminated from the winery [1] and their permanence in nature induced genetic changes that were not found among a control group of isolates that derived from clonal expansion of the commercial “mother” strain [2]. The objective of the present study was to evaluate genome variations among four isolates of the commercial strain *S. cerevisiae* Zymaflore VL1 that were re-isolated from vineyards surrounding the wineries where this strain was applied, in comparison to the commercial “mother” strain, by the use of comparative genome hybridization on array. These approaches were carried out as described [3]. Data analysis showed genetic differences among the recovered isolates in comparison with the “mother” strain. Amplification (between 1 and 2 fold changes) of 14 genes were detected, related with mitosis (*SHE1*) or meiosis (*HFM1*), lysine biosynthesis (*LYS14*), galactose (*GAL1*) and asparagine catabolism (*ASP3-2*). *ASP3-2* amplification is in agreement with the previously shown increased expression during nitrogen starvation. This might occur as an adaptation to natural environments with poor yeast-utilizable nitrogen sources. Eight Ty elements were also amplified, whereas each of the recovered strains had a unique pattern of amplifications. Phenotypic screening was performed considering 28 physiological tests. Seven phenotypic traits distinguished recovered strains from the “mother” strain which was unable to grow at 18 °C, but evidenced some growth in the presence of CuSO<sub>4</sub> 5 mM and SDS 0.01%. Variable growth patterns were found for NaCl 1.5 M, KHSO<sub>3</sub> (300 Mg/L) and wine supplemented with glucose (0.5% and 1% w/v). We hypothesize that the transition from nutrient-rich musts to nutritionally scarce natural environments induces adaptive responses and microevolutionary changes promoted by Ty elements. These changes (and possibly others as well) may contribute to intra-strain phenotypic variability.

[1] Schuller et al., 2007. *Yeast* 24:625-636 [2] Valero et al., 2005. *FEMS Yeast Research* 5:959-969 [3] Carreto et al., 2008. *BMC Genomics* 9:524. This work was funded by the fellowship SFRH/BD/48591/2008 and by the projects PTDC/BIA BCM/64745/2006, PTDC/AGR-ALI/103392/2008 and FP7/2007-2013 (n° 232454).

S1: 2

## Adaptive response to acetic acid in the highly resistant yeast species *Zygosaccharomyces bailii*, revealed by quantitative proteomics

**Joana F. Guerreiro, Nuno P. Mira, Isabel Sá-Correia**

Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Technical University of Lisbon, 1049-001 Lisboa, Portugal

*Zygosaccharomyces bailii* is the most tolerant yeast species to acetic acid-induced toxicity, being able to grow in the presence of concentrations of this food preservative close to the legal limits. For this reason, *Z. bailii* is the most important microbial contaminant of acidic food products. However, the mechanisms behind this intrinsic resistance to acetic acid are very poorly understood. The experimental model *Saccharomyces cerevisiae* is also a contaminant of acidic foods and genome-wide approaches have been explored to unveil global mechanisms underlying yeast response and adaptation to acetic acid [1-3]. An expression proteomic approach, based on quantitative two-dimensional gel electrophoresis (2-DE), was explored to gain mechanistic insights into the adaptive response and intrinsic tolerance to acetic acid in *Z. bailii*. The experimental strategy detailed in [4] was used to identify alterations occurring in cellular protein content in response to sudden exposure to an inhibitory, but sub-lethal, concentration of acetic acid, following the supplementation of a minimal medium containing glucose with the acid or during the growth curve in this supplemented medium. The response to acetic acid was found to involve an increased activity of carbohydrate metabolic processes, in particular, the TCA cycle when glucose is present and gluconeogenic and pentose phosphate pathways when acetic acid is the only carbon source. Increased ATP production, required to assure acetate catabolism and cell detoxification, is also suggested, as well as the activation of oxidative and general stress responses. All these indications may be useful to guide the rational design of more effective strategies to control food spoilage by this highly acetic acid resistant yeast species.

[1] Teixeira, M.C. et al. (2011) Curr Opin Biotechnol 22, 150-156. [2] Mira, N.P. et al. (2010) Microb Cell Fact 9, 79. [3] Mira, N. P. et al. (2010) OMICS 14, 587-601. [4] Sá-Correia, I. and Teixeira, M. C. (2010) Expert Rev Proteomics 7, 943-953.



S1: 3

## Enhancement of rate and extension of enzymatic hydrolysis of cellulose by high pressure pre-treatments

Ana R.F.C. Ferreira, Andreia B. Figueiredo, Dmitry V. Evtuguin, Jorge Manuel Alexandre Saraiva

Universidade de Aveiro, Portugal

The inevitable reduction of dependence from fossil fuels for energy and chemicals production has increased attention to the use of renewable biomaterials for these processes, as is the case of the conversion of cellulose from plant resources into bioethanol and chemicals synthesis, via bioprocessing of fermentable sugars. However, the structural arrangement and tight packing of cellulose chains hinders its hydrolysis, being this an obstacle for a successful use of these naturally abundant renewable sources for production of second-generation fuels and chemicals. High pressure (HP) is now an established technology for food preservation, with potential also to modify the properties of macromolecules, by action on non-covalent bonds. A previous work of our research team showed that HP treatments of cellulosic pulps are a promising tool for non-degradative modification of cellulose fibres properties, such as rearrangement of elementary fibrils in cellulosic fibres, in such a way that those become less aggregated and more hydrated (containing increased amounts of strongly bound water), leading to improvement of cellulose accessibility towards hydrolysis with diluted sulphuric acid. Based on these results and following the same rational strategy, the effect of HP, as a pre-treatment, on the subsequent enzymatic hydrolysis of bleached kraft *Eucalyptus globulus* cellulosic fibres by cellulase, from *Trichoderma viride*, was evaluated in the present work. Results showed that pressure pre-treatments of 300 and 400 MPa during 5-45 min, lead to both an increased rate and degree of hydrolysis, reaching values ranging from 1.5- to 1.9-fold, quantified by the formation of reducing sugars. Both the pressure and time under pressure influenced the enzymatic enhanced "hydrosability" of the cellulosic pulps, with the former being more important. A kinetic effect of HP treatment time on hydrosability of cellulose was verified. These results open promising possibilities, to contribute to overcome conventional limitations of enzymatic cellulose hydrolysis, by enhancement of both rate and yield of hydrolysis of cellulose. The results are also of interest for the preparation of "pressure engineered" cellulose, with respect to the desired hydrolysis extent and profile, with tailored hydrolysis patterns and possible different properties for new applications. Optic microscope images of the hydrolysed fibres corroborate this possibility.

S1: 4

## Evaluating food safety management performance in an Irish milk pasteurising facility using a microbiological assessment scheme

### Thomas Kennedy

Department of Agriculture, Food and the Marine, Kildare Street, Dublin 2, Ireland

Milk and milk products are a heterogeneous group of food products. Depending on the heat treatment applied during production, different pathogens pose risks. The pathogens of concern are *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* spp, *Staphylococcus aureus* and *Escherichia coli* since these may survive pasteurisation treatments. The performance of a food safety management system (FSMS) in a drinking milk pasteurisation establishment was measured using a microbiological assessment scheme (MAS). The MAS consisted of multiple sampling locations along the processing line consisting of high-risk raw materials, the processing environment, process water and end products. A total of 1268 samples were analysed over an 18-month-period. Nine microbial parameters (*Salmonella* spp., *Listeria* spp., *B. cereus*, *S. aureus*, Total Bacterial Counts (TBC), *Enterobacteriaceae*, *E. coli*, faecal enterococci and coliforms) were assessed using standard methods. Results were benchmarked against legal, industry and best practice norms. 100% ( $n_0=233$ ) of raw milk samples met the EU TBC standard of  $< 10^5$ CFU/MI, however, *Listeria innocua* was isolated in 3% ( $n_1=134$ ) of raw milk samples. *Listeria* spp. ( $n_2=128$ ), *Salmonella* spp. ( $n_3=118$ ), *S. aureus* ( $n_4=118$ ), *Enterobacteriaceae* ( $n_5=114$ ), *B. cereus* ( $n_6=38$ ) and *E. coli* ( $n_7=23$ ) were not detected in any end products. *Listeria welshimeri* (a poor hygiene indicator) was identified in 2% ( $n_8=153$ ) of environmental samples. *Salmonella* was not isolated in any of 63 environmental samples. 6% and 1% of operator hand swabs ( $n_9=100$ ) had TBC and *Enterobacteriaceae* counts respectively in excess of best practice norms of  $10^2$  cm<sup>-2</sup> and  $10^1$  cm<sup>-2</sup> respectively. One (2.2%) water sample ( $n_{11}=46$ ) had a coliform count of 201 CFU/MI whereas five samples (11%) had TBC counts above acceptable norms. The results indicate that the FSMS is producing a safe product. The MAS is an effective risk assessment tool that is useful to assess the overall performance of the FSMS and allows a more targeted use of resources to implement improvement. Satisfactory end product microbiological results indicate that cold chain control, post pasteurisation contamination from dry ingredients (e.g. buttermilk cultures), packaging or unsanitary pipe work are not issues for this plant. However, the prerequisites of environmental sanitation, raw material supply and control, water treatment and storage and staff hygiene are the areas within the FSMS that pose the greatest risks.

# Environmental Microbiology and Biotechnology

*Thematic symposium 2*

**Keynote speakers:**

*Célia Manaia  
Gilda Carvalho*

**Oral presentations:**

*Isabel Henriques  
Cátia Fidalgo  
Ademola Olaniran  
Marta M Neto*

**Keynote speaker / S2: 5**

## **Antibiotic resistance and the urban water cycle**

**Célia Manaia**

Escola Superior de Biotecnologia, Portugal

Antibiotic resistance (AR) is a natural property of bacteria. Over the last 70 years, the massive use of antibiotics contributed to invert the natural equilibrium between fully susceptible and resistant bacteria. Gradually, AR became more prevalent and diversified, reaching new habitats and new drugs. Nowadays, high levels of AR are not confined to hospitals and health care facilities. Levels above the expectable are reported in wild animals, surface waters or agriculture soils, allegedly in association with anthropic pressures. Water environments are important sources for AR dissemination [1]. In this respect, ubiquitous bacteria, which can colonize different types of water and be transmitted from and to humans, are of special interest. In the urban water cycle, sewage treatment plants, which receive high doses of antibiotics and antibiotic resistant bacteria, are important sources of contamination, mainly of surface waters (humans-to-environment). On the other hand, antibiotic resistant bacteria present in surface or drinking waters can be transmitted to humans (environment-to-humans). Wastewater treatment lead to bacterial removal rates around 1.5-2 log units. Nevertheless, it is estimated that at least  $10^7$ - $10^{10}$  culturable bacteria, discharged per day per inhabitant equivalent in the final effluent, will have any kind of acquired AR [2]. During wastewater treatment, gene mutation, horizontal gene transfer or bacterial community rearrangements may occur, eventually leading to significant increases of AR. This is exemplified for quinolone resistant *Escherichia coli* and faecal enterococci [3, 4]. In general the predominant bacterial genera and species in wastewaters are different from those observed in surface and drinking waters. Nevertheless, surface and drinking waters are also colonized by antibiotic resistant bacteria. Although drinking water disinfection may impose a bottleneck on the species diversity and be apparently efficient on the removal of some antibiotic resistant bacteria (e.g. aeromonads), tap water can present high levels of AR bacteria of environmental origin (e.g. sphingomonads) [5, 6]. Indeed, similar antibiotic resistance phenotypes and genotypes have been detected in waste, surface and/or drinking water. Although specific processes of resistance acquisition may be involved, it is hypothesized that the dynamics of bacterial populations has a major role on the dissemination of antibiotic resistance in aquatic environments.

[1] Baquero Fet al (2008). *Curr Opin Biotechnol*, 19(3): p. 260-5. [2] Novo A et al (2010) *Appl Microbiol Biotechnol*, 87(3): p. 1157-66..[3] Ferreira da Silva M et al (2006) *FEMS Microbiol Ecol*, 55(2): p. 322-9. [4] Ferreira da Silva M et al (2007) *FEMS Microbiol Ecol*, 60(1): p. 166-76.[5] Vaz-Moreira I et al (2011) *Appl Environ Microbiol*. 77(16):5697-706. [6] Figueira V et al (2011). *Water Res* doi:10.1016/j.watres.2011.08.021.

**Keynote speaker / S2: 6**

## **Identification and carbon source preference of the microbial communities in a polyhydroxyalkanoate-producing mixed culture from fermented molasses**

**Gilda Carvalho**

REQUIMTE/CQFB, Portugal

Polyhydroxyalkanoates (PHAs) are biologically synthesised polymers that have gained increasing attention as bio-based and biodegradable plastics. PHA production from wastes using mixed microbial cultures (MMC) is a promising alternative to costly pure culture processes. The key to optimise PHA production efficiency in MMC processes is the selection of efficient PHA-storing organisms. However, still little knowledge is available on the factors governing community selection in waste-based PHA-producing systems. This study focused on the analysis of the microbial communities enriched with a feast and famine sequencing batch reactor fed with fermented molasses, and the study of their competition for different carbon sources using molecular techniques. The microbial population was characterised through a 16S rRNA gene clone library and fluorescence *in situ* hybridisation (FISH), coupled with microautoradiography (MAR). The microbial enrichment was found to be composed of PHA-storing populations (84% of the microbial community) comprising members of the genera *Azoarcus*, *Thauera* and *Paracoccus*. The dominant PHA-storing populations ensured the high functional stability of the system, which was characterised by high PHA storage efficiency (up to 60% PHA content). The fermented molasses contained primarily acetate, propionate, butyrate and valerate. The substrate preferences were determined by MAR-FISH and differences in the substrate uptake capabilities for the various probe-defined populations were found. The results also showed that in the presence of multiple substrates, the microbial populations specialised in different substrates, thereby co-existing in the SBR by adapting to different niches. *Azoarcus* and *Thauera*, primarily consumed acetate and butyrate, respectively. *Paracoccus* consumed a broader range of substrates and had a higher cell-specific substrate uptake. The relative microbial composition and their substrate specialisation were reflected in the substrate removal rates of different carbon sources in the SBR reactor. This study established, for the first time to our knowledge, a link between the phylogeny and substrate uptake preference of PHA-producing organisms. This knowledge can be incorporated in metabolic models for improved performance prediction of PHA-production mixed culture systems.

S2: 1

## Antibiotic resistance in a portuguese lotic ecosystem: focus on clinically relevant extended-spectrum beta-lactamases

Marta Tacão, Isabel Henriques, António Correia

CESAM & Biology Department, University of Aveiro, Portugal

The main goals of this study are to compare the prevalence and diversity of cefotaxime resistant (CTX<sup>R</sup>) Gram-negative bacteria and to characterize clinically relevant extended-spectrum beta-lactamases (ESBLs) within polluted (P) and unpolluted (UP) lotic ecosystems. Twelve rivers from the Vouga River basin were sampled: 9 sites classified as UP and 3 as P, based on physico-chemical and microbiological parameters of water quality. Bacterial counts were  $1.9 \times 10^6$  CFU/L in P and  $0.68 \times 10^6$  CFU/L in UP streams, of which 8.8% and 0.6%, respectively, grew on cefotaxime-supplemented agar. Clonal relationships among CTX<sup>R</sup> isolates were assessed by BOX-PCR and the phylogenetic affiliation determined by 16S rDNA sequencing. Among strains from P waters (n= 61), 42.6% are *Pseudomonas* spp., 34.4% affiliated with *Enterobacteriaceae* members and 21.3% with *Aeromonas* spp. In UP waters isolates (n=94) *Pseudomonas* spp. adds 62.7%, *Enterobacteriaceae* and *Aeromonas* spp. with 9.6% and 1.1% respectively and *Acinetobacter* sp. appears as the second most abundant genus in these samples, with 26.6%. Antimicrobial susceptibility testing against 16 antibiotics of 6 different classes revealed that 60% and 40% of strains isolated from P and UP sites, respectively, were multiresistant (resistant to 3 or more classes of antibiotics). ESBL production was detected on 39 isolates (27 from P and 12 from UP sites). The presence of integrons and ESBL genes encoding TEM, SHV, CTX-M, GES, PER, VEB and OXA was inspected by PCR. The CTX-M gene was the most dominant (n=18) followed by TEM (n=10). On 6 strains it was detected both CTX-M and TEM. Int1 gene was identified in 18 isolates (17 from P and 1 from UP sites). Since CTX-M gene was the most frequent and considering its clinical relevance, later work has been focused in further characterizing these ESBLs. Sequence analysis identified CTX-M from group 1 (CTX-M-1, -3, -15 and -32) and from group 9 (CTX-M-14). Their genomic environment is presently under study. Also to compare the diversity of this ESBL gene in both P and UP sites, clone libraries of gene fragments amplified from environmental DNA have been constructed and analyzed. Results here presented show clear differences between polluted and unpolluted lotic ecosystems, concerning prevalence, phylogenetic diversity and antimicrobial susceptibility profiles of CTX<sup>R</sup> bacteria and also the production of clinically relevant ESBL and the presence of integrons.

S2: 2

## Unveiling cadmium resistance mechanisms in an extremophilic yeast

**Cátia Fidalgo, Ana Tenreiro, Rogério Tenreiro, Rui Malhó, Sandra Chaves**

Universidade de Lisboa, Faculdade de Ciências, Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG). Edifício ICAT, Campus da FCUL, Campo Grande, Lisboa, Portugal

Heavy metal resistant microorganisms are often associated with acidic environments due to the facilitated solubilization of metals in acidic milieus. A novel yeast species from *Cryptococcus* genus was isolated in distinct acidic environments: a heavy metal rich acid mine drainage (Portugal) and a volcanic river with low metal content. (Argentina). This species is an acidophilic microorganism (optimum pH 2.5-4) and the two selected strains, one from each environment, endure high concentrations of different metals (e. g. As, Cd, Cu) despite their different ecological background. Cadmium is a nonessential heavy metal considered to be very toxic for most organisms even in low concentrations. Both *Cryptococcus* sp. strains were grown in the presence of different cadmium concentrations and a resistance level up to 1088 times greater than the 0.05-2 mM inhibitory concentrations range described for the model yeast *Saccharomyces cerevisiae* was detected. In lower eukaryotes, thiol-mediated vacuole accumulation is reported to be the predominant cadmium resistance mechanism. This mechanism comprehends cytoplasmic metal chelation by thiolated peptides (e. g. glutathione) and subsequent accumulation in the vacuole, mediated by tonoplast transporters (e. g. Ycf1). To assess the presence of this mechanism in the selected strains, dot blot hybridization was performed with the *ycf1* gene homologue from *C. neoformans*, the type species of the genus. The results point to the presence of a *ycf1* homologue gene in both strains. However, assessment of thiol accumulation in cells grown in presence vs. absence of cadmium by fluorescence microscopy with a thiol-specific probe (5-chloro-methyl fluorescein diacetate) only revealed differential cell staining for the Argentinean strain. This result is in agreement with the cytoplasmic chelation hypothesis for this strain, as the higher fluorescence intensity in cadmium grown cells points to the cellular accumulation of thiolated peptides. However, the cadmium resistance mechanism for the Portuguese strain probably does not rely primarily on this type of cytoplasmic accumulation. Differentially expressed genes in presence vs. absence of cadmium were also identified by a transcriptomic approach based on suppression subtractive hybridization, further elucidating the resistance mechanisms that operate in this unique yeast species.

S2: 3

## Bioremediation of water co-contaminated with 1,2-dichloroethane and arsenic or lead

Ashmita Arjoon, Ademola Olaniran, Balakrishna Pillay

Discipline of Microbiology, School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture, University of KwaZulu-Natal (Westville Campus), Durban, South Africa

Improper disposal practices and accidental spills of 1,2-dichloroethane (1,2-DCA) have made it a common contaminant of soil and groundwater. This priority compound continues to be a cause for concern, worldwide, due to its toxicity, persistence and bioaccumulation in the environment. Although microorganisms have been shown to degrade 1,2-DCA under both aerobic and anaerobic conditions, sites co-contaminated with heavy metal pollutants pose a greater challenge, since the two pollutants have to be treated differently. Therefore, this study was undertaken to quantitatively assess the effects of cadmium (Cd) and arsenic (As) on 1,2-DCA degradation and to investigate the use of biostimulation, bioaugmentation and dual bioaugmentation for enhanced 1,2-DCA degradation in co-contaminated water. Both heavy metals were found to be inhibitory, leading to a significant ( $p < 0.05$ ) 18.65 and 15.76% decrease in 1,2-DCA degradation, in the presence of As and Pb, respectively after 28 days. All microcosms undergoing one of the bioremediation treatments displayed higher degradation rate constant ( $k_1$ ), ranging from 0.047-0.058  $d^{-1}$  and 0.067-0.093  $d^{-1}$  in As and Pb- co-contaminated water, respectively compared to the untreated control. Dual bioaugmentation, bioaugmentation and biostimulation of co-contaminated water resulted in 1.76-, 1.6- and 1.42-fold and 2.1, 1.71- and 1.5-fold increase in  $k_1$  in the presence of As and Pb, respectively. Dual bioaugmentation was most effective, exhibiting 22.43 and 30.49% increase in 1,2-DCA degradation after 28 days in microcosms co-contaminated with As and Pb respectively. Dominant bacterial strains obtained from the co-contaminated microcosms were identified to belong to the genera, *Burkholderia*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Bradyrhizobiaceae* previously reported to degrade 1,2-DCA and other chlorinated compounds. PCR-DGGE analysis revealed variation in microbial diversity over time in the different co-contaminated microcosms. DGGE profiles revealed that in dual bioaugmentation, heavy metal resistant strains offered protection from metal stress to the indigenous bacterial population, since no decrease in band intensity was observed in microcosms over time. In conclusion, remediation approaches utilized in this study proved effective in the bioremediation of water co-contaminated with 1,2-DCA and heavy metals water and may provide the foundation for new and innovative treatment strategies for co-contaminated sites.



S2: 4

## **Protozoa grazing evaluation: a novel way to assess wastewater treatment performance?**

**Marta Martins Neto, David Ribas, Nelson Lima, Manuel Mota, Ana Nicolau**

IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Portugal

Protozoa are recognized as essential to reach high-standard performances in wastewater treatment plants (WWTP), namely activated-sludge, by drastically reducing the number of dispersed bacteria and therefore the turbidity of the final effluent. Moreover, protozoa are sensitive to environmental variations and changes in these populations are known to affect the whole food-web, thus affecting the performance of the wastewater treatment plant. The analysis of the protozoa populations is currently used to assess the performance of WWTP and the impact of protozoa grazing on the survival of particular groups of bacteria has been studied. Nevertheless, no studies exist on the possibility of simply evaluating the grazing rate of protozoa to assess the ecosystem health and therefore the WWTP performance. The results obtained in the present study suggest that protozoa grazing reflect the health of the whole community inhabiting the WWTP aeration tank and therefore can be used to evaluate the performance of the treatment system. Grazing was assessed by determining the ingestion of marked GFP (Green Fluorescent Protein) *E. coli* by the sessile ciliate *Epistylis* sp. using fluorescence microscopy. The samples were also inspected for the determination of the Sludge Biotic Index (SBI), routinely used to evaluate WWTP performance. The grazing rate clearly and significantly reflected the SBI evaluation. The study stands for the possibility of using grazing assessment as an alternative to the highly-expertise skills required in SBI determination.

# Health Microbiology and Biotechnology

*Thematic symposium 3*

**Keynote speakers:**

*Fani Sousa*

*Mário Ramirez*

**Oral presentations:**

*Joana Rolo*

*Andreia Couto*

*Nuno Bernardes*

*Adelaide Almeida*

**Keynote speaker / S3: 5**

## **Plasmid DNA for gene therapy applications: from the purification to the *in vitro* evaluation**

**Fani Sousa**

Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Covilhã, Portugal

The success attained in biotechnological investigation has significantly contributed to the expansion of biopharmaceutical products application in diverse biological and clinical fields. The therapeutic application purpose of plasmid DNA (pDNA) requires its recovery as a highly pure product, aiming to accomplish the strict quality criteria established by regulatory agencies. The challenges related to the purification of plasmids motivate the exploitation of the unmatched selectivity of affinity techniques. In fact, a new amino acid-based affinity chromatography [1] was implemented as a novel chromatographic approach to purify pDNA, using histidine [2], arginine [3] or lysine [4] as specific ligands. For beyond the possibility of these affinity matrices to isolate the major plasmid isoforms (open circular and supercoiled (sc) pDNA), the applicability of these matrices to efficiently purify sc pDNA from host impurities present in a clarified *E. coli* lysate was further demonstrated. Recently, the arginine chromatography was applied in an integrative approach designed for the purification of the sc isoform of a p53 tumor suppressor encoding plasmid, which was further encapsulated in nanoparticulated carriers [5]. Our findings showed that the sc isoform is recovered under mild conditions with high purity and structural stability. Regarding the encapsulation efficiency, it was also verified that higher levels are obtained for the sc pDNA (>75%). Moreover, *in vitro* transfection experiments confirmed the reinstatement of the p53 protein expression and most importantly, the sc pDNA transfected cells exhibited the highest p53 expression levels when compared to other formulations. Overall, given the fact that sc pDNA topoisomere indeed enhances transgene expression rates this approach might have a profound impact on the development of a sustained nucleic acid-based therapies.

[1] Sousa F et al. 2008. *Trends Biotechnol.* 26: 518-525 [2] Sousa F et al 2005. *Anal Biochem.* 343: 183-185. [3] Sousa F et al 2009. *J Gene Med.* 11: 79-88 [4] Sousa A et al 2011. *J Chromatogr B* (in press). [5] Gaspar VM et al 2011. *J Control Release* (in press). This work was supported by the Portuguese Foundation for Science and Technology (FCT), (PTDC/EQU-EQU/65492/2006 and PTDC/EBB-BIO/114320/2009).

**Keynote speaker / S3: 6**

## **More than meets the eye in relationship between pneumococci and its bacteriophages**

**Mario Ramirez**

FMUL, Portugal

*Streptococcus pneumoniae* is a major human pathogen causing a variety of diseases that range from milder infections such as sinusitis and otitis media to life threatening infections such as pneumonia, bacteremia and meningitis. Most *S. pneumoniae* isolates causing human infections are lysogenic, that is they carry temperate bacteriophages inserted in their genome. It is known that pneumococcal lysis plays a key role in pathogenesis by releasing important virulence factors and pro-inflammatory molecules. Since temperate bacteriophages can enter the lytic cycle spontaneously during pneumococcal growth their presence could contribute to the virulence of this important pathogen. On the other hand phages rely on efficient progeny release for their dissemination on bacterial populations. Here we will explore the mechanisms used by temperate pneumococcal phages to release their progeny at the end of the lytic cycle and the consequences that phage carriage has on biofilm formation, an important bacterial phenotype for bacterial persistence in the human host. Data will be presented on how temperate pneumococcal phages do not have an absolute requirement for a functional phage endolysin but engage the bacterial autolysin to accomplish the timely and efficient release of phage progeny. The enhanced biofilm capacity of lysogenic pneumococci and the mechanisms underlying it will also be discussed.

S3: 1

## High genetic diversity of community-associated (CA) *Staphylococcus aureus* in Europe

**Joana Rolo<sup>1</sup>, Maria Miragaia<sup>1</sup>, Agata Turlej<sup>2</sup>, Joanna Empel<sup>2</sup>, Ons Bouchami<sup>1,3</sup>, Nuno Alexandre Faria<sup>1</sup>, Ana Tavares<sup>1</sup>, Waleria Hryniewicz<sup>2</sup>, Ad C. Fuit<sup>4</sup>, Hermínia de Lencastre<sup>1,5</sup>, and the CONCORD working group<sup>6,7</sup>**

<sup>1</sup>Laboratory of Molecular Genetics, Instituto de Tecnologia Química e Biológica (ITQB), Oeiras, Portugal; <sup>2</sup>National Medicines Institute, Division of Clinical Microbiology and Infection Prevention, Warsaw, Poland; <sup>3</sup>Laboratory of the National Bone Marrow Transplantation Centre, Bab Saadoun, Tunis, Tunisia; <sup>4</sup>Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands; <sup>5</sup>Laboratory of Microbiology, The Rockefeller University, New York, USA; <sup>6</sup>D. Nashev, Bulgaria; O. Melter, H. Zemlicková, M. Fridrichova, Czech Republic; H. Westh, Denmark; S. Salmenlinna, Finland; G. Lina, France; I. Spiliopoulou, E. Drougka, Greece; K. Kristóf, F. Rozgonyi, Hungary; <sup>7</sup>G. Raponi, M.C. Ghezzi, Italy; M. Wulf, The Netherlands; I. Codita, G. Ionescu, Romania; A. Lišková, Slovak Republic; P. Ruiz-Garbajosa, R. Cantón, M. Angeles Dominguez, Spain; A.C. Petersson, Sweden; R. Walker, R. Anderson, J. Andrews, United Kingdom.

The worldwide emergence of infections caused by methicillin-resistant *S. aureus* (MRSA) in healthy individuals is worrisome. Nonetheless, a global picture of the epidemiology of this pathogen in Europe remains unclear. In this study, we aimed to describe the population structure of CA-SA and to shed light on the origin of MRSA in this continent. A total of 569 *S. aureus* isolates recovered in 16 European countries, from outpatients or patients attending hospitals within the 48 hours of hospitalization, were analyzed. The genetic background of isolates was characterized by standard typing techniques (*spa* typing, PFGE and MLST) and the presence of PVL (Panton-Valentine leukocidin) and ACME (arginine catabolic mobile element) was tested by PCR. MRSA were further characterized by SCC*mec* (staphylococcal cassette chromosome *mec*) typing. We found that 61% of all isolates were related with community-associated genetic lineages. Most MRSA were related with USA300 (ST8-IVa and variants) (37%), followed by the European clone (ST80-IVc and derivatives) (36%) and the Taiwan clone (ST59-IVa and related clonal types) (13%). A total of 50% of MRSA carried PVL and 13% carried ACME. Surprisingly, we found a high genetic diversity among MRSA clonal types (ST-SCC*mec*), Simpson's Index of Diversity (SID)=48.7% (32.7-64.7). Specifically, about half of the isolates showed associations between genetic background and SCC*mec* that were not described before. Analysis of *spa* types by eBURP showed that some CA-MSSA and CA-MRSA isolates were highly related, suggesting the *in loco* acquisition/loss of SCC*mec*. In particular, Southwest Pacific (ST30-IVc) and Queensland (ST93-IVa) clones, appear to have been imported, while USA300 (ST8-IVa), USA700 (ST72-IVc) and Taiwan (ST59-V; ST59-IVa; ST338-V), may have emerged in Europe from already existing MSSA isolates by the acquisition of SCC*mec*. A wide genetic diversity was found among CA-MRSA in Europe, in striking contrast with the reality observed in the USA. The conditions, which favored this wider variety of clonal types still, remains to be clarified, but might be related to the eclectic nature of Europe in what respects to infection control and antibiotic prescription policies. Our data also suggest that while some CA-MRSA might have emerged and evolved in Europe, other seem to have been imported.

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S3: 2

### An HIV-1 gp120 loop grafted in a single domain VL antibody inhibits HIV-1 infectivity and is a potential HIV-1 vaccine

**Andreia Couto**<sup>1,2</sup>, **Lídia M.D. Gonçalves**<sup>3</sup>, **Lara Figueiredo**<sup>3</sup>, **Catarina Santos**<sup>1,2</sup>, **António J. Almeida**<sup>3</sup>, **João Gonçalves**<sup>1,2</sup>

<sup>1</sup>URIA-CPM, Faculdade de Farmácia de Lisboa, Portugal; <sup>2</sup>IMM, Instituto de Medicina Molecular, Universidade de Lisboa.; <sup>3</sup>Faculdade de Farmácia de Lisboa, Portuga

The fusion process of the Human Immunodeficiency Virus type 1 (HIV-1), is mediated by the gp120 surface protein and the gp41 transmembrane protein. To facilitate viral entry, gp120 must bind to cell-surface CD4, alter its conformation to reveal a site for co-receptor attachment, and trigger conformational rearrangements in gp41 to mediate fusion of viral and host cell membranes. Therefore the gp120-CD4 interaction is critical for virus-cell fusion. The gp120 region that binds CD4 is the target of the broadly neutralizing antibody B12. The B12 antibody recognizes a highly conserved epitope overlapping the CD4-binding region of gp120. This antibody is one of the four known human monoclonal antibodies that can efficiently neutralize a broad array of primary isolates of HIV-1 *in vitro* and can protect against viral challenge *in vivo*. The goal of this work is to evaluate the potential of virus-cell fusion inhibition and vaccine antigen properties, of the gp120 loop recognized by the B12 antibody. This will be done using the 23 amino acids loop of gp120, grafted at the CDR1 of a highly stable single domain VL antibody, designated VL-B12. The potential of this VL-B12 construct was tested for CD4 binding by FACS analysis using Jurkat cells, a Human T lymphocyte cell line, and appears to have a high binding to CD4. The inhibition assays were performed in TZM-bl, a HeLa cell clone engineered to express CD4 and CCR5 and contains an integrated reporter gene for firefly luciferase under the control of HIV-1 LTR. The VL-B12 shows HIV-1<sub>NL4-3</sub> inhibition with an IC<sub>50</sub> = 3.89 microM, indicating that VL-B12-CD4 binding alone is sufficient to block virus-cell fusion. For the vaccine evaluation the mucosal immunity was tested by using encapsulated VL-B12 in nanoparticles of chitosan in comparison with subcutaneous routes with aluminium adjuvant and without adjuvant. Th1 or Th2 responses were measured by cytokine profiles produced by antigen-stimulated cells. IL-4 and IL-6 production was evaluated as a marker for Th2 type response and IFN- $\gamma$  and IL-2 were measured as Th1 cytokines. The ratio of antigen-specific IgG2a-IgG1 in the serum samples was calculated, specific IgA was also evaluated in order to assess mucosal immunity. The HIV1 neutralization assay using serum from different groups to evaluate the VL-B12 as potential vaccine antigen is ongoing. In conclusion, VL-B12 inhibits HIV-1 by binding to CD4 and is also being evaluated as potential vaccine antigen.

S3: 3

### Bacterial protein azurin as a new candidate drug to treat P-cadherin-overexpressing breast cancer

**Nuno Bernardes<sup>1,2</sup>, Ana Sofia Ribeiro<sup>2</sup>, Bruna Mota<sup>1</sup>, Rute G. Matos<sup>3</sup>, Cecilia M Arraiano<sup>3</sup>, Raquel Seruca<sup>2</sup>, Joana Paredes<sup>2</sup>, Arsénio M Fialho<sup>1</sup>**

<sup>1</sup>Institute for Biotechnology and Bioengineering/Center for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Lisboa, Portugal; <sup>2</sup>Institute of Molecular Pathology and Immunology, University of Porto, Porto, Portugal; <sup>3</sup>Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Oeiras, Portugal

Azurin is a low molecular weight (14kDa), water-soluble protein produced by the bacterium *Pseudomonas aeruginosa*. Azurin has a particular ability to mediate high-affinity interactions with unrelated proteins relevant in cancer, conferring on it the property of a natural scaffold for therapeutic purposes. This effect has been already proven for the receptor tyrosine kinase EphB2-mediated cell signaling, since azurin, due to its interaction with this receptor, prevents its binding to the ligand ephrinB2. In the present work, we further extend the azurin interaction studies to the Cadherin superfamily, with a special emphasis to a specific type of cadherins, the P-cadherin. P-cadherin overexpression occurs in about 30% of all breast carcinomas and has been shown to promote invasion, motility and migration of breast cancer cells, which in part is due to a soluble form of this protein (sP-cadherin). We hypothesized that azurin could be a scaffold against P-cadherin, antagonizing its pro-invasive effects. Protein-protein docking results and SPR experiments have validated this approach confirming an interaction between azurin and P- and E-cadherins in the nanomolar range ( $K_D$  of 20nM and 42nM, respectively). *In vitro*, azurin treatments of breast cancer cell models expressing different levels of P-cadherin caused a specific decrease of this protein shown by western blot analysis, whereas levels of E-cadherin remain unaltered. Additionally, the soluble form of P-cadherin was reduced after azurin treatment. These results were further confirmed by immunofluorescence assays. Moreover, Matrigel invasion assays were performed to test whether azurin can affect the invasiveness of P-cadherin overexpressing breast cancer cells. The results obtained demonstrate that azurin can significantly reduce the invasive phenotype of the cells. Studies are in progress aiming to elucidate the molecular mechanism(s) by which azurin interferes with P-cadherin in breast cancer cells.

S3: 4

### **Photodynamic inactivation of pathogenic microorganisms by tetrapyrrolic photosensitizers**

**Adelaide Almeida, Liliana Costa Costa, Eliana Alves Alves, Ângela Cunha, Newton Gomes, Carla Carvalho, João Tomé, Augusto Tomé, Amparo Faustino, Graça Neves, José Cavaleiro**

University of Aveiro, Portugal

The emergence of microbial resistance to many of the major classes of antimicrobial drugs is becoming an increasing problem in the clinical area. As such, the discovery of drugs with novel modes of action will be vital to meet the threats created by the multidrug resistant microorganisms. Antimicrobial photodynamic therapy (aPDT) is receiving considerable attention for its potentialities as a new form of antimicrobial treatment. It is a non-antibiotic approach that combines a nontoxic photosensitizer (PS), with visible light to generate singlet oxygen and free radicals that oxidize microbial vital constituents resulting in lethal damage. In this presentation an overview of the advances performed by the University of Aveiro group on the inactivation of microorganisms by tetrapyrrolic photosensitizers in the clinical area is presented. The results obtained by this group show that 1) cationic porphyrins efficiently inactivate Gram negative and Gram positive bacteria, bacterial endospores, viruses and fungi at micromolar concentrations under different light conditions, 2) aPDT efficiently inactivates multidrug resistant bacteria overcoming the problem related to bacterial resistance, 3) the PS efficiently inactivates bacteria and viruses without the possibility of recovery of viability after one week of dark incubation, 4) bacteria and viruses that surviving aPDT treatments in the presence of 5 mM of PS do not develop resistance after 10 cycles of aPDT, 5) in the presence of ampicillin and chloramphenicol the efficiency of the PS against Gram negative bacteria is increased even when antibiotic concentration is below the minimum inhibitory concentration (MIC), improving the action of classical antimicrobial compounds, without the use of large dosages.



# Next Generation Sequencing, comparative Genomics and Evolution

*Thematic symposium 4*

**Keynote speakers:**

*Gabriela Moura*

*Pedro Santos*

**Oral presentations:**

*Teresa Nogueira*

*Marco A Coelho*

*Catarina L Santos*

*Rita G Sobral*

**Keynote speaker / S4: 5**

**The role of transfer RNAs on gene evolution – insights from the fungal *Saccharomycotina* subphylum**

**Gabriela Moura**

Department of Biology & CESAM, University of Aveiro, Portugal

The genetic code is one of the most conserved features of life. Almost all organisms use the same amino acid codon assignments and flexibility in the genetic code is introduced by genetic code degeneracy only (most codons code for more than one amino acid), which allows for evolution of codon utilization biases. How these biases arise and are maintained in genes has been intensively studied but one does not yet fully understand their biological roles. In this talk I will address the role of tRNAs on the evolution of codon usage biases in the fungal *Saccharomycotina* subphylum. We have chosen this phylogenetic group because some of these species of the so called CTG-clade use a unique genetic code alteration that involves the decoding of leucine (Leu) CUG codons as serine (Ser) by a novel transfer RNA (tRNA<sub>CAG</sub><sup>Ser</sup>). This CUG reassignment erased the cognate tRNA<sub>CAG</sub><sup>Leu</sup> gene and thousands of Leu CUG codons from the genome of the CTG-clade ancestor, creating a situation where CUGs were allowed to re-emerge with a new identity. Among the CTG-clade, *Candida cylindracea* is a very poorly studied species that incorporates 100% of serine at CUG codons, while others incorporate lower levels of serine. In order to understand the evolution of CUGs in this species we have sequenced its transcriptome using RNAseq. The data shows a dramatic effect of this codon reassignment event on codon usage. In most CTG clade species, Ser CUG codons are rarely used and their tRNA<sub>CAG</sub><sup>Ser</sup> decoder is a low abundance tRNA (as expected), but *C. cylindracea* is remarkably different as CUGs are the most frequently used Ser codons. This raises various questions, namely how are these highly used codons being decoded? Is the tRNA<sub>CAG</sub><sup>Ser</sup> being expressed at high level in *C. cylindracea*? And how was this accomplished? I will address these questions and will also explain how tRNA decoding plays a critical role on gene evolution.

**Keynote speaker / S4: 6**

## **Exploring the genome catalogs of *Pseudomonas* using next generation sequencing technologies**

**Pedro M. Santos**

CBMA, University of Minho, Portugal

Bacteria belonging to the *Pseudomonas* genus possess an intricate ability to tolerate and thrive in very challenging environments such as the Human lungs, the deep sea or highly polluted soils. Such competence resides in their wide nutritional and metabolic versatility and in an intricate network of adaptation mechanisms. In other words, the necessary code to efficiently cope with a wide variety of different substrates, biotic and/or abiotic stress conditions is included in their genomic catalog. Therefore, they hold great potential, as a source of novel biomolecules and cell factories, for different biotechnological applications, namely in the fields of biocatalysis, biosensors, bioremediation, and biomedicine. The main goal of our group is to develop the necessary knowledge base and analytical resources to establish selected *Pseudomonas* spp. and/or their biomolecules as biotechnological tools, using an integrative biology approach by the application of high-throughput technologies. In this context, we have used Next Generation Sequencing technologies to decode the genome catalog of three selected *Pseudomonas* isolates. The environmental *Pseudomonas* sp. M1 strain was selected due to its remarkable ability to metabolize a wide variety of xenobiotics/recalcitrants whereas two *Pseudomonas aeruginosa* clinical isolates were used to shed some light into the *P. aeruginosa* accessory genome (mobilome) and how genomic diversity translates into phenotypic diversity. Details on the strategies used to reconstruct and decipher the genome of the selected *Pseudomonas* isolates, and on the current status of each project will be presented. Future research approaches will also be addressed mainly focused on an extensive exploitation of the genomic potential of the three *Pseudomonas* strains, envisaging the understanding of the genomic complexity of these bacteria and its full biotechnological/biomedical potential.

[1] Santos P.M et al (2007) *J. Biotechnol* 131:371-378. [2] Santos P.M., et al (2007) *OMICS* 11:233-251. [3] Santos P.M et al (2009) *Proteomics* 9:5101-11. [4] Soares-Castro et al (2011) *J. Bacteriol.* 19:5573 This work was supported by FEDER through POFC – COMPETE, by national funds from FCT - project PEst-C/BIA/UI4050/2011 and by the project PTDC/EBB-BIO/104980/2008 (FCT).

S4: 1

## **Bacterial sociality and virulence are mainly encoded on mobile non-core regions of the genome**

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Bacteria are social organisms. Although they can have a solitary life, they usually live within communities where they interact with each other, by releasing to the environment proteins essential for foraging, shelter, microbial warfare, freely released for the profit of their neighbours. They can also interact with their hosts, as they can secrete virulence factors, in the context of the infection. The bacterial genomes are also very dynamic which allows them to be versatile, and colonize almost all niches on earth, where they have a variety of different lifestyles, encompassing from commensals to pathogenic, or even enabling them to engage cooperative behaviours. The core genome is the set of genes that are in common in all genomes of a clade (group of organisms believed to share a recent common ancestor, or that are genetically related). On the other hand, the pan genome is defined as the total of non-orthologous genes of all genomes, and so that enables the expression of all phenotypes and lifestyles of a clade. In this context, we would expect to find the genes coding for the housekeeping proteins and the primary metabolism, located in a more stable core genome. Similarly, we would also expect to find the genes coding for the more adaptive and plastic traits, placed in the more mobile and dynamic non-core genome (i.e., the set of genes that are missing from at least one strain of the clade). The aim of this work is to demonstrate that the bacterial sociality and virulence are mainly encoded on mobile non-core regions of the genome. Our dataset is composed of nearly 200 completely sequenced bacterial genomes, both Firmicutes and Proteobacteria. We have characterized the core and pan genome of 24 bacterial clades, and have addressed a sub-cellular localization for nearly half of them. This allowed us to disentangle the intracellular proteome from the secretome (the set of proteins that are either targeted to be active at the extracellular milieu, or that are exhibited at the external side of the cell). Here we show evidence that genes involved in bacterial cooperation and virulence are over-represented on mobile elements, mainly placed on non-core regions.

*T.N. was a recipient of a grant from FCT (SFRH/BPD/41863/2007)*

## S4: 2

**Evidence of maintenance of sex determinants but not of sexual stages in red yeasts****Marco A. Coelho, Paula Gonçalves, José Paulo Sampaio**

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal

The red yeasts are an early-diverged group of basidiomycetes comprising sexual and asexual species that are completely intermingled and classified in the order *Sporidiobolales*. Sexuality in this group is based on two compatible mating types (or sexes) and sexual identity is determined by *MAT* loci that encode homeodomain transcription factors (*HD1/HD2*), peptide pheromones and their receptors [1]. The co-occurrence in the same phylogenetic group of a set of species sharing similar ecological and physiological properties, allowed us to investigate if the asexual species are truly asexual by focusing on the search for evidence for the presence of *MAT* genes, and for selective pressure acting on those genes. We surveyed 18 sexual heterothallic and self-fertile (homothallic) species and 16 asexual species. Apparently functional pheromone receptor homologues (*STE3.A1* and *STE3.A2*) were retained in multiple isolates of most of the sexual and asexual species and, for each of the two mating types, sequence comparisons with whole-genome data indicated that synteny tended to be conserved along the pheromone receptor region. For the *HD1/HD2* homologues obtained from sexual and asexual representatives of this group, likelihood methods suggest that diversifying selection acting on the self/non-self recognition region promotes diversity in sexual species, while rapid evolution seems to be due to relaxed selection in asexual strains. Our results indicate that events of loss of sexuality seem to be very recent because of the lack of a footprint of gene loss and decay. However, these events seem to be frequent and not uniformly distributed within the *Sporidiobolales*. This, together with the existence of asexual strains within sexual species, makes the separation between sexual and asexual species imprecise. Loss of sex associated with relaxed selection on *MAT* genes could promote the emergence of asexual lineages derived from ancestral sexual stocks, eventually leading to speciation, but not to the generation of exclusively asexual phylogenetic lineages that persist for a long time.

[1] Coelho MA et al (2010) *PLoS Genet* 6:e1001052. This work is supported by an FCT research grant (project PTDC/BIA-GEN/112799/2009) and PhD grant SFRH/BD/29580/2006.

S4: 3

### An Actinobacterial census: the diversity and ecological context of quorum-sensing LuxR regulators

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The bacterial ability to communicate through the synthesis and binding of molecular signals is known as quorum-sensing (QS), and is associated with the regulation of many genes coding for ecologically and medically important traits. One of the best studied QS circuits involves a LuxI synthase, which produces a QS signal, and a LuxR regulator, which senses and responds to that signal. In order to activate LuxR, the QS signal needs to reach a certain concentration threshold, which is usually dependent on the number of cells producing that specific signal. Although many studies focused on this QS circuit have been performed using Gram-negative models, its presence in the Gram-positive *Actinobacteria* phylum is still poorly explored. This study aimed to address the diversity and evolutionary stability of LuxI/LuxR-encoding genes in Actinobacteria, as well as to disclose significant relationships between their distribution/function(s) and microbial ecological features. A domain-based approach using two different algorithms was employed to detect putative LuxR regulators among Actinobacteria. Interestingly, we were able to identify 991 proteins having at least one LuxR domain among 53 completely sequenced actinobacterial species. However, a similar search for LuxI homologues yielded no hits, suggesting that LuxR regulators in Actinobacteria respond to signals other than those involved in the above described density-dependent circuits. Moreover, in 59% of the identified LuxR sequences an additional domain is present, usually in the N-terminal. In most cases (52%) this domain is REC (RECeiver, a CheY-like phosphoacceptor), suggesting that LuxR regulators in Actinobacteria may function either as single transcription factors or as two-component regulators. Adding to this, the frequency of LuxR regulators is positively correlated with the genome size, the ability to sporulate, the arrangement of cells in pluricellular forms and the possibility of having plants as hosts, whereas it is negatively correlated with the host dependency. Additionally, an *in silico* functional annotation of the LuxR regulators was carried out, and the results suggest a significant role of these proteins in the actinobacterial pathogenic phenotypes.

S4: 4

### Genome comparison of a *Staphylococcus aureus* mutant showing decreased antibiotic resistance and its revertant strain

**Rita Gonçalves Sobral**<sup>1,2,5</sup>, **Michael Mwangi**<sup>3,5</sup>, **Ana Madalena Ludovice**<sup>2,4</sup>, **Raquel Pereira Portela**<sup>1,2</sup>, **Hermínia de Lencastre**<sup>2,3</sup>, **Alexander Tomasz**<sup>3</sup>

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The level of expression of methicillin resistance (i.e., MIC value) in *Staphylococcus aureus* depends not only on the expression of the acquired resistance determinant *mecA* but also on the functioning of several housekeeping genes, some of which code for cell wall synthetic proteins, such as PBP2, FemA/B or MurF. We constructed an insertional mutant F9 in the *murF* determinant resulting in a massive decrease in oxacillin MIC value from 800 to about 4 mg/ml. Stable revertant subpopulations, designated F9H emerged with high frequency from the insertional mutant. We used whole genome sequencing to detect differences between the parental strain COL, insertional mutant F9 and a stable revertant derivative F9H2, selected for survival and growth at 200-400 mg/ml oxacillin. We detected only a single nucleotide change in F9H2 genome compared to F9: a missense mutation (P175L) in the a chain of phenylalanyl-tRNA synthetase. This subunit catalyzes the conversion of phenylalanine and ATP into the stable intermediate phenyl-adenylate. The substituted proline residue belongs to a  $\alpha$ -helix located at the bottom of a solvent-exposed cleft which harbors the catalytic site. Such pocket engulfs the ATP molecule, a large portion of the tRNAPHE and at the bottom part, phenylalanine. As proline either breaks or kinks a helix, its substitution for leucine may result in a strong structural modification and in reduced binding affinity to phenylalanine. Two additional mutations were also detected in the genomes of both F9 and F9H2: a synonymous mutation occurred in a RNA helicase gene and a missense mutation occurred in a transcription termination factor. We are presently constructing a conditional mutant for the tRNAPHE synthetase gene in the background of the F9 strain. Characterization of this double mutant should reveal if this single nucleotide difference is responsible for the reestablishment of the high and homogeneous resistance phenotype. To our best knowledge, our findings represent the first demonstration of an aminoacyl-tRNA biosynthesis pathway contributing to beta-lactam resistance. The multifactorial nature of oxacillin resistance in this strain suggests a complex reprogramming of cell physiology to survive in the face of drug challenge.

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# Bioprocess engineering

*Thematic symposium 5*

**Keynote speakers:**

*Gabriel Monteiro*

*Lígia Rodrigues*

**Oral presentations:**

*Ana J Cavaleiro*

*Belinda P Bibbins*

*Carla A Santos*

*Luís Borlido*



**Keynote speaker / S5: 1**

## **Rational engineering of *E. coli* strains and plasmids for improved manufacturing of plasmid biopharmaceuticals**

**Gabriel Monteiro**

IBB - Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal

Plasmids are being used as tools to non-viral gene therapy and DNA vaccination but one of the key challenges that could affect the success of the plasmid biopharmaceutical market is the development of cost effective manufacturing. Even plasmid downstream processing is a key issue the upstream plasmid production strongly impacts in plasmid production [1]. Most plasmids and the producer *Escherichia coli* host strains were originally developed for cloning or protein expression applications. So, we have rationally engineered 1) specific chromosome genes in order to develop a host capable of synthesizing large amounts of supercoiled plasmids at a high plasmid/impurities ratio suitable for large scale downstream purification and 2) re-design minicircle plasmids less prone to nucleases activities, less potential instability regions and capable of improved yields in downstream purification. Plasmid DNA over synthesis alters levels of gene expression and carbon flux. Gene knockout and overexpression strategies can improve plasmid DNA production in *E. coli*. Altering central metabolism to produce more energy and reducing power could improve plasmid yields. Also relevant is the regulation of genes from the pentose phosphate pathway which is responsible for biosynthesis of the nucleotide precursors. The minicircles are generated *in vivo* in *E. coli* DH5 $\alpha$  from a parental plasmid containing a recombinase under tight transcription control of an induced promoter. After *in vivo* recombination results in two circular DNA molecules, one containing the eukaryotic expression cassette (minicircle) and the other containing the plasmid bacterial backbone (miniplasmid) which need to be separated.

[1] Geisa A. L et al., *Biotechnol. J.*, DOI:10. 1002/biot.201100062

**Keynote speaker / S5: 6**

## **New strategies for the production of biosurfactants towards biomedical applications**

**Lígia Rodrigues**

IBB – Centre of Biological Engineering, University of Minho, Braga, Portugal

Microbial adhesion to biomaterial surfaces and subsequent biofilm formation has been observed on nearly all medical devices with severe economic and medical consequences. The significant resistance of biofilms to conventional antibiotic therapies has encouraged the development of new biomaterials and coatings. Biosurfactants represent an interesting approach as they can be used to modify the surface properties conferring it an anti-adhesive and antimicrobial activity, leading to new and effective means of combating colonization by pathogenic microorganisms without the use of synthetic drugs and chemicals. These microbial compounds constitute a diverse group of surface-active molecules occurring in a variety of chemical structures. Biosurfactants from lactic acid bacteria have been used as a strategy to avoid microbial colonization of silicone rubber voice prostheses. Also, they were found to be active against several bacteria and filamentous fungi responsible for diseases and infections in the urinary, vaginal and gastrointestinal tracts, and in the skin. Nevertheless, it is important to stress that the insufficient data on their toxicity for humans, as well as their high costs of large-scale production, have been restraining their commercialization and use in most medical applications. Many biotechnological strategies have been pursued to reduce the biosurfactants production costs including the use of agro-industrial wastes as substrates, optimization of medium and culture conditions, and efficient recovery processes. However, the improvements obtained from these strategies are marginal and to successfully compete with synthetic surfactants, novel microorganisms must be designed. The use of hyper-producer strains allows increasing the production yields and consequently reducing costs. These strains can be screened from the natural environment, or engineered using synthetic biology approaches. Hence, data on the genes involved on the production of biosurfactants is critical for designing organisms with improved features. Once the genes have been identified and isolated, they can be expressed in other microorganisms, or they can be modified or placed under regulation of strong promoters to increase their expression and so enhance production. This knowledge will also allow the production of novel biosurfactants with specific new properties for different industrial applications. Further advances in genetic engineering of the known biosurfactant molecules could yield potent biosurfactants with altered antimicrobial profiles and decreased toxicity against mammalian cells.

S5: 2

## Innovative start-up strategies for optimal methane production from lipids in anaerobic bioreactors

**Ana Júlia Cavaleiro, Diana Sousa, Madalena Alves**

IBB – Centre of Biological Engineering, University of Minho, Braga, Portugal

Lipids and long-chain fatty acids (LCFA) are energy-rich compounds that can be used as carbon and energy source by anaerobic microbial communities. Theoretically, large amounts of methane, a valuable energy carrier, may be generated during this process. However, operational problems, mainly associated with LCFA accumulation onto the sludge, have limited the use of anaerobic technology to produce methane from LCFA. In this work, two novel start-up strategies were tested for optimal methane production from LCFA: (i) bioreactor start-up using an intermittent feeding strategy, and (ii) bioreactor bioaugmentation with a LCFA-degrading bacterium. Intermittent feeding start-up resulted in efficient continuous methane production from high LCFA loads (up to 21 kgCOD m<sup>-3</sup> day<sup>-1</sup>, 50% COD as oleate (unsaturated LCFA, C18:1)). Alternating continuous bioreactor feeding and batch degradation periods, during bioreactor start-up, was crucial for sludge acclimation and contributed to the development of a metabolically specialized anaerobic microbial community that was able to efficiently convert oleate to methane. After intermittent feeding bioreactor start-up, methane yields higher than 70% were achieved, and neither LCFA nor VFA accumulated in the system. Bioaugmentation experiments were performed using *Syntrophomonas zehnderi*, a bacterium able to degrade saturated and unsaturated LCFA [1]. Anaerobic sludge amended with active and inactive *S. zehnderi* was incubated with 1 mM oleate as sole carbon and energy source. Methane production from oleate in bioaugmented batches was faster and high methane yields (89 ± 5%) were achieved. This work highlights the importance of the start-up strategy for the development of balanced syntrophic communities specialized in methane production from LCFA. Intermittent feeding and bioaugmentation with LCFA-degrading bacteria may be applied as alternative or complementary strategies.

[1] Sousa et al (2007) *Int J Syst Evol Microbiol*, 57: 609 – 615. Part of this study has been funded by FEDER, through the COMPETE program, and by Portuguese funds, through Portuguese Foundation for Science and Technology (FCT), in the frame of the project FCOMP-01-0124-FEDER-014784. Financial support from FCT and the European Social Fund (ESF) through the PhD grant SFRH/BD/24256/2005 attributed to AJ Cavaleiro is also acknowledged.

S5: 3

### **High xylitol bioproduction by *Debaryomyces hansenii* immobilized in alginate beads in an airlift bioreactor operated in fed-batch mode**

**Belinda Pérez Bibbins, Noelia Rodríguez Pazo, Ana María Torrado Agrasar, Jose Manuel Domínguez González**

Universidad de Vigo, Spain

The use of microorganisms for the production of additives for the food, pharmaceutical and cosmetic industries has interesting advantages over the chemical synthesis, as milder conditions of operation and higher selectivity, which facilitates the purification stage. However, the economic viability of the biotechnological processes is often a critical aspect for their industrial implementation. As well as the search for high producing and secure microorganisms, the development of high cell density cultures by means of cell immobilization constitutes a good strategy to reach high productivities, also enhancing cell stability and resistance to inhibitory factors. Nevertheless, high cell density cultures require the use of adequate bioreactors that allow good mass and heat transfer while keeping the integrity of the microorganism and the support. This work deals with the bioproduction of xylitol from xylose by *Debaryomyces hansenii* immobilized in alginate beads, using a 3.5 L airlift bioreactor operated in fed-batch mode to avoid the shear stress due to mechanical agitation and the inhibition caused by high substrate concentrations. A first assay was performed starting with 30 g/L of xylose and feeding at the same level when the sugar concentration was around 5 g/L. Considering the need of a microaerobic environment for the xylitol production by this yeast, fluidization was made by bubbling air at the lowest necessary flow rate (0.4 mL/min). The results showed the suitability of this bioreactor and mode of operation for this bioproduction and the viability of the culture, which kept productive during 85 days and reached 170 g/L of xylitol with yields higher than 0.6 g/g. Nevertheless, the production and the substrate consumption rates decreased after 20 days of fermentation. A batch assay in erlenmeyer flasks was then performed in presence of increasing xylitol levels added at 24 h of incubation at concentrations from 0 to 165 g/L. The results pointed out the existence of product inhibition for concentrations higher than 100 g/L as one of the reasons that would explain the decreasing efficiency of the fed-batch culture at long times of incubation. A last fed-batch culture was done to enhance the productivity of the bioreactor by means of increasing the initial concentration of xylose and the feeding to 60 g/L with the aim of improving the growth rate. This way the xylitol production rate was doubled and 120 g/L of xylitol were achieved in half of time (14 days).

S5: 4

## Microalgae fermentation to produce biodiesel and simultaneously improving autotrophic growth

**Carla Amarelo Santos<sup>1</sup>, Leonor Caldeira<sup>1</sup>, Julio Maggiolly Novais<sup>2</sup>, Alberto Reis<sup>1</sup>**

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Microalgae are the only renewable resource of oil to produce biodiesel capable of being produced accordingly to demand with the advantage of not producing carbon dioxide. Algae are capable of many kinds of trophic (nourishment) centred on both major forms of nutrition, namely autotrophy (phototrophy) and heterotrophy, of which autotrophy is by far the most important. However, some microalgae assimilate and thus utilize organic carbon and energy sources for growth in the dark. The heterotrophic culture mode circumvents technical and physiological difficulties associated with the supply and distribution of light and carbon dioxide involved in photosynthetic mode of cultivation. It offers the possibility of increasing cell concentration and productivity [1]. The microalgae *Chlorella protothecoides* was chosen for this work due to its ability of growing under different nutritional modes (photoautotrophic, heterotrophic and mixotrophic), attaining a high biomass productivity and lipid content, suitable for biodiesel production [2]. The exit gas phase of a fermentor (2 L) was connected to a vertical alveolar panel (VAP) photobioreactor (3 L) to aerate the autotrophic cultivation of algae. The algae was grown in the fermentor with a simple organic medium in the dark. In the photobioreactor a complex inorganic medium for algae was used with low light conditions [3]. The maximum microalgae productivity in the fermentor was 1.4 g/(L.d) at the end of 3 days cultivation and the fatty acid (FA) content in this biomass was 18% (dry wt). The fatty acid productivity was 0.35 g/(L.d). A comparison from heterotrophic to autotrophic *Chlorella protothecoides* fatty acids reveals that heterotrophic biomass has the highest lipid content (26% FA/DW) and these lipids had the most suitable fatty acid profile (42% Oleic acid, 30% Linoleic acid, 16% Palmitic acid) to produce a biodiesel with high quality according to EN 14214 (high monounsaturated (~50%), 20% or less of saturated FA and 30% or less of polyunsaturated (dienoic-, trienoic-)). The autotrophic microalgae cultivation was improved achieving a microalgae productivity of 0.141 g/(L.d) compared with a cultivation that was aerated with air 0.099 g/(L.d). Further improvement on the fermentation of algae can be applied to optimize the actual productivity, but these results are promising, as the fermentation process is much easier to scale up compared to autotrophic cultivation.

[1] Grobberlaar J.U. (2004) Richmond Amos (ed) *Handbook of microalgal Culture: Biotechnology and applied phycology*, Blackwell, London, pp 97 -114. [2] Miao X, Wu Q. (2006) *Biore. Technol.*, 97:841-846. [3] Santos et al (2011) *J Ind Microbiol Biotechnol* 38:909-917.

S5: 5

## Potential of boronic acid magnetic particles in the direct purification of human monoclonal antibodies from CHO cell supernatants

**Luís Borlido<sup>1</sup>, A.M. Azevedo<sup>1</sup>, A.C.A. Roque<sup>2</sup>, M.R. Aires-Barros<sup>1</sup>**

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Magnetic adsorbents offer fast and gentle separation conditions with the potential for high binding capacities. With the pressing demand for high amounts of monoclonal antibodies (mAbs) and the inability of Protein A chromatography to directly purify samples with high mAb titers (titers greater than 10 g/L are now possible), alternative and more cost effective purification processes are needed. The boronic acid (BA) ligand is a fully synthetic ligand capable of binding to cis-diol containing molecules such as glycoproteins. Antibodies are a part of this group of proteins as they bear oligosaccharides in both the Fv and Fc regions. In this work we have evaluated the potential of boronic acid functionalized magnetic particles for the one-step capture of a human monoclonal antibody (mAb) from a chinese hamster ovary (CHO) cell culture supernatant. For comparison, Protein A coated magnetic particles were also used. The most important factor influencing the overall process yield and product purity in boronic acid particles was found to be the binding pH. Basic pH values promoted higher purities while resulting in decreased yields due to the competing effects of molecules such as glucose and lactate present in the cell culture supernatant. After optimization, the particles were successfully used in a multi-cycle purification process of the mAb from the CHO feedstock. Boronic acid particles were able to achieve an average overall yield of 86% with 88% removal of CHO host cell proteins (HCP) when the binding was performed at pH 7.4, while at pH 8.5 these values were 58% and 97%, respectively. In both cases, genomic DNA removal was in excess of 97%. Comparatively, Protein A particles recorded an average overall yield of 80% and an HCP removal greater than 99%. Boronic acid based purification processes can offer a cost-effective alternative to Protein A as the direct capturing step from the mammalian cell culture.

[1] L. Borlido et al, *J. Chromatogr. A* (2011) doi:10.1016/j.chroma.2011.08.084.

# Molecular Microbiology and Microbial Physiology

*Thematic symposium 6*

**Keynote speakers:**

*Luísa Figueiredo*

*Vitor Costa*

**Oral presentations:**

*Luis G Gonçalves*

*Carlos São-José*

*Gabriel Paiva*

*Júlia Santos*

**Keynote speaker / S6: 5**

## **The role of chromatin in antigenic variation of African Trypanosomes**

**Luísa Figueiredo**

IMM, Universidade de Lisboa, Portugal

*Trypanosoma brucei* is a unicellular parasite that causes the fatal disease African sleeping sickness in humans. *T. brucei* evades the host immune system through a mechanism known as antigenic variation, in which it periodically changes a dense, uniform coat of variant surface glycoproteins (VSGs). For a VSG to be transcribed, it must be positioned at a specialized subtelomeric locus called a bloodstream expression site (BES). There are 15 BES in the genome, but only one at a time is transcriptionally active. What determines this singularity remains unknown, but published work implicates epigenetic factors. We and others have recently shown major structural differences between the chromatin of active and silent BESs, with the active BES being essentially devoid of regularly spaced nucleosomes. In our lab, we are interested in understanding how this chromatin structure affects VSG regulation. Our working hypothesis is that the “open” and “closed” chromatin structures at BESs result from the presence of different structural components and the action of chromatin modifying or remodelling enzymes that alter the accessibility of DNA to the transcription machinery. Currently, we are using RNA interference to knock down and characterize the phenotype of selected candidate genes, such as histone H1. We will discuss the organization of histone H1 gene family in *T. brucei* and their role in VSG gene regulation.



**Keynote speaker / S6: 6**

## **Sphingolipid signaling, redox homeostasis and lifespan in yeast**

**Vitor Costa**

Instituto de Biologia Molecular e Celular, Porto, Portugal

Sphingolipids are ubiquitous structural components of cell membranes but a number of studies revealed that ceramides and other bioactive sphingolipids play important roles as signaling and regulatory molecules. These lipids bind target proteins, including receptors, protein kinases and phosphatases that in turn modulate signal transduction pathways. Ceramides can be produced by *de novo* biosynthesis, through acylation of a long chain sphingoid base derived from serine and palmitoyl-CoA, or generated through hydrolysis of complex sphingolipids, mediated by sphingomyelinases. Stress conditions, such as ionizing radiation, DNA damage, heat stress and oxidants, activate sphingomyelinases. The resulting ceramides induce cellular responses ranging from apoptosis and cell cycle arrest to cell survival and proliferation. In the yeast *Saccharomyces cerevisiae*, complex sphingolipids (inositol phosphosphingolipids) are hydrolysed by Isc1p, an orthologue of the mammalian neutral sphingomyelinase 2. Our studies have shown that Isc1p plays a key role in oxidative stress resistance and chronological lifespan, modulating mitochondrial function, redox homeostasis, iron levels and cell death by caspase-dependent apoptosis. Isc1p finely tunes ceramide levels during cell aging and, therefore, controls ceramide signaling pathways. Indeed, Isc1p is an upstream regulator of Sit4p, the catalytic subunit of type 2A ceramide-activated protein phosphatase that has been functionally linked to the regulation of cell cycle, nutrient signaling and cell functions that depend on protein kinase C. Our current understanding of the role of Isc1p, ceramide and Sit4p in cell signaling and modulation of mitochondrial function will be presented.

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S6: 1

## Evolution of the biosynthesis of di-*myo*-inositol phosphate, a marker of adaptation to hot marine environments

**Luis G Gonçalves<sup>1</sup>, Nuno Borges<sup>1</sup>, François Serra<sup>2</sup>, Pedro L Fernandes<sup>3</sup>, Hernán Dopazo<sup>2</sup>, Helena Santos<sup>1</sup>**

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The synthesis of di-*myo*-inositol phosphate (DIP), a common compatible solute in hyperthermophiles, involves the consecutive actions of inositol-1-phosphate cytidyltransferase (IPCT) and di-*myo*-inositol phosphate synthase (DIPPS). In most cases, both activities are present in a single gene product, but separate genes are also found in a few organisms. Genes for IPCT and DIPPS were found in the genomes of 33 organisms, all with thermophilic/hyperthermophilic lifestyles. Phylogeny of IPCT/DIPPS revealed an incongruent topology with 16S RNA phylogeny, thus suggesting horizontal gene transfer. The phylogenetic tree of the DIPPS domain was rooted by using phosphatidylinositol phosphate synthase sequences as outgroup. The root locates at the separation of genomes with fused and split genes. We propose that the gene encoding DIPPS was recruited from the biosynthesis of phosphatidylinositol. The last DIP-synthesizing ancestor harboured separated genes for IPCT and DIPPS and this architecture was maintained in a crenarchaeal lineage, and transferred by horizontal gene transfer to hyperthermophilic marine *Thermotoga* species. It is plausible that the driving force for the assembly of genes encoding IPCT and DIPPS in the early ancestor is related to the acquired advantage of DIP producers to cope with high temperature. This work corroborates the view that Archaea were the first hyperthermophilic organisms.

Gonçalves LG *et al* (2011). Environmental Microbiology in press.

S6: 2

## Identification of the navigation system of bacteriophage SPP1: a single tail protein that finds and binds two receptors of the host cell surface

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Phage SPP1 targets two receptors of the *Bacillus subtilis* surface to initiate infection, the cell wall glucosylated teichoic acids (Glu-WTA) and the membrane protein YueB. The calcium-dependent SPP1 interaction with WTA is reversible but it greatly increases the chances of the phage to find and bind to YueB by “guiding” the virus particle to the cell location where the membrane receptor concentrates, the cell poles [1,2]. SPP1 binding to YueB triggers phage DNA ejection to the *B. subtilis* cytoplasm, which preferentially occurs near or at cell poles [2]. The SPP1 virion protein(s) responsible for recognition of both receptors (virion receptor-binding protein(s), RBP) were unknown. Here we show the studies of two collaborating labs on the identification of SPP1 RBP(s) as well as our current model for the events leading to SPP1 DNA entry into *B. subtilis*. Extracts containing SPP1 tail proteins were run through a YueB affinity matrix leading to the capture of gp21, a putative component of the SPP1 tail spike. To assess the significance of this interaction, we developed a strategy to select mutant phages specifically affected in YueB binding. The mutants carried a single mutation in the gp21 C-terminus. Immunoelectron microscopy located the gp21 N-terminus (gp21N) in the SPP1 tail cap and upper region of the tail spike, in agreement to recent structural data [3], whereas the C-terminus (gp21C) was mapped further down in the spike structure. *In vitro* fluorescence studies revealed that anti-gp21C antibodies (a-gp21C) interfered with phage/receptor binding and subsequent viral DNA ejection, whereas a-gp21N antibodies did not affect these events. In addition, at high antibody concentrations a-gp21C was sufficient to trigger SPP1 DNA ejection in contrast to a-gp21N. The results point to a major role of the gp21C in YueB binding and trigger of SPP1 DNA ejection. An analogous approach was followed to identify RBP(s) binding Glu-WTA. Interestingly, we also found that the C-terminal half of gp21 is responsible for the interaction with WTA. In conclusion, our results indicate that a single RBP (gp21), located in the SPP1 tail spike, is designed to recognize the particular topology of phage receptors on the *B. subtilis* surface, which determine the site of phage DNA entry.

(1) Baptista et al., 2008. *J. Bacteriol.* 190: 4989-96 (2) Jakutyte et al., 2011. *J. Bacteriol.* 193: 4893-903 and unpublished results (3) Goulet et al., 2011. *J. Biol. Chem.* 286: 25397-405

S6: 3

**Production of nematocidal extracellular proteases by the nematode-associated bacterium *Serratia* sp. A88C13****Gabriel Pajva<sup>1</sup>, Diogo N. Proença<sup>1</sup>, Susana S. Santos<sup>1</sup>, Luis Fonseca<sup>1</sup>, Isabel M.O. Abrantes<sup>1,2</sup>, Paula Veríssimo<sup>1,2</sup>, Paula V. Morais<sup>1,2</sup>**<sup>1</sup>IMAR-CMA, 3004-517 Coimbra, Portugal; <sup>2</sup>Department of Life Sciences, FCTUC, University of Coimbra, 3001-401 Coimbra, Portugal

The genus *Bursaphelenchus xylophilus*, the pine wood nematode, is one of the most interesting genera of nematodes since it is a pathogen of trees and is the causal agent of pine wilt disease. Several studies have shown that bacteria from various genera could be isolated associated with *B. xylophilus*. In Portugal, healthy pine trees of the species *Pinus pinaster* have a diverse endophytic microbial community. One of the last works published on bacteria associated to the nematode showed that in recently invaded areas by *B. xylophilus*, nematodes isolated from infected trees showed associated bacteria that were from different species according to the area where the nematodes were isolated. The nematocidal ability was analyzed for 46 strains isolated associated to nematodes from trees with PWD. Screenings of these isolates reveal the presence of bacterial strains producing different extracellular products. Only 7 strains did not showed toxicity against Bx and the only genus with all the strains non-toxic was *Burkholderia*. All strains of the genus *Pseudomonas* except one strain of *P. putida* showed toxicity against the nematodes. The genus *Serratia* included the strains more toxic to the nematodes: all except one strain showed the highest toxicity level. The bacterial strains more active in killing nematodes were selected and the bacterial products produced during growth studied, in order to determine their activity. The objective of the present work was the identification and characterization of hydrolytic enzymes produced by the isolates and their nematocidal activity. The toxicity assays revealed that only fractions with proteases, separated by FPLC, showed nematocidal activity comparable to the whole extract. These fractions showed the higher toxicity rates and included different concentrations of 2 different proteases. The use of inhibitors selective to serine proteases or to metalloproteases demonstrated that the serine protease was the more responsible for the toxicity of the supernatant. Understanding the infection process and all the factors involved is important in order to develop strategies to control the *B. xylophilus* dispersions.

S6: 4

**Ammonium is toxic for amino acid-starved yeast cells under extreme calorie restriction, inducing cell death through the regulation of PKA, TOR and Sch9 activities****Júlia Santos<sup>1,2</sup>, Maria João Sousa<sup>3</sup>, Cecília Leão<sup>1,2</sup>**

<sup>1</sup>Life and Health Sciences Research Institute (ICVS), School of Health Sciences University of Minho, Braga, Portugal; <sup>2</sup>ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal; <sup>3</sup>Molecular and Environmental Research Centre (CBMA)/ Department of Biology, University of Minho, Braga, Portugal

We have previously shown that ammonium, a nitrogen source commonly used for yeast growth, stimulates cell death in amino acid-deprived auxotrophic *Saccharomyces cerevisiae* cells (aa-starved cells) after they are transferred to water. Ammonium induced cell death was accompanied by an initial small increase of apoptotic cells followed by extensive necrosis. The occurrence of necrosis was confirmed by the observation of the nucleo-cytosolic translocation of Nhp6Ap, which nuclear release is considered a marker of necrosis. Ammonium toxicity decreased CLS and induced cell death in aa-starved cells but not in nitrogen-starved cells (N-starved cells), suggesting that the ammonium effect was dependent on the inappropriate arrest of the aa-starved cells. Autophagy was inhibited by ammonium, but this blockage did not cause decrease in cell viability. Activation of PKA stimulated ammonium induced decrease of CLS consistent with the observation that deletion of *MEP1*, *MEP2*, *RAS2* or *TPK1* partially reverted the ammonium effect. Deletion of *TOR1* also significantly rescued the ammonium effect and decreased PKA activation. However, PKA activation in response to ammonium was completely abolished by *SCH9* deletion, but even so, could not revert the shortening in CLS, indicating that PKA inactivation cannot extend CLS in the absence of Sch9p, that plays a pro-survival role in the process.

JS holds a PhD fellowship (SFRH/BD/33314/2008) from FCT.

# Cell and Tissue Engineering and Biomaterials

*Thematic symposium 7*

**Keynote speakers:**

*Helena S Azevedo*

*Susana Rosa*

**Oral presentations:**

*Andreia Madeira*

*Wojciech Szymczyk*

*Catarina Madeira*

*Claudia C. Miranda*

**Keynote speaker / S7: 5**

## **Biomimetic extracellular matrices as artificial environments for cell culture**

**Helena Sepúlveda Azevedo**

3B's Research Group, University of Minho, Portugal

The development of 3D culture models that allows studying cell behavior in physiological and pathological conditions in the context of the extracellular matrix (ECM) would be extremely useful to investigate specific cell-cell and cell-ECM interactions, as well as the effects of specific factors. Compared with culturing cells on plastic, cell culture in 3D environment provides another dimension for external mechanical and chemical inputs, which dramatically affects integrin ligation, cell contraction and associated intracellular signaling. By binding to a wide variety of soluble growth factors and other signaling molecules, native ECM provides their controlled diffusion and serves to regulate their local concentrations and gradients. The diversity of the (often unknown) cues that are present in native ECM, strongly contributes for the difficulty to realize such complexity *in vitro*. Naturally-derived ECMs provide a wide spectrum of chemical and physical cues that are known to influence the function of many cells, but their variability in composition and mechanical properties are clearly a disadvantage when trying to isolate the effects of specific factors. Advances in synthetic biomaterials are converging to allow the creation of *in vitro* models that capture some complex features of the *in vivo* environment. Synthetic matrices, that are tailored to mimic specific ECM properties, are being implemented to provide well controlled and reproducible cellular environments. Moving to well-defined synthetic 3D systems is however more challenging and requires control not only of cell adhesion sites and matrix viscoelasticity, but of nano and microporosity (which regulates cell motility and the transport of soluble molecules), growth-factor binding and matrix degradation. Consequently, the need for matrices that combine the benefit of natural and synthetic materials has become apparent. This talk will focus on our efforts to develop bioactive matrices for applications in tissue regeneration strategies, in particular for cartilage and skin, but also as *in vitro* model systems for studying cell behavior and discover new therapies. These matrices are formed by self-assembly when combining designed peptide structures with natural polymers, and can capture many aspects of the structure and function of natural ECM.

**Keynote speaker / S7: 6**

## **Stem cells and biomaterials for the treatment of ischemic diseases**

**Susana Rosa**

Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal

Ischemic diseases such as myocardial infarction and chronic wounds cause a high morbidity with important social impact. Two major angiogenic therapies are being tested to treat patients suffering from these diseases based on the delivery of growth factors and/or cells (e.g. vascular cells, vascular progenitor cells and stem cells). The transplantation of vascular cells could be a potential platform for the rapid formation of blood vessels after an ischemic insult. Potential sources of vascular cells are human pluripotent stem cells (human embryonic stem cells and human induced pluripotent stem cells) and umbilical cord blood (UCB). Indeed, recent studies have demonstrated that the transplantation of vascular cells in animal models of hindlimb ischemia and diabetic chronic wounds significantly increases the vascularization of ischemic tissues. However further research is necessary in order to identify the mechanisms underlying the regenerative potential of stem cells, and also to improve the survival and retention of stem cells after transplantation. Our research group aims, by intersecting biomaterials and stem cells, to develop new platforms to retain cells at the injured site in order to improve their engraftment and therapeutic effect. I will focus my presentation in two technologies recently developed by our group. I will show that the delivery of  $\beta 4$  (T $\beta 4$ ), a pro-survival and pro-angiogenic factor together with vascular cells derived from human embryonic stem cells (hESCs) in a bioactive hydrogel, to the infarcted area of animal model, improved cardiac performance and structure by promoting structural organization of native endothelial cells and *de novo* capillaries formation. In addition, I will show a methodology to improve the survival, vascular differentiation and regenerative potential of umbilical cord blood (UCB)-derived hematopoietic stem cells (CD34<sup>+</sup> cells). The methodology is based in a co-culture system of the stem cells with CD34<sup>+</sup>-derived endothelial cells (ECs), in a 3D fibrin gel. Our results indicated that the pro-survival effect of CD34<sup>+</sup>-derived ECs on CD34<sup>+</sup> cells is mediated, at least in part, by bioactive factors released from ECs. This effect likely involves the secretion of novel cytokines, including interleukin-17 and interleukin-10, and the activation of the ERK 1/2 pathway in CD34<sup>+</sup> cells. The regenerative potential of this co-culture system was demonstrated in a chronic wound diabetic animal model.



S7: 1

## Molecular and cellular networking underlying the *ex-vivo* expansion of human mesenchymal stem cells revealed by 2-DE based quantitative proteomics

**Andreia Madeira**<sup>1,2</sup>, **Francisco dos Santos**<sup>1,2</sup>, **Cláudia Lobato da Silva**<sup>1,2</sup>, **Emílio Camafeita**<sup>3</sup>, **Joaquim M S Cabral**<sup>1,2</sup>, **Isabel Sá-Correia**<sup>1,2</sup>

<sup>1</sup>Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal.; <sup>2</sup>Department of Bioengineering, Instituto Superior Técnico, Lisboa, Portugal.; <sup>3</sup>Unidad de Proteómica, Centro Nacional de Investigaciones Cardiovasculares, CNIC, Madrid, Spain.

Mesenchymal stem cells (MSC) have become one of the most promising candidates for tissue engineering and regenerative medicine applications. In order to meet the clinically relevant cell dosage, a fast *ex-vivo* expansion process is required. The main goal of this study was to address the impact of the extended *in vitro* cultivation involving consecutive passaging on the molecular and cellular networking of human bone marrow (BM) MSC using a quantitative proteomics approach based on two-dimensional gel electrophoresis (2-DE) as detailed in [1,2]. To this end, Passage 2 (P2) BM MSC were thawed and collected at P3 (7 days of cultivation) and P7 (35 days of cultivation). Immunophenotypic characterization and multilineage differentiation analyses were performed and the effect of cell passaging was assessed in terms of specific growth rate, population doublings, clonogenic and differentiation potential. P3 and P7 proteomes were separated by IEF/SDS-PAGE 2-DE and stained with fluorescent dye. A total of 79 proteins whose content was statistically different in P3 and P7 were identified by mass spectrometry. Of these, 30 are unique protein forms while the remaining correspond to multiple isoforms, namely the cytoskeleton components  $\beta$ -actin (7) and vimentin (24). The most differently expressed proteins belong to the categories: Structural Components and Cellular Cytoskeleton; Folding and Stress Response Proteins; Energy Metabolism; and Apoptosis. The most significant network identified for the proteins whose content is altered in P7 compared to P3 includes proteins involved in "Cell death, gene expression, cellular growth and proliferation". The transcription factors MAZ, SP1 and AP1 are the suggested regulators of genes encoding the proteins present in the dataset. Overall, this work provided comprehensive data at a molecular systems biology level on how human MSC lose their proliferative and clonogenic potential with extended *ex-vivo* cultivation.

[1] Santos PM et al (2009) *PROTEOMICS*, 9, 657-70. [2] Sá-Correia I et al (2010) *Expert Rev Proteomics*, 7, 943-53.

S7: 2

## **Fabrication and evaluation of scaffolds for small diameter blood vessel engineering**

**Wojciech Szymczyk<sup>1,2</sup>, Albino Pereira Martins<sup>1,2</sup>, Vitor Manuel Correlo<sup>1,2</sup>, Nuno Neves<sup>1,2</sup>, Alexandra Pinto Marques<sup>1,2</sup>, Rui Luís Reis<sup>1,2</sup>**

<sup>1</sup>3B's Research Group / University of Minho, Portugal; <sup>2</sup>3B's - PT Government Associate Laboratory, Braga / Guimarães, Portugal

Diseases of the blood vessels, particularly of small diameter arteries are responsible for most of deaths in developing and developed countries. The number of operations using arterial prostheses and of small calibre vascular bypass reaches enormous values every year. With the limited availability of functional autologous vessels the need for new strategies to develop artificial blood vessels is increasing. The objective of this study was to develop a scaffold model aiming at fabricating small diameter blood vessel grafts with distinct surface properties. This study was designed to evaluate the influence of the scaffold properties on endothelial and smooth muscle cells. The scaffolds consisted of either a polycaprolactone (PCL) nanofibre mesh (NF) layer fabricated by means of electrospinning or a PCL membrane fabricated by solvent casting (SC); and a second layer prepared from a mixture of  $\beta$ -glycerol phosphate salt (GP) and chitosan (Ch). Scaffold characterisation was performed in terms of surface topography (SEM) and mechanical properties (tensile, Young's tensile and yield stress; and strain at break). For the biological evaluation endothelial and smooth muscle cells isolated from the vein of human umbilical cord, respectively HUVECs and HUVSMCs, were used. Single cell cultures were established for both cell types and both scaffolds up to 7 days. Cell behaviour was evaluated after DNA quantification, alkaline phosphatase activity, methylene blue staining and SEM analysis. The tensile strength values for both SC PCL and NF PCL scaffolds exceed the one of natural artery (15MPa vs. 3MPa vs. 1MPa). As expected no alkaline phosphatase activity was detected in the cultures. Moreover, HUVECs attachment and proliferation rate was significantly higher on the SC PCL layer while for HUVSMCs the opposite was observed and the NF PCL layer was the preferable substrate for adherence and growth. Scaffolds with mechanical properties capable of withstand the physiological vascular conditions were obtained. The GP layer did not cause any sign of calcification which constitutes a good indicator for its incorporation within the blood vessel scaffold. The selective response of each cell type to a specific surface topography allows the definition of the design of a blood vessel graft combining HUVECs and HUVSMCs in the opposite layers.

S7: 3

## Non-viral gene delivery strategies using minicircles into neural stem cells

**Catarina Madeira, Mónica S.C. Reis, Filipa F.C.G. Ferreira, Carlos A.V. Rodrigues, Margarida M. Diogo, Joaquim Cabral**

Department of Bioengineering, Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Technical University of Lisbon, Lisboa, Portugal

Neural stem cells (NSCs) are multipotent stem cells that can differentiate into neurons, astrocytes and oligodendrocytes. These cells can be obtained directly from the Central Nervous System (CNS) or derived from embryonic stem cells (ESCs) or Induced Pluripotent Stem Cells (iPSC) and demonstrate the ability to engraft within the CNS, migrate to CNS pathology and in certain scenarios to reconstitute injured or diseased nervous system. These cells can be genetically modified *in vitro* to express desired transgenes for improved expandability, as well as for *in vivo* cellular carrier of toxic payloads for tumor elimination or growth factors for cellular regeneration in the CNS. These cells are often genetically modified by viral vectors, but due to safety concerns non-viral based methodologies are preferred in clinical settings. Non-viral gene delivery strategies are based on the use of plasmid DNA vectors (pDNA) carrying the gene of interest but recently a new generation of vectors lacking bacterial backbone, named Minicircles (mC), have been tested in mammalian and stem cells with superior results in terms of transfection efficiency and long-term expression of the transgene, when compared to conventional plasmids. Actually, in Gene Therapy, when transient expression of a specific therapeutic protein more advantages may be obtained when its expression occurs for a longer period of time after using a vector that did not hamper cell viability or proliferation. The main purpose and novelty of this work focused on evaluation of the effect of mC on NSCs envisaging a safe strategy for future gene/cell therapy applications for treatment of neurological diseases. We verified that mC encoding GFP are more efficiently transferred into NSC by microporation when compared to cationic liposomes (80% versus 25% transfected cells, respectively). When using both methodologies cell viability was 85-90% and overall transfection yield of 65% was achieved with microporation. We verified that increasing DNA amount does not induce major protein expression although an increasing DNA amount can be found inside the nucleus. Interestingly, when using similar number of molecules of mC (0.5 mg) and pDNA (0.8 mg), mC promoted higher number of NSC expressing GFP, higher levels of GFP expression and higher cell viabilities, when compared with pDNA. Accordingly, our results provide evidence that mC are more efficient gene carriers to NSC when compared to the respective conventional plasmid.

S7: 4

## **Towards fully defined culture systems for human induced pluripotent stem cell expansion**

**Tiago Fernandes, Carlos A.V. Rodrigues, Claudia C. Miranda, Margarida M. Diogo, Joaquim Cabral**

Department of Bioengineering, Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Technical University of Lisbon, Lisboa, Portugal

The generation of induced pluripotent stem (iPS) cells has changed the technological paradigm in stem cell biology. Nowadays, there is the hope that in a few years personalized treatments for degenerative diseases may rely on cellular therapies. The use of iPS cell technology will potentially allow increasing the probability of success of such therapies, as these cells can be generated from the patient himself reducing the risk of immune rejection. This work aimed to analyze important aspects in the establishment of protocols for human iPS cell culture, namely evaluation of different feeder-free, xeno-free culture systems by examining different culture media and cell adhesion matrices suitable for iPS cell maintenance. The experimental work was focused on the analysis of the influence of different culture conditions, either using feeder layers or feeder-free coating substrates, such as the composition of the respective media. Cell growth was assessed for every condition, and pluripotency maintenance verified through immunocytochemistry and flow cytometry analysis following staining with intracellular and surface markers. The cells were successfully adapted to feeder-free culture conditions using Matrigel™ coating and xeno-free medium composed of several growth factors (bFGF and TGFβ) and GSK3 inhibitor. In the end, the most promising system was shown to comprehend a Matrigel™ matrix with TeSR™2 medium, under 20% oxygen, which allowed a higher cell expansion in combination with the expression of the pluripotency extracellular markers TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4, as well as the intracellular marker Sox2. Under those culture conditions, more than 95% of Oct4 and Nanog positive cells were obtained within the population. Also, single-cell passaging using blebbistatin as a non-muscle myosin II inhibitor and a cell density of 50,000 cells/cm<sup>2</sup> seems to be the culture condition with the highest potential to scale up the expansion process. Overall, several culture protocols were applied with the final aim of achieving a serum-free, xeno-free system that is capable of expanding human iPS cell cultures while maintaining the pluripotent state. However, the therapeutic potential of human iPS cells still remains unfulfilled. In order to be able to generate specific cell types from human iPS cells, further optimization is needed, especially by maximizing the cell growth in controlled culture systems (e.g. bioreactors) at an early stage of the process.

# Functional Genomics and Systems and Synthetic Biology

*Thematic symposium 8*

**Keynote speakers:**

*Isabel Rocha*

*Miguel C Teixeira*

**Oral presentations:**

*Nuno P Mira*

*Rosa Grenha*

*Rita Branco*

*André da Costa*

**Keynote speaker / S8: 5**

## **New tools for the simulation and optimization of microbes in metabolic engineering problems**

**Isabel Rocha**

IBB – Centre of Biological Engineering, University of Minho, Braga, Portugal

Industrial Biotechnology is increasingly replacing chemical processes in numerous industrial sectors since it allows the use of renewable raw-materials and provides a more sustainable manufacturing base. The field of Metabolic Engineering (ME) has thus gained a major importance since it allows the design of improved microorganisms for industrial applications, starting with wild-type strains that usually have low production capabilities in terms of the target compounds. The ultimate aim of ME is to identify genetic manipulations *in silico* leading to improved microbial strains, that can be implemented using novel molecular biology techniques. This task, however, is a complex one, requiring the existence of reliable (genome-scale-) metabolic models for strain simulation and robust optimization algorithms for target identification. Strain simulation is usually performed by using Linear or Quadratic Programming methods that assume a steady state over the intracellular metabolites. However, there is no guarantee that the engineered cells actually function according to the optimal pathway predicted by these methods. In this scope, we have been working towards the use of a modification of the concept of Control Effective Fluxes to be able to find Metabolic Engineering solutions that couple growth with product formation while considering optimal, as well as sub-optimal routes and their efficiency. Regarding strain optimization, the most common task is to solve a bi-level optimization problem, where the strain that maximizes the production of a given compound is sought, while trying to keep the organism viable. Several different algorithms have been proposed to address this problem, namely mixed integer linear programming. More recently, we have proposed the use of stochastic meta-heuristics, such as Evolutionary Algorithms (EAs) and Simulated Annealing (SA). These approaches allow to solve the Metabolic Engineering problem in a considerable shorter time, originating a family of (sub)optimal solutions. Moreover, they are quite flexible regarding the use of non-linear objective functions. However, so far optimization approaches have been limited to the tasks of selecting the best set of genes to knockout from an organism. To extend the manipulation possibilities, we have been using both dynamic and steady-state models in modified formulations to account for gene over and under expression. In this way, it is possible to indicate the set of genes that should be modified, the type of modification that should be performed and the degree of over and underexpression. These algorithms have been validated with different case-studies, namely the production of lactic and succinic acid with *E. coli* and *S. cerevisiae* and some are already available in the open source and user friendly software tool Optflux.

1 - Burgard AP, et al., *Biotechnol Bioeng*, 84:647–657, 2003, 2 – Patil K, et al. *BMC Bioinformatics*, 6(308), 2005., 3 - Rocha M. et al. *BMC Bioinformatics* 9:499, 2008., 4 - Rocha I. et al. *BMC Systems Biology*, 4(45), 1-12, 2010.

**Keynote speaker / S8: 6**

## **Role and regulation of the drug:H<sup>+</sup> antiporter family: from *S. cerevisiae* to *C. glabrata***

**Miguel Teixeira**

IBB- Center for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal, Portugal

The widespread emergence of multidrug resistance (MDR) poses a severe problem, with repercussions in human health, biotechnology and in the environment. MDR often results from the action of drug efflux pumps from the ABC (ATP-Binding Cassette) and MFS (Major Facilitator Superfamily) superfamilies. However, the role of the putative drug:H<sup>+</sup> antiporters (DHA) from the MFS in eukaryotes has largely escaped characterization. Upon the release of the *S. cerevisiae* genome sequence, efforts made to characterize the role and regulation of DHA transporters have highlighted their importance in the context of multidrug resistance [1]. The extrapolation of this knowledge to the pathogenic yeast *Candida glabrata* is being undertaken and, so far, we have implicated three of these uncharacterized DHA transporters, CgQdr2, CgAqr1 and CgTpo2/3, in antifungal drug resistance. CgQdr2 and CgAqr1 are localized to the plasma membrane, similar to what was registered for their *S. cerevisiae* homologs. Their deletion increases *C. glabrata* tolerance towards azole drugs, but also, in the case of CgAqr1, to amphotericin B and to flucytosine. The participation of CgTpo2/3 in azole drug extrusion was demonstrated. The heterologous expression of these transporters in *S. cerevisiae* is being used to test their ability to complement the susceptibility exhibited in the absence of the corresponding *S. cerevisiae* homologs. Given the fact that acquired MDR can be seen as a genetic stabilization of the transcriptional up-regulation of MDR transporters observed under stress, the transcriptional control of DHA genes is also being analyzed. Particularly, a systems biology approach, combining mathematical modeling and experimental verification, was used to unveil the regulatory network controlling the *S. cerevisiae* FLR1 gene under stress induced by the fungicide mancozeb [2]. The devised network is now being extended to the *C. glabrata* homolog of this gene, which was also found to confer mancozeb resistance. Altogether, the obtained results are expected to improve current understanding of multidrug resistance in fungal pathogens and to guide the design of new tools for the diagnosis and treatment of multi-resistant *Candida* infections.

[1] Sá-Correia et al, 2009, *Trends Microbiol*, 17, 22-31; [2] Teixeira et al., 2010, *Mol Biosyst*, 6: 2471 – 2481.

S8: 1

## Haa1p-dependent regulatory network of the yeast response to acetic acid stress

**Nuno P Mira<sup>1</sup>, Silvia F Henriques<sup>1</sup>, Greg Keller<sup>2</sup>, Miguel C Teixeira<sup>1</sup>, Rute G Matos<sup>3</sup>, Cecília M Arraiano<sup>3</sup>, Dennis R Winge<sup>2</sup>, Isabel Sá-Correia<sup>1</sup>**

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The understanding of the molecular mechanisms underlying *Saccharomyces cerevisiae* adaptation and tolerance to acetic acid is required for the rational engineering of more robust industrial yeast strains for processes limited by this weak acid's toxicity, in particular wine making and bioethanol production from lignocellulosic hydrolysates [1,2]. A major acetic acid-resistance determinant, identified through the exploitation of a molecular systems biology approach, is the transcription factor (TF) Haa1 [3,4]. Haa1 was found to be a key player in the reprogramming of yeast genomic expression under acetic acid stress by regulating, directly or indirectly, the expression of approximately 80% of the acetic acid-responsive genes [4]. Mapping of the promoter region of one of the Haa1-activated genes, *TPO3*, allowed the identification of an acetic acid responsive element (ACRE) to which Haa1 binds in vivo [5]. A putative Haa1-Responsive Element (HRE), 5'-GNN(G/C)(A/C)(A/G)G(A/G/C)G-3' was hypothesized based on the *in silico* analysis of the promoter regions of the genes of the Haa1-regulon. Using surface plasmon resonance (SPR) and electrophoretic mobility shift assays, it was demonstrated that Haa1 interacts with high affinity with the HRE motif present in the ACRE region of *TPO3* promoter. Using site-directed mutagenesis and SPR, the minimal binding sequence for Haa1 was proposed, 5'-(G/C)(A/C)GG(G/C)G-3' [5]. This motif is present in approximately 55% of the genes transcriptionally activated by Haa1 in response to acetic acid stress [5]. The genes indirectly regulated by Haa1p are documented targets of Msn4p, Fkh2p or Mcm1p TFs, whose transcriptional regulation is Haa1p-dependent [4]. A putative transcriptional regulatory network controlled by Haa1 in response to acetic acid stress was proposed using the results of this research and the tools available in the YEASTRACT database [6].

[1] Teixeira M et al., *Curr Opin Biotechnol*, 22:1-7, 2010 [2] Mira NP et al., *OMICS*, 14:525-40, 2010 [3] Mira NP et al., *Microb Cell Fact*, 9:79, 2010 [4] Mira NP et al., *OMICS*, 14:587-601, 2010 [5] Mira NP et al., *Nucleic Acids Res*, 16:6896-907, 2011 [6] Teixeira et al., *Nucleic Acids Res*, D132-6, 2008



S8: 2

**Structural study of PlcR, a quorum sensing effector of *Bacillus cereus*****Rosa Grenha<sup>1</sup>, Sylvie Nessler<sup>1</sup>, Didier Lereclus<sup>2</sup>**<sup>1</sup>LEBS-CNRS, Gif-sur-Yvette, France; <sup>2</sup>GME-INRA, Guyancourt, France

Quorum sensing is a decision-making system in bacteria for processes such as sporulation, production of virulence factors, induction of competence, etc. Most are initiated when a threshold level of a regulatory protein is attained in the cell upon triggering by a cascade of partners activated by external signals. In most Gram-positive bacteria, the signal molecule consists of a secreted peptide acting indirectly via a two-components type system after re-importation. Sporulation initiation in *Bacillus anthracis* is one example of these systems [1]. In some cases however, the regulatory protein interacts directly with the signalling peptide [2,3]. These receptors have been classified in a single family called RNPP from the first identified members Rap, NprR, PlcR and PrgX [4]. PlcR, which stands for Phospholipase C Regulator, is a master regulator of most known virulence factors in *Bacillus cereus* [5] and the center of our study. It is an HTH-type transcription factor activated upon binding of its cognate signalling peptide PapR-7 [2,3]. Functional studies of the PlcR virulence regulon allowed the characterization of its target sequence [5,2], the palindromic "PlcR box". In order to fully understand the activation mechanism of PlcR, a closer look at the conformation changes induced upon binding of PapR-7 is essential. We will unveil our recently solved crystal structure of the ternary complex between the regulatory protein PlcR, its target DNA and the cognate peptide, PAPR-7 (ADLPFEF). Comparison with the structure of the PlcR:PapR-7 binary complex allows us to describe how the HTH-type DNA-binding domains of the PlcR dimer undergo a striking conformational change upon DNA binding. We also propose a mechanism explaining how peptide binding activates the transcriptional activity of PlcR.

(1). Grenha, R., et al. *JBC*, 2006. 281(49) (2) Slamti, L. & D. Lereclus *EMBO J*, 2002. 21 17 (3.) Bouillaud, L., et al. *Nucleic Acids Res*, 2008. 36,11, (4). Declerck, N., et al. *PNAS USA*, 2007. 104, 47 (5). Gohar, M., et al. *PLoS One*, 2008. 3, 7

S8: 3

### Whole-cell biosensors for the detection of chromate in environmental samples

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Contamination of soils and water supplies with chromium compounds is considered a serious environmental issue. Water soluble heavy metals components, including the chromate compounds are, nowadays, of particular concern for drinking water and agricultural water quality. Detection of the presence of low concentrations of chromate is mandatory to identify contaminated locations and to control the progress of remediation efforts. Although traditional methods, such as chemical procedures, can detect and quantify metal concentrations in environmental samples, they often use potent chemicals and are more laborious. Whole cell microbial biosensors offer excellent potentialities for assaying the bioavailable portion of pollutants in contaminated environmental samples, which currently cannot be easily attended. On the other hand, they allow sensitive determinations of the bioavailable contaminant without expensive equipment or specialized training. This work describes the application and evaluation of a microbial biosensor strain designed to detect the bioavailability of chromate in contaminated water samples. First, based on the green fluorescent protein (GFP) a bacterial biosensor for the detection of bioavailable chromate was constructed and characterized. The biosensor strain pCHRGFP1 *Escherichia coli* DH5a was developed based on the expression of *gfp* under the control of the *chr* promoter and the *chrB* gene of Tn*OtChr* determinant from *Ochrobactrum tritici* 5bv11. This reporter was specific and sensitive for very low concentrations of chromate (nanomolar and micromolar range) and also revealed no reaction with the other heavy metals and chemical compounds analysed. A strain of the bacterium *O. tritici* was also engineered to fluoresce in the presence of micromolar levels of chromate. Both chromate reporter strains were effective for discriminating contaminated from uncontaminated water samples collected in a Portuguese river. This work shows the first biosensor to be created for specific and selective detection of chromate and its applicability in environmental samples, even using cryo-preserved cells, which represents an easy and practical strategy. In contrast to other chromate detection methodologies, these reporter strains can provide on-demand usability in the field, and in a near future it might be a powerful tool in identification of chromate-contaminated sites.

S8: 4

### Solvent-cast films of an elastin-like polymer fused to an antimicrobial peptide, ABP-CM4, exhibits high antibacterial activity against *Pseudomonas aeruginosa*

**André da Costa, Raul Machado, Margarida Casal**

CBMA, Universidade do Minho, Portugal

The nosocomial infections grew significantly in the last years and became a worldwide problem. Antimicrobial peptides (AMPs) arise as a good treatment to these infections, since traditional antibiotics have become useless against resistant hospital strains. AMPs exhibit a broad range of antimicrobial activity but antitumoral and antiviral activities have also been found. AMPs are usually small, cationic molecules that occur as part of the innate defense mechanism in many organisms, even in microbes and virus. The combination of AMPs with recombinantly produced polymers, such as the Elastin-like Polymers (ELPs), inspired in the mammalian elastin, could improve medical equipment, such as catheters, to overcome infections and biofilms formation. In this work we describe the cloning and recombinant production in *Escherichia coli* BL21(DE3) of ABP-CM4, a cationic AMP from *Bombyx mori*, fused to an ELP, consisting of 200 repeats of the pentamer VPAVG (A200). This ELP exhibits thermoresponsive properties, exploitable as a purification method. The morphological characteristics as well as its antibacterial activity of this hybrid polymer were studied as essential for the applicability in medical devices. The ABP-CM4 gene was chemically synthesized, with the inclusion of a formic acid cleavage site, and fused in frame with the N-terminus of the gene coding A200. Production of the recombinant polymer in *E. coli* BL21(DE3) was achieved and purification was based on the use of the inverse transition cycling method. Formic acid treatment allowed tag removal and obtention of the soluble protein. The hybrid polymer, CM4::A200, and the cleaved ABP-CM4 were tested for its antimicrobial activity in liquid form. Solvent-cast films of CM4::A200, using formic acid as solvent, were tested for the antibacterial activity against *Pseudomonas aeruginosa* comparing with A200 polymers containing different contents of positively charged aminoacids. The hybrid polymer presented similar morphological and physicochemical features to A200. The cleaved recombinant ABP-CM4 and CM4::A200 showed low levels of inhibition against *P. aeruginosa* in the liquid form but, in the solvent-cast film form, the inhibition of growth was almost 100%. This result reveals very good perspectives for the use of these polymers in the medical equipment.

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# Cellular Microbiology and Pathogenesis

*Thematic symposium 9*

**Keynote speakers:**

*João Gonçalves*

*Kimberly Jefferson*

*Margarida Saraiva*

**Oral presentations:**

*Palmira Barreira-Silva*

*Christian G Ramos*

*Ana Almeida*

**Keynote speaker / S9: 4**

## **The interplay of restriction and helper factors during HIV-1 infection in T-lymphocytes.**

**João Gonçalves**

URIA/IMM, Lisboa, Portugal

HIV-1 is a sophisticated retrovirus that has adapted to utilize the host machinery for its own propagation. A dynamic interaction between the virus and host systems is observed at every step of the HIV-1 lifecycle. For successful infection HIV-1 must use cellular functions and proteins, which by being more stable and less prone to mutations than viral proteins could be promising alternative targets for antiviral therapy. Recently, several reports have identified potential host cellular cofactors for HIV-1 replication by silencing gene expression, transiently with siRNA or stably with shRNA, in genome screenings. However, the identities of these genes are highly divergent with less than 7% of overlap. This is mainly due to differences in experimental conditions such as cell type, time-points analyzed and filtering of thresholds used. Moreover by silencing cellular genes all these screens aimed to uncover new helper factors and not restriction factors. We previously identified new 14 different proteins essential to HIV-1 replication in T- lymphocytes that do not affect cell viability. This group of proteins includes kinases belonging to MAPK, JNK and ERK pathways, a phosphatase involved in pleiotropic cellular functions and proteins implicated in DNA repair. Besides helper factors, the identification of cellular restrictions factors is advantageous because it provides a direct pathway to discover therapeutic targets, and could be accomplished by systematic overexpression of every gene in an HIV-1 permissive human cell. We used libraries of engineered polydactyl zinc-finger transcription factors to approach this challenge. When fused to transcriptional activators these domains upregulate specific genes. Libraries of zinc-finger proteins have proven to be useful for the selection of endogenous gene regulators. When combined with an appropriate selection system, the use of libraries should result in identification of new genes that directly or indirectly regulates HIV-1 replication. One of the most important viral restriction factors is APOBEC3G which is counteracted by HIV-1 Vif protein. This factor inhibits HIV replication, by creating high levels of G to A mutations in the viral genome. Since its identification, the study of A3G and its mode of action against HIV as became a priority. Understanding A3G restriction and how the virus escapes it is of extreme importance for the development of drugs with the ability to block viral replication. In conclusion, the discovery of novel host proteins key to the viral lifecycle could result in the development of a new class of HIV-1 drugs since these proteins could serve as targets for the development of traditional small molecule drugs. It is anticipated that the results of this investigation will provide researchers with novel pathways to study the interplay between HIV-1 and the host.

**Keynote speaker / S9: 5**

## **Unraveling the etiologic mystery of bacterial vaginosis**

**Kimberly Jefferson**

VCU, Richmond, VA, United States of America

Bacterial vaginosis (BV) has remained an etiologic mystery for over half a century and the lack of knowledge about its cause and progression has interfered with efforts to improve therapeutic intervention. *Gardnerella vaginalis* was originally implicated as the causative agent of BV, but subsequent evidence cast doubt on this claim. BV involves the proliferation of a wide variety of anaerobic bacteria, the majority of which are neutralophiles and therefore fail to grow when a normal healthy flora composed of acid-producing lactobacilli is present. Recent studies suggest that rather than being the causative agent of the symptoms and complications of BV, *G. vaginalis* may instead serve a role as the precipitating factor of the disorder through the formation of a tenacious biofilm on the vaginal epithelium and possibly by increasing the pH. Understanding how *G. vaginalis* and other BV-associated species interact with each other and with the vaginal epithelium is essential to the prevention of BV and maintenance of vaginal health. Advances in the use of molecular approaches such as next-generation sequencing have enabled the development of a more comprehensive picture of all of the bacterial species, even uncultivated species, involved in BV. Through the Human Vaginal Microbiome Project, we have begun to identify bacterial species that exhibit a greater predictive value for BV and for specific symptoms of the disorder. Whole genome sequencing through the Microbiome Project has led to the prediction of growth requirements and virulence determinants of poorly characterized species. Together we hope that these advances will help to clarify the role of the many species involved in BV, and provide the knowledge to better maintain vaginal health.

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**Keynote speaker / S9: 6**

## **Recognition of *Mycobacterium tuberculosis* by macrophages and dendritic cells: implications for IL-10 gene regulation**

**Margarida Saraiva**

ICVS-3Bs Laboratório Associado, Braga, Portugal

*Mycobacterium tuberculosis* (Mtb), the causative agent of Tuberculosis (TB), establishes a chronic lung infection, with a balanced immune response that prevents both pathogen growth and tissue damage. One of the mechanisms limiting the effector activity of phagocytes and tissue damage is the expression of the anti-inflammatory cytokine IL-10. The role of IL-10 in TB pathogenesis remains controversial. However, there is compelling evidence that in humans, increased IL-10 expression during the chronic phase of TB has a limiting effect on the immune response. Thus, understanding the molecular mechanisms regulating IL-10 expression during infections by Mtb may reveal novel pathways that can be targeted to modulate the expression of this cytokine and improve the immune response. We are interested in understanding the molecular mechanisms of IL-10 gene regulation in macrophages and dendritic cells (DC) infected with live Mtb, as both cell types play critical roles in the establishment of an immune response to Mtb. Macrophages are within the first cells, in the lung, to sense, phagocyte and try to kill Mtb. DC also sense and phagocyte Mtb in the lung, but then migrate to the lymph nodes where they participate in the differentiation of effector T cell responses. Our data shows that the expression of IL-10 by macrophages and DC is differentially modulated, as a result of transcriptional and post-transcriptional mechanisms. We found that in macrophages, depending on the stimulation of specific Toll-like receptors (TLR), the expression of IL-10 can be post-transcriptionally controlled. Providing that TRIF signals are present, a prolonged activation of the MAPK p38 is observed, which allows for the stability of the IL-10 mRNA, thus promoting a higher secretion of active IL-10. This study reveals a novel intracellular pathway involved in the regulation of IL-10 secretion and suggests that differential recognition of pathogens by TLR has an impact on the amount of IL-10 produced by innate immune cells.

S9: 1

## Thymic atrophy upon infection with a highly virulent strain of *Mycobacterium avium*

**Palmira Barreira-Silva**<sup>1,2,3</sup>, **Margarida Borges**<sup>3</sup>, **Manuela Florido**<sup>3</sup>,  
**Margarida Correia-Neves**<sup>1,2</sup>, **Rui Appelberg**<sup>3</sup>

<sup>1</sup>Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; <sup>2</sup>ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal; <sup>3</sup>IBMC-Institute for Molecular and Cell Biology, University of Porto, Porto, Portugal

Premature thymic atrophy occurs as a consequence of several pathological situations like stressful conditions, treatment with dexamethasone or estrogen, exposure to ethanol or pesticides and systemic infections. Thymic atrophy has been shown also to occur in mice during infection by several pathogens. This accelerated infection-induced thymic atrophy has been associated with different mechanisms that are not mutually exclusive like increased apoptosis in the thymus, associated or not with increased production of glucocorticoid, and premature output of not fully differentiated T cells from the thymus. *Mycobacterium avium* strain ATCC 25291 SmT causes premature thymic atrophy upon systemic infection of C57BL/6 mice. A less virulent strain, the strain 2447 also infects the thymus but does not cause premature atrophy. Taking this into account and since understanding the mechanisms responsible for thymic atrophy is essential to be able to prevent it, as well as to find a way to recover normal thymic activity, we made use of these two *M. avium* strains to dissect the mechanism responsible for infection-induced thymic atrophy. We have previously shown that the systemic infection of C57BL/6 mice with the highly virulent strain of *M. avium* leads not just to thymic atrophy but also peripheral depletion of T and B cells. Peripheral lymphopenia has been shown to be dependent on the production of IFN- $\gamma$ . Our results show that the corticosterone serum levels are increased on mice infected with *M. avium* strain 25291 but not on the ones infected with strain 2447. However the glucocorticoid receptors are decreased and the expression of Cyp11b1 (a gene coding for an enzyme required for glucocorticoid production) is maintained in the thymus during infection by strain 25291. In other hand we have observed that the number and the percentage of thymocyte populations (DN, DP, CD4SP and CD8SP) are altered by the infection with the highly virulent strain but not with the less virulent strain. In addition our results show that the most immature thymocytes (DN subpopulations and early thymic precursors) are also altered what may indicate a role of bone marrow on premature thymic atrophy. As future work we aim to make use of both strains of *M. avium* in parallel to further explore the bone marrow precursor alterations and the apoptosis pathways within the thymus to understand the mechanism responsible for infection-induced thymic atrophy.



S9: 2

## Hfq-like RNA chaperones and sRNAs: A new layer of complexity in virulence regulation

**Christian G Ramos<sup>1</sup>, Paulo JP da Costa<sup>1</sup>, André M Grilo<sup>1</sup>, Jörg D Becker<sup>3</sup>, Leonilde M Moreira<sup>1,2</sup>, Jorge H Leitão<sup>1,2</sup>**

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Bacterial virulence in most pathogens is a multifactorial phenomenon, requiring a wide array of distinct effectors [1]. Over the last few years, bacterial virulence has been increasingly shown to be regulated at the post-transcriptional level by small non-coding RNAs (sRNAs), mediated by Hfq-like RNA chaperones [2]. *Burkholderia cepacia* complex (Bcc) bacteria are opportunistic multidrug resistant human pathogens, which present an unusually large genome (~7000 genes), as well as a surprisingly high number of predicted sRNAs [1]. sRNAs generally require the action of the ubiquitous RNA chaperone Hfq. In the case of Bcc bacteria, we have recently shown that two distinct and differentially expressed RNA chaperones, Hfq and Hfq2, are required for full virulence [3]. Transcriptional profiling of *B. cenocepacia* J2315 and its derivatives *hfq* and *hfq2* mutant strains, showed that the deletion of *hfq* affects ~800 genes, while a deletion of the *hfq2* gene affected the expression of approximately 3000 genes. Interestingly, the group of genes that is most affected by mutations in RNA chaperones are key regulators of gene expression, such as several sigma factors and quorum-sensing regulators. In addition, the expression of a relevant number of virulence-associated genes is altered. We have recently found that *hfq* is post-transcriptionally regulated by an Hfq2-dependent sRNA, while *hfq2* is regulated by a cis-encoded sRNA, whose stability depends on the presence of Hfq. This complex regulatory circuit, mediated by Hfq and Hfq2, will be presented and the implications of these findings on virulence will be discussed.

[1] Leitão et al. (2010). Appl Microbiol Biotechnol **87**, 31-40. [2] Ramos et al. (2010) *Small non-coding RNAs in Prokaryotes: Roles in Pathogenesis and Potential Therapeutic Targets* Saarbrücken, Germany, Lambert Academic Publishing, pp. 75. [3] Ramos et al (2011). J Bacteriol **193**, 1515-1526. Funding is acknowledged to FCT (contract PTDC/EBB-BIO/098352/2008 and a Doctoral grant to AMG), and a Doctoral grant to CGR from Fundação Calouste Gulbenkian.

S9: 3

## **A murine model of human rhinoscleroma identifies specific Mikulicz cells as atypical inflammatory monocytes**

**Ana Almeida<sup>1,2</sup>, Philippe Sansonetti<sup>1,2</sup>, Régis Tournebise<sup>1,2</sup>**

<sup>1</sup>Pathogénie Microbienne Moléculaire, Institut Pasteur, France; <sup>2</sup>INSERM U786, Paris, France

Rhinoscleroma is a human specific chronic disease caused by the bacterium *Klebsiella pneumoniae* subsp. *rhinoscleromatis* (KR) and characterised by the formation of granuloma in the upper airways. A hallmark of this pathology is the formation of atypical large macrophages called Mikulicz cells (MCs), into which KR is able to survive. Very little is known about the molecular and cellular mechanisms underlying this disease. The precise description of MCs is not known, nor how they are recruited and matured. A major goal of our work is to understand why KR causes a chronic infection while *Klebsiella pneumoniae*, a genetically very closely related species causes acute infections. In order to characterise the interaction between the bacteria and the host, our lab developed a new mouse model recapitulating the appearance of MCs. We observed that these cells are atypical inflammatory monocytes recruited to the lungs upon infection in a CCR2-independent manner. Interestingly, the inflammatory reaction caused by KR is characterised by a strong production of interleukin-10 (IL-10) concomitant to the appearance of MC. As this important anti-inflammatory cytokine is known to inhibit both radical oxygen species and nitric oxide production in macrophages, we propose that IL-10 plays an important role in the establishment of a proper environment for the formation of MCs and KR survival. To address the role of some bacterial virulence factors, we are developing a capsule mutant of KR in order to investigate the role of this polysaccharide structure on the bacterium phagocytosis and its implication in MCs maturation and physiology. We are now focusing on the cellular characterisation of MC, trying to understand how inflammatory monocytes can phagocytose KR and the reason why they cannot eliminate the bacteria. This new animal model of a neglected infectious disease will allow us to further decipher the molecular and cellular mechanisms used by KR to cause rhinoscleroma and how a bacterium can switch from causing an acute infection to a chronic one.

# Nanotechnology

*Thematic symposium 10*

**Keynote speakers:**

*Pedro Fernandes  
Sara Beirão da Costa*

**Oral presentations:**

*Romeu M Francisco  
Sofia MA Martins  
Orfeu Flores  
Ana IF Martins*

**Keynote speaker / S10: 5**

## **Miniaturization in bioprocesses: a resilient approach or just another fad?**

**Pedro Fernandes**

Instituto Superior Tecnico, Portugal

The development of biotechnological processes involves a wide array of variables and various stages, which harmoniously combined result in an optimized process, displaying high yield and high productivity. Miniaturization bestows a tool to increase the pace of bioprocess development significant and reliably. Thus, this approach allows high throughput, with minimal requirements of chemicals and biologicals; is prone to automation and to provide high data density; and suitable methodologies have been developed to enable reproducibility of data throughout scales, an issue that is being improved. Besides, the use of miniaturized devices led to a paradigm shift in production strategies, by introducing the concept of scaling-out, in opposition to the standard scale-up approach, thus ruling out the need for complex engineering work for effective scale-up, which despite extensive efforts, not always proves totally effective. Furthermore, continuous operation is clearly favored under miniaturized environment. The last decade has brought along representative examples of the viability and relevance of the use of miniaturized devices, and guidelines for their application are being defined. At the same time, some pitfalls and limitations have been identified. The current presentation aims therefore to provide an updated overview of the role of miniaturized devices in bioprocess development. Within such scope, the different designs, configurations and modes of operation of miniaturized devices, with particular focus on microreactors (viz. microstructured and microbioreactors) will be addressed, but reference to microfluidic devices in downstream processing will also be included. The feasibility of the use of the diverse configuration of miniaturized devices in either process development or production scale will be described, and current challenges to more effective devices (viz. integration with analytics or reversible biocatalyst immobilization) will be discussed. All of these will be illustrated with relevant examples. Further perspective developments within the overall concept addressed in the presentation will be considered. It is therefore expected that the presentation will deliver a sound and realistic perspective on the role of miniaturized devices in bioprocess development and intensification, where initial promises, current situation and foreseeable evolution will be clearly established.

Keynote speaker / S10: 6

## Micro- and nanotechnology in the food field

### Sara Beirão da Costa

CEER – Biosystems Engineering. ISA. Technical University of Lisbon, Portugal

Nanotechnology, the ability to work at molecular and (sub)atomic levels to produce and use structures, tools and systems at the nanometer scale, finds a broad field of applications, from electronics to medicine, from biomaterials to energy. The new about *nano* is that size matters! In fact, structures at sizes lower than 100 nm show different physical, biological and chemical properties. As generally happens, nature came first! Many are the nanostructures naturally occurring in nature and, consequently, on food. Likewise, traditional food processing practices also induce the production of micro/nano structures imparting determinant properties to food products. Those structures, produced from different components, like proteins, starch or fats, may be present as *is* in the final product or as *intermediates* to further developed final components. In the food industry, many are the potential uses: development of new products, improving the shelf life, improving food safety by microbial development control or imparting similarity to textural and sensorial properties of lipid rich products. Micro/nano encapsulation finds in food field one of the largest areas of application. This technology enables the entrapment of materials (core) into another material(s) system (wall) aiming the protection of sensitive ingredients. In novel food, micro/nano encapsulation allows also naturally food ingredients to be adapted for better taste and digestion. Several processes are available for micro/nano encapsulation conducting to wall materials with different morphological and physical properties. These properties will have an active role on *how*, *when*, *where* and *at what rate* the core material will be delivered. Micro/nano technology may also be an important tool for developing packaging films with different layers and functionalities. Also, separation membranes with micro/nanosized pores enabling fractionation of solutes at molecular level are being applied in the food industry. So, applications of novel micro-nanotechnologies to food are likely to bring large benefits to food processes and regarding health effects to consumers. This impact will be high, for instance, in the development of novel microprocesses, the creation of new textures and tastes or in the design of less calorie-dense foods, with increased nutritional value. However we need to increase our understanding of unintended consequences, as well as the answers to many questions that still remain such as: how created food structures are formed and broken down, digested and absorbed? How can they migrate from packaging materials into foods? May nanoparticles pass the blood-brain barrier and is this passage harmful? Likewise, much of the regulatory aspects still remain in a dark box. Nevertheless we should be optimistic since a lot of them were already found, anticipating promising developments and applications for the food industry.

S10: 1

## Chromium remediation by extracellular phosphate nanoparticles induced by iron in *Ochrobactrum tritici* 5bv11

**Romeu M. Francisco<sup>1</sup>, Pedro M. de Abreu<sup>1</sup>, Bradley A. Plantz<sup>4</sup>, Vicki L. Schlegel<sup>3</sup>, Rui A. Carvalho<sup>2</sup>, Paula V. Morais<sup>1,2</sup>**

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Cr(VI) is a carcinogenic environmental contaminant which remediation can be achieved by using microorganisms to reduce Cr(VI) to the less toxic and less soluble Cr(III). Several Cr(VI)-resistant bacteria species have been isolated in recent years. Cr(VI)-resistance is a consequence of the summed effects of various strategies, such as: Cr(VI) reduction; protein, lipid and DNA repair; free-radical scavenging enzymes and the efflux of Cr(VI) from the cytoplasm. Cr(VI) efflux is performed by the membrane transporter ChrA, coded by the *chrA* gene, present in *Pseudomonas aeruginosa* (pUM505), in *Cupriavidus metallidurans* (pMOL28) and in *Ochrobactrum tritici* 5bv11 (TnOtChr). Among the most resistant bacteria known are the strains *Brevibacterium* sp. CrT-13, *O. intermedium* SDCr-5 and *O. tritici* 5bv11. Metal sequestration and precipitation occurs in several bacteria, and is important in uranium or chromium bioremediation strategy, sometimes occurring as extracellular phosphate complexes. Recently, after Cr(VI) reduction by bacterial consortia, Cr(III) was found coordinated octahedrally to phosphate groups. In 2005, polyphosphate was found in abundance in marine sediments, and suggested to originate from benthic microorganisms in response to redox potential changes, which affect metal solubility and oxidation state. There is however a lack of evidences linking polyphosphates to the metal-phosphate extracellular aggregates produced by bacteria. Due to its Cr(VI) resistance, *O. tritici* strain 5bv11 was used in this work as a model. Under Cr(VI) stress, siderophore production by this strain was enhanced. The presence of Fe(III) decreased Cr(VI) toxicity, increasing cell growth and Cr(VI)-reduction rates in cultures, and decreasing the amount of damaged cells. Moreover, the formation of insoluble phosphate nanoparticles responsible for chromium removal from solution was observed by SEM-EDS and found to depend of both Cr(VI) and Fe(III). In presence of both metals, nanoparticle formation occurred together with enhanced inorganic phosphate uptake by cells and increased polyphosphate kinase activity. <sup>31</sup>P-NMR analysis of the particles detected polyphosphate and phosphonate together with orthophosphate. FT-IR supported these results and detected coordinated Cr(III). This work demonstrated that *O. tritici* 5bv11 possesses mechanisms against Cr(VI) toxicity that may open new perspectives for remediation of metal contaminated environments.

S10: 2

**Microfluidic cell chips: monitoring GPCR activation****Sofia de Medina Aires Martins<sup>1,2,3</sup>, João Trabuço<sup>1,2</sup>, Gabriel A Monteiro<sup>1,2</sup>, João P Conde<sup>1,3</sup>, Virginia Chu<sup>3</sup>, Miguel DMF Prazeres<sup>1,2</sup>**

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Background: G-protein coupled receptors (GPCRs) constitute a class of cell receptors that play a major role in pharmaceutical research and approximately 30% of the approved drugs in the market selectively target members of this family. Although several formats for the study of GPCRs are currently available, new approaches for the identification of novel modulators continue to be developed. Indeed, current financial pressures on the pharmaceutical industry demand for the development of high-throughput technologies for the identification of new relevant targets, in a faster and cost-effective manner. This paradigm represents the perfect opportunity for the development of microfluidic-based systems that hold the potential for minimum reagent's consumption through miniaturization and increased multiplex capability. Objectives: The goal of this work is thus to develop a cell-based microfluidic device capable of supporting cell signaling through GPCR activation, aiming the identification of potential drug candidates. Methods: The Gq signaling pathway, leading to intracellular calcium release, was the chosen GPCR activation model. Polydimethylsiloxane (PDMS) microfluidic structures were fabricated using soft lithography techniques and computational fluid dynamics (CFD) was used to optimize the structures' geometries. HEK 293 cells were loaded into the devices and stained with the calcium sensitive Fluo4 dye. Endogenous Muscarinic M1 GPCR was stimulated by the addition of the target carbamoylcholine. Results: Two potential microstructures were identified: Rectangular microchannels (500  $\mu\text{m}$  width x 60  $\mu\text{m}$  height) and a hydrodynamic chamber (1 mm diameter x 100  $\mu\text{m}$  height), providing respectively 600 and 78 nL of culture volume, demonstrated to be compatible with cell viability and adhesion. The use of CFD allowed the improvement of operational conditions regarding flow velocities and shear stresses. An optimized adhesion protocol was established comprising the surface coating with fibronectin (100  $\mu\text{g}/\text{mL}$ ) and 1 h adhesion time. Endogenous activation of M1 GPCR was confirmed using fluorescence microscopy and by following in real-time the rise in cell's fluorescence upon addition of the target carbamoylcholine (0-1.5 mM). In this way, calcium dynamic curves could be extracted, hence confirming that the technology presents the potential to be applied in the monitoring of GPCR signaling processes.

S10: 3

**Nanoparticles for theragnostics of Alzheimer disease.****Orfeu Flores**

STAB VIDA, Portugal

Alzheimer's disease (AD) is the most common neurodegenerative disease among the elderly population. Substantial genetic and biochemical evidence points to amyloid- $\beta$  ( $A\beta$ ) as the trigger of the disease. Recent evidence highlighted the toxicity associated to soluble  $A\beta$  oligomers formed on the pathway to the mature amyloid fibrils.  $A\beta$  aggregates are therefore considered as possible targets for diagnosis and therapy of AD, which still represents unmet needs. STAB VIDA is a biotech start-up performing research in consortium with academic partners for AD theragnostics. NAD is an EC-funded project (7<sup>th</sup>FP) aiming at the development of nanoparticles (NPs) specifically engineered for targeting  $A\beta$ , for both diagnostic and therapeutic purposes. NPs are suitable vehicles for imaging probes and therapeutic agents and also offer the possibility to be functionalized on their surfaces with target-specific ligands. Moreover, the presence of multivalent interactions may strikingly increase their affinity for the targets. Possible  $A\beta$  ligands being developed by STAB VIDA within the NAD project is represented by monoclonal anti- $A\beta$  antibodies ( $A\beta$ -MAbs). Here we report the development of a novel  $A\beta$ -MAb, as well as the preparation and the *in vitro* characterization of liposomes decorated with it. In particular, we analyzed their physicochemical features and their  $A\beta$ -binding properties; either *in vitro* or *in post-mortem* brain samples from AD patients. Results will be presented and discussed.



S10: 4

## On the development of a plasmid DNA probe for interaction force measurements and characterization of membrane adsorbers by AFM

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The atomic force microscope (AFM) is a powerful tool for chemical and bioengineering process improvement. This technology has been an invaluable means of obtaining three-dimensional topography images of biological specimens, with resolution at, or near the atomic level, and of quantifying the interaction forces between colloidal probes and planar surfaces of materials, in liquid or gaseous environment, in the order of  $10^{-12}$  N. For this particular application, specific colloidal-coated probes must be developed. This approach played a key role in the development of fouling-resistant membranes for ultra- and nanofiltration processes. This work describes the use of AFM for the characterization of chromatographic membrane adsorbers, with special focus on the measurement of interaction forces between chromatographic ligands at the surface of membrane adsorbers and plasmid DNA (pDNA) molecules, as a function of the distance between the tip of an AFM cantilever and the membrane surface. *Escherichia coli* DH5 $\alpha$  hosting the pVAX1-lacZ was cultured in LB medium in an orbital incubator (250 rpm; 37°C). Afterwards, cells were harvested, washed, disrupted by alkaline lysis and the plasmid purified by isopropanol precipitation, HIC and SEC sequential steps. For pDNA probe assembling, amine pre-functionalized monodispersed silica microspheres were covalently immobilized with pure pDNA (up to 35 pDNA molecules per  $\mu\text{m}^2$  of probe surface), through NHS-psoralen that inserts in DNA double strands after UV irradiation ( $\lambda=365$  nm). Thereafter, a single pDNA-immobilized silica microsphere was fixed on a tipless AFM cantilever and the construct (probe) used for the interaction studies. The chromatographic membrane adsorbers selected for characterization were of HIC type, one with alkyl-like ligands, previously prepared in our laboratory, and another with phenyl ligands, kindly provided by Sartorius Stedim Biotec GmbH. The interaction forces were analysed in dry state and moistened with buffer Tris-HCl pH 8. AFM topographic studies allowed developing the pDNA immobilization procedures, as it permitted nanoscale scanning images of the probes surface and pDNA structure assessment. The force measurements helped interpretation of chromatographic behaviours of membrane adsorbers and will foster membrane adsorber design.

# European Consortium of Microbial Resources Centres

*EMbaRC symposium*

**Keynote speaker:**  
*Paul de Vos*

EMbaRC: 1

**European Consortium of Microbial Resources Centres (EMbaRC): Secure the future of microbial resources at laboratory scale**

**Sylvie Lortal<sup>1</sup>, Chantal Bizet<sup>1</sup>, Christiane Bouchier<sup>1</sup>, Erko Stackebrandt<sup>1</sup>, David Smith<sup>1</sup>, David R. Arahall<sup>1</sup>, Philippe Desmeth<sup>1</sup>, Nelson Lima<sup>1,2</sup>, Gerard Verkleij<sup>1</sup>, Joost Stalpers<sup>1</sup>, Paul De Vos<sup>1</sup>, Stéphane Declerck<sup>1</sup>**

<sup>1</sup>EMbaRC Consortium; <sup>2</sup>IBB/Centre of Biological Engineering, Braga, Portugal

EMbaRC is an EU project which aims to improve, coordinate and validate microbial resource centre (MRC) delivery to the European and International researchers. To ensure harmonisation of the quality of MRCs, EMbaRC plans to take the current OECD best practice guidelines and emerging national standards for Biological Resource Centres (BRCs) to the international level. Outreach and training activities will ensure that not only the consortium but that all European collections operate to the standards required to deliver products and services of consistent quality thus meeting customer needs. A one-stop access to the collections of EMbaRC and the wider European BRC community via a searchable web portal building on the outcomes of the CABRI and EBRCN is being developed. Access and high-quality support and training to research teams is provided via calls for access and selected participants can spend time at partner institutions gaining access to resources, technologies and expertise. This project is a mixture of networking, training and research. Research output will deliver new methods for strain and DNA preservation, novel techniques for identifying species. The networking elements will give better access to authentic microorganisms and validated associated data and provide a set of business models for the self-sustainability of BRCs. This project creates the European platform of the OECD envisaged Global Biological Resource Centre Network. Amongst its activities the consortium is developing strategies to protect investment in research by ensuring preservation of key biological material holdings on research laboratories. Specific activities of the project have included improving protocols for the authentication and preservation of cultures and the provision of an EMbaRC quality manual to enable collection operators to identify and move towards the implementation of best practices. Working with scientists and journal editors the improvement of access to strains cited in publications is being addressed. Within the area of compliance with regulatory requirements, emphasis is laid on the development of a Biosecurity Code of Conduct. These and many other areas impacting on the handling, storage and distribution of microbial strains have been discussed, studied and mechanisms elucidated.

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# Industrial and Food Microbiology and Biotechnology

*Poster session thematic symposium 1*

*Guimarães | Conde | Todorov | Sousa |  
Collins | Pedro | Cobas | Duarte | Mendes | Smenia Jr |  
Pintado | Nóbrega | Martins | Mussato | Moreira |  
Padrão | Garcia-Fraga | Lopes | Aguilár | Vasconcelos  
Mendes | Cerqueira | Santos | Carneiro | Arantes |  
Dragone | López-Seijas | Nunes | Genisheva | Cruz |  
Reto | Silva | Dinis | Araújo | Madureira | Cruz | Aires |  
Rocha | Inês | Soares | Aranda | Manga | da Silva |  
Magalhães | Pereira | Rustiguel | da Silva | Campos-  
Takaki | Pires | Braga | Martins | Batista | Dinis |  
Moreira | Santos | Lemos | Saraiva | Vilela-Moura |  
Lopes | Franco | Estevinho | Santos | Araujo |  
Fernandes | Martins | Rêgo | Rigueiro | Souza | Couto*

PS1: 1

### Laccases from *Streptomyces coelicolor*

**Rodrigo Soares Guimarães<sup>1,2</sup>, Andrea Zille<sup>1</sup>, Pedro Moradas-Ferreira<sup>1,3</sup>, Marta Vaz Mendes<sup>1</sup>**

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The search for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace the conventional non-biological methods. Among the different existing oxidant enzymes, laccases (EC 1.10.3.2) have been the subject of intensive research in the last decades due to their low substrate specificity. Laccases are oxidoreductases that catalyse the oxidation of a variety of organic and inorganic compounds while concomitantly reduce molecular oxygen to water making them one of the most effective enzymes to be used for biotechnological applications. To date, laccases for biotechnological applications have mostly been isolated from fungi, but several studies have shown that bacterial laccases show some functional advantages when compared with fungal enzymes, such as a wide range of optimum pH, temperature and substrates specificity. Several studies have shown the ability of some Actinobacteria, in particular some *Streptomyces* strains, to produce laccases. This potential has been reinforced by the analysis of the complete genomes of several *Streptomyces* strains that present several putative multicopper oxidases encoding genes. In this work *Streptomyces coelicolor* A3(2) was chosen as model organism due to the fully sequenced and annotated genome. The *loci* annotated as encoding putative multi-copper oxidases (*SCO2081*, *SCO3439*, *SCO3440* and *SCO6712*) have been cloned in an expression vector and heterologously expressed in *E. coli*. Once purified, the proteins were biochemically characterized using the previously characterized laccase from *S. coelicolor* (SLAC; *SCO6712*) as control. The ultimate goal of the work is to assemble a multi-copper oxidase protein collection that will be used in the future as a working-tool to generate tailor-made bacterial laccases with new and improved catalytic properties, meant for biotechnological applications.

PS1: 2

### Mannitol transport and oxidation are synchronized in *Olea europaea* under salt and drought stresses

**Artur Conde<sup>1,2</sup>, Paulo Silva<sup>1,2</sup>, Alice Agasse<sup>2</sup>, Carlos Conde<sup>3</sup>, Hernâni Gerós<sup>1,2</sup>**

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The intracellular accumulation of organic compatible solutes acting as osmoprotectants, such as polyols, is an essential response mechanism of several plants to drought and salinity. In *Olea europaea* a mannitol transport system (OeMaT1) has been previously characterised as a key player in plant response to salinity, and here, heterotrophic sink models, such as olive suspension-cultured cells and fruit tissues, and source leaves were used for analytical, biochemical and molecular studies focused on mannitol metabolism to better understand its involvement in drought and salt stress tolerance. The kinetic parameters of mannitol dehydrogenase (MTD) determined in mannitol-growing cells, at 25 °C and pH 9.0, were as follows:  $K_m$ , 54.5 mM mannitol and  $V_{max}$ , 0.47  $\mu\text{mol h}^{-1} \text{mg}^{-1}$  protein. The corresponding cDNA was cloned and subsequently named *OeMTD1*. *OeMTD1* expression was correlated with MTD activity, *OeMaT1* expression and carrier-mediated mannitol transport, in both mannitol- and sucrose-growing cells. Moreover, sucrose-growing cells showed only residual OeMTD activity, even though high levels of *OeMTD1* transcription were observed. The results support that OeMTD is regulated at both transcriptional and post-transcriptional levels due to substrate influence. Remarkably, MTD activity and *OeMTD1* expression were dramatically repressed after  $\text{Na}^+$ ,  $\text{K}^+$  and PEG treatments, both in mannitol- and sucrose-growing cells. In contrast, salt and drought significantly increased mannitol transport activity and *OeMaT1* expression, thus allowing for the intracellular accumulation of this polyol. Altogether, the results strongly suggest that olive tree copes with drought and salinity by tightly coordinating mannitol transport with intracellular metabolism. Taking into account the protective role of mannitol in olive tree, it would be extremely interesting to investigate if a criterious exogenous addition of this polyol during usual agricultural practices could be beneficial for olive tree development and productivity, and consequently for the whole olive-based food industry.

Conde et al (2011) Plant Cell Physiol. doi: 10.1093/pcp/pcr121 (in press). This work was supported by the Fundação para a Ciência e Tecnologia (FCT) (research project ref. PTDC/AGR-ALI/100636/2008 and A. Conde grant ref. SFRH/BD/47699/2008)

PS1: 3

### Bacteriocin production by *Pediococcus pentosaceus* ST3HA isolated from commercial smoked salmon

**Svetoslav Todorov**

Universidade de São Paulo, Brazil

Bacteriocins of lactic acid bacteria (LAB) are ribosomally synthesized antimicrobial peptides. Their bactericidal mechanisms may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis. This research is on isolation of the anti-*Listeria* bacteriocin producing strain; characterization of the genetic determinates for bacteriocin production and studies some aspects of the bacteriocin mode of action. Strain ST3HA, isolated from commercially available smoked salmon was identified as *Pediococcus pentosaceus* based on biochemical tests, sugar fermentation reactions (API 50CHL), PCR with species-specific primers and 16S rDNA sequencing. Strain ST3HA produces a 4.5kDa class IIa bacteriocin, active against lactic acid bacteria, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*. The peptide is inactivated by proteolytic enzymes, but not when treated with  $\alpha$ -amylase, Triton X-100, Triton X-114, SDS, Tween 20, Tween 80, urea and EDTA. No change in activity was recorded after 2h at pH values between 2.0 and 12.0, and after treatment at 100°C for 120 min or 121°C for 20 min. The mode of action against *Listeria ivanovii* subsp. *ivanovii* ATCC19119 is bactericidal, resulting in cell lyses and enzyme- and DNA-leakage. The highest level of activity ( $1.6 \times 10^8$  AU/ml) was recorded when cells were grown at 37°C or 30°C in MRS broth (pH 6.5). Peptide ST3HA adsorbs at high levels (6400 AU/ml) to producer cells. Based on PCR analysis, strain ST3HA harbour a 1044 bp fragment corresponding in size to that recorded for pediocin PA-1. Sequencing of the fragment revealed a gene identical to *pedB*, reported for pediocin PA-1. The combined application of the low levels (below MIC) of ciprofloxacin and bacteriocin ST3HA results in the synergetic effect in the inhibition of *L. ivanovii* subsp. *ivanovii* ATCC19119. *Pediococcus pentosaceus* ST3HA is a potential producer of the chromosomally associated pediocin PA-1-like bacteriocin based on the high similarity of the sequence *pedB*. Bacteriocin ST3HA is highly active against *L. ivanovii* subsp. *ivanovii* ATCC19119 and exhibit a synergetic effect in the inhibition of this test-microorganism when applied with the sublethal doses of ciprofloxacin.

PS1: 5

## Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced cell death in *Saccharomyces cerevisiae*

**Marlene Rodrigues Sousa<sup>1</sup>, Susana Chaves<sup>1</sup>, Cecília Leão<sup>2,3</sup>, Manuela Côrte-Real<sup>1</sup>, Maria João Sousa<sup>1</sup>**

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Acetic acid is a weak organic acid that is a normal by-product of the alcoholic fermentation carried out by *Saccharomyces cerevisiae*. It is a common toxic agent present in different biotechnological processes, such as in wine or bioethanol production, and is frequently associated with impairment of fermentation<sup>1,2</sup>. On the other hand, its toxic effects have prompted its use as a food preservative, but the resistance of some spoilage yeast to this acid limits its action, causing great economic losses<sup>3</sup>. The more recent finding that acetic acid triggers a programmed cell death (PCD) process provides a new basis for future breeding strategies of industrial strains with improved cell survival, and may also give clues for more efficient food preservation strategies. Therefore, we aimed to identify genes involved in the execution and regulation of acetic acid-induced PCD through a genome-wide analysis. To this end, we optimized and carried out a functional analysis of the Euroscarf knockout mutant collection. For the phenotypic screening, deletion strains representing haploid (viable) single deletions of approximately 4800 yeast ORFs were grown in YPDA media for 48 hours. Cells were then replicated into 96-well plates with YPD medium and grown for an additional 24 hours at 30°C, after which they were inoculated in YPD medium (pH 3.0) with 400 mM acetic acid. After 100, 300 and 330 minutes, cells were replicated into 96-well plates containing YPD medium without acetic acid, and optical density (640 nm) was read to assess growth after two days of incubation at 30°C. This screening uncovered a set of genes involved in resistant and sensitive phenotypes that were clustered according to biological function and known physical and genetic interactions. The results obtained contribute to further characterize acetic acid-induced PCD, and provide information on new putative targets for its control.

(1.) Vilela-Moura et al (2011) Appl Microbiol Biotechnol 89, 271–280. (2.) Palmqvist et al (2000) Bioresour Technol 74, 17-24. (3.) Mollapour et al, (2008) Yeast 25, 169-77. This work was financed by FEDER through POFC-COMPETE and national funds from FCT PEst-C/BIA/UI4050/2011 and PTDC/AGR-ALI/102608/2008.



PS1: 6

**NMR solution structure of a cold-adapted thiol-disulfide oxidoreductase.****Tony Collins<sup>1</sup>, Manolis Matzapetakis<sup>2</sup>, Tiago Pais<sup>2</sup>, Pedro Lamosa<sup>3</sup>, Helena Santos<sup>2</sup>**<sup>1</sup>Centre of Molecular and Environmental Biology (CBMA), University of Minho, Braga, Portugal;<sup>2</sup>Instituto de Tecnologia Química e Biológica (ITQB), Univ. Nova de Lisboa, Oeiras, Portugal;<sup>3</sup>Centro de Ressonância Magnética António Xavier (CERMAX), ITQB, Univ. Nova de Lisboa, Oeiras, Portugal

Psychrophilic enzymes produced by cold-adapted micro-organisms have successfully overcome the low temperature challenge and adapted to maintain high catalytic rates in their permanently cold environments. The current consensus is that this high activity at low temperatures is mainly achieved through an increase in the flexibility of the protein structure, thereby allowing for the molecular motions necessary for activity in their low thermal energy environment. The actual molecular basis for the adaptation is still however only poorly understood and direct evidence of the proposed increased flexibility is scant, with previous attempts to demonstrate this leading to conflicting results. In an attempt to better understand strategies of cold adaptation we have determined the NMR solution structure of the reduced form of a cold adapted thiol disulphide oxidoreductase (DsbA) isolated from an Antarctic bacterium. While a number of crystal structures for cold adapted enzymes have been published, this is the first report of an NMR structure of these enzymes and thereby opens up a new dimension in the study of cold adaptation. In particular, the potential power of NMR to monitor both local and global motions over a large range of time scales should allow for a better understanding of the role of dynamics in protein adaptation to temperature. The gene encoding the cold-adapted enzyme has been isolated and the protein overexpressed in *E. coli* with both unlabelled and labelled (<sup>15</sup>N<sup>13</sup>C, <sup>15</sup>N) protein being purified from the periplasmic extracts. NMR data were acquired on a Bruker Avancell+ 800 MHz spectrometer and the solution structure of the reduced form of this cold adapted oxidoreductase determined and compared to that of its mesophilic homolog from *Vibrio cholerae*. In addition, the temperature dependence of activity and stability of both the psychrophile and mesophile have been ascertained and compared. Here, the results of the NMR structure determination and the comparative structural and physicochemical studies of the cold adapted DsbA with its mesophilic homolog will be presented.

PS1: 7

### **Occurrence and survival/growth of *Listeria monocytogenes* in live bivalves stored at refrigeration temperatures**

**Ana Sofia Pinto<sup>2</sup>, Joana Torres<sup>2</sup>, Sónia Pedro<sup>1</sup>**

<sup>1</sup>INRB, I.P./L-IPIMAR, Portugal; <sup>2</sup>FCUL, Portugal

The occurrence of *Listeria monocytogenes* was studied in live bivalve samples placed in the Portuguese market. None was positive for *L. monocytogenes* but 3 % were contaminated with this genus. *L. monocytogenes* was able to survive in spiked live bivalves stored under two refrigeration temperatures, but no growth was observed. Nevertheless, levels of 100 ufc *L. monocytogenes* /g were maintained in this food item until the end of its shelf-life. So, *Listeria monocytogenes* can survive in live bivalves and pose a health risk for susceptible consumers.

PS1: 8

### **Synthesis, water solubility, antioxidant activity and antimicrobial capacity of Schiff base chitosan derivatives**

**Alejandra Cardelle-Cobas, Alexandra Macedo, Márcia A. M. Correia, Maria Manuela Estevez Pintado**

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Several strategies have been applied in order to expand the range of solubility and functional properties of chitosan and consequently applicability. One of the most used is the introduction of hydrophilic residues in the chitosan molecule, through the formation of covalent bonds with the reactive amino groups. Schiff bases of low molecular weight chitosan (LMWC) were synthesized by the reaction of chitosan with mono- (galactose, glucose, fructose) and disaccharides (lactose and lactulose). The procedure of obtaining these LMWC derivatives was applied to obtain complexes with a degree of substitution (DS) up to 20%. Properties such as water solubility, water and fat binding capacities, total antioxidant capacity and antimicrobial activity were studied. Experimental results indicated that the solubility of all chitosan derivatives was enhanced comparing to the native chitosan and the Schiff-base typed chitosan-fructose derivative showed the best performance. Binding capacities of water and fat were also improved and the Schiff-base typed chitosan-fructose and chitosan-lactulose showed the best results. Studies of Total Antioxidant Capacity (TAC) by the ABTS•+ and ORAC methods showed different behaviors for the derivatives. Thus, with the ABTS•+ method, native chitosan showed a lower antioxidant activity, which was markedly enhanced using the derivatives, especially using the Schiff-base typed chitosan-lactulose. Results obtained with ORAC indicated that the substitution of free amino groups of chitosan under the conditions carried out in this study (DS up to 20%) do not influence the antioxidant capacity of the chitosan molecule. Antimicrobial studies showed that all the Schiff base derivatives possessed antimicrobial activity upon the six tested microorganisms. These activities were, in all cases, equal or better than those of native chitosan. MICs and MLCs depended largely on the type of bacterium and on the structure of the substrate. Values obtained for the Schiff based derivatives were ranged from 0.03 to 0.2 % (w/v).

PS1: 9

### The genotypic and pheno-metabolomic landscape of a *Saccharomyces cerevisiae* strain collection

**R Duarte<sup>1</sup>, I Mendes<sup>1</sup>, CC Castro<sup>2</sup>, J Silva<sup>2</sup>, A Xavier<sup>1</sup>, J Drumonde-Neves<sup>1,3</sup>, RC Martins<sup>1</sup>, JM Oliveira<sup>2</sup>, AC Ferreira<sup>4</sup>, D Schuller<sup>1</sup>**

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Genome sequencing is essential to understand individual variation and to study the relationship between genotype and phenotype. Recently, large-scale sequencing projects of *Saccharomyces cerevisiae* revealed the existence of a few well defined lineages and some mosaics of that lineages, suggesting two domestication events during the history of association to human activities, one for sake strains and one for wine strains. Nevertheless, scarce information is available regarding phenotypic variability among strains used for different biotechnological applications. The objective of the present work was to undertake high-throughput approaches for a combined genetic, phenotypic and metabolomic evaluation of 187 *S. cerevisiae* strains. 100793 data points were generated and data analysis was performed using Principal Component Analysis (PCA). Phenotypic screening considered 28 physiological traits with biotechnological relevance. Resistance to ethanol (14 and 16% v/v), growth in the presence of potassium bisulfite and sulfur dioxide and the capacity to grow at 40°C contributed most to the highest variance (strain variability). Genetic characterization was performed using eleven highly polymorphic *S. cerevisiae* specific microsatellite loci. More than 200 different alleles were obtained, being around 30 responsible for the highest strain variability. All strains were used for fermentations with must of the grape variety Loureiro. When glucose concentration was below 5 g/L, samples were collected and used for fiber optics spectroscopy and bioanalytical analysis. HPLC was used to quantify primary fermentation products and organic acids. Fructose, glucose and ethanol concentrations contributed to the highest variance. Relevant volatile compounds that account for inter-strain differences were determined by GC-MS. PCA revealed ethyl acetate, 1-hexanol and 2-methylpropanol as the ones with highest weight. Globally, only spectral analysis (UV-VIS-NIR) showed correspondence between PCA segregation of strains and their technological use. Computational analyses are now underway to combine the strain's metabolic signatures with phenotypic and genetic data, to obtain a holistic overview of the pheno-metabolomic landscape and to develop tools for the prediction of a strain's biotechnological potential.

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PS1: 10

### **An improved microbiological water quality monitoring enzymatic kit for commercial use**

**Diana Mendes<sup>1</sup>, Lígia Lima<sup>2</sup>, José A. Teixeira<sup>1</sup>, Lucília Domingues<sup>1</sup>**

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Outbreaks of waterborne diseases – such as cholera, typhoid fever and bacillary dysentery – remain a major challenge to public health providers, claiming millions of lives annually, worldwide. The need for more rapid, sensitive and specific tests is essential; not only for water industry, but for a better public safety. Total coliforms and *Escherichia coli* are widely accepted as indicators and its presence can provide information about the efficacy of the treatment, cleanliness and integrity of the systems of distribution of water (total coliforms), as well as indicate that a faecal contamination has occurred and security measures should be taken in order to protect public health (*E. coli*) since pathogens may be present. Therefore, the purpose of this research was to design an enzymatic commercial kit, at request and in collaboration with a Portuguese company, capable of detecting *E. coli* and total coliforms in water samples. In order to be competitive in the market, it was sought distinction from other existing commercial kits for being fast to obtain the results, of simple handling, low cost, high efficiency and sensitivity (1 CFU). The detection was based on the enzymes  $\beta$ -galactosidase, which catalyses the hydrolysis of a chromogenic substrate into a yellow coloured product indicating the presence of total coliforms; and  $\beta$ -glucuronidase (specific for *E. coli*), which catalysis the hydrolysis of a fluorogenic substrate into a product capable of exhibiting blue fluorescence when exposed to UV light, indicating the presence of faecal coliforms on the sampled water and, consequently, the possible presence of pathogenic organisms. It was achieved a medium capable of simultaneous detection of total coliforms and *E. coli* in 14 h to 18 h with a sensitivity of 1 cell per sample. This medium was successfully tested on different types of water: drinking water (public distribution, treated with chlorine), river water (Rio Este) and sea water (Praia de Esposende), with efficient differentiation of the indicators. Due to the inhibitory effect of the high concentration of sodium chloride in sea water, the fluorescence on this type of water is comparatively fainter than the observed on other sources of sampling and some adjustments should be made in order to improve the visibility of detection. A kit for microbiological water quality monitoring was successfully developed, with the competitive advantage of providing results considerably faster than other commercial options.

PS1: 11

**Antimicrobial activity of sterols and triterpenes from *Ganoderma* spp.****Elza Fatima Albino Smania<sup>1</sup>, Fabio Albino Smania<sup>1</sup>, Franco Delle Monache<sup>2</sup>,  
Artur Smania Junior<sup>1</sup>**<sup>1</sup>Universidade Federal de Santa Catarina, Brazil; <sup>2</sup>Università di Roma, Italy

Increase in antibiotic resistance due to multiple factors increases risk of death, and is often associated with prolonged hospitalizations, which incited many researchers to focus on the investigation of natural products as source of new antibiotics. The aim of this study was to investigate the antimicrobial activity of sterols and triterpenes isolated from species of fungi belonging to the genus *Ganoderma* (*G. applanatum*, *G. australe*, *G. subamboinense*, *G. annulare*, and *G. resinaceum*). The dried fruiting body was extracted with organic solvents and subjected to silica gel column chromatography. Eight compounds were identified as lanostanoid on the basis of their spectral data, respectively sterols 5a-Ergost-7en-3b-ol, 5a-ergost-7,22-dien-3b-ol, and 5,8-epidioxy-5a,8a-ergost-6,22-dien-3b-ol; and the acid triterpenes: applanoxidic acids A, C, F, G, and H. The isolated compounds were solubilised in DMSO and their antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) using the microdilution broth method against *Escherichia coli* and *Staphylococcus aureus*, and against the fungi *Candida albicans* and *Trichophyton mentagrophytes*. The Gram-positive strain *S. aureus* was slightly more sensitive than the Gram-negative strain *E. coli*, and the dermatophyte *T. mentagrophytes* was slightly more sensitive than the yeast *C. albicans* to the compounds tested. For both groups of microorganisms, the MIC obtained varied between 500 and >2.000 µg/ml. Although the antimicrobial activities found for these compounds are not comparable with that of antibiotics commonly used in the treatment of infectious disease, their intriguing structures may encourage further modification studies to increase their antimicrobial activity.

PS1: 13

### Development and activity of a chitosan based mouthwash

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In our study we aimed to develop a mouthwash based on non-altered acid soluble chitosan. Chitosan mouthwash formulations were developed using high molecular weight (HMW), low molecular weight (LMW) and combinations of both molecular weights in combination with sodium chloride and sodium citrate. Stable formulations were then assessed for antimicrobial activity against several oral pathogens among which *Streptococcus mutans*. Formulations presenting better results were then compared against a commercially available mouthwash and the one that presented the best results was selected. The capacity of this formulation to interfere with biofilm formation by *Streptococcus mutans*, *Lactobacillus acidophilus* and mixture of both was then assessed. Finally, aromatic flavors, stabilizers and sweetener were added and the antimicrobial activity was again assessed in addition to a basic sensorial analysis. Results showed that a formulation containing a combination of both HMW and LMW presented antimicrobial activity even at low concentrations of mouthwash. The addition of the aromatic flavors, stabilizer and sweetener did not reduce the antimicrobial activity of the formulation. *S. mutans*, *L. acidophilus* and a mixture of both strains presented a greatly reduced capability (over 70 % of reduction when compared to a control) of biofilm formation in the presence of the formulation. These results were even more relevant when compared to the commercially available mouthwash, which presented weaker results in all assays.

PS1: 14

### **Differential expression of food *enterococci* in response to distinct growth environments**

**Cláudia Nóbrega, Sara Correia Santos, António Barreto, Teresa Semedo-Lemsaddek**

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Enterococci contribute to the organoleptic characteristics of several fermented foods, but during the last decades they have emerged as increasingly important causes of healthcare-associated infections. In the present investigation, in order to assess for the role of environmental cues in the modulation of gene expression, we compared two dairy isolates from Portuguese ewe's milk (LN11) and cheese (QSE123) and a clinical isolate (V583), after growth in environments related to colonization and infections sites, using two complementary approaches: transcriptome and proteome analysis. To study the transcriptome, we performed a RNA arbitrarily primed PCR (RAP-PCR) based on the use of an arbitrary primer and low annealing temperature for cDNA synthesis reactions. In order to select the best primer and conditions, RNA from LN11 grown in distinct conditions was isolated and used in preliminary experiments. The amplification products obtained for primers GTG5, M13, OPC15, OPC19 and pH, were resolved in polyacrylamide gels. Analysis of the RNA-fingerprints led to the selection of OPC-19 for further analysis, with a total of 45 differential expressed products, in comparison to an average of 21 observed for the other primers.

Subsequently, the dairy isolates LN11 and QSE123 were grown in all the conditions under study. Analysis of the corresponding RAP-profiles revealed a higher percentage of differentially expressed bands for LN11 (54%) in comparison to QSE123 (18%), pointing to a superior ability of LN11 to respond to the growth conditions under analysis and modulate gene expression accordingly. Regarding proteome analysis, preliminary experiments analyzed whole protein extracts of LN11, QSE123 and V583 by SDS-PAGE. Comparison of the patterns obtained led to the selection of growth in urine to be further analyzed by 2D-electrophoresis (2DE). 2DE performed with proteins extracts obtained after growth in urine showed that 68% of the 59 protein spots were shared by the three strains while 32% were differentially expressed. Among these differential proteins, half were common to QSE123 and V583, pointing towards similar responses of a dairy and a clinical enterococci to a condition simulating an infection setting and suggesting once again the versatility of enterococci to adapt to harsh environments. Overall, dairy and clinical enterococci modulated gene expression accordingly to the growth environment, which suggests the pathogenicity potential of food isolates.



PS1: 15

**Enzymatic sustainable eco-friendly processes related with lignin-based compounds****Ligia O Martins<sup>1</sup>, Tania Rosado<sup>1</sup>, Pedro Bernardo<sup>1</sup>, Kamila Koci<sup>1</sup>, Ana V Coelho<sup>1</sup>, Maria Paula Robalo<sup>2</sup>**<sup>1</sup>Universidade Nova de Lisboa, Portugal; <sup>2</sup>Instituto Superior de Engenharia de Lisboa

The pulping industry releases annually large amounts of lignin that constitute an immense quantity of biomass partially usable for applied purposes, with the advantage of decreasing the industrial dependence from petrochemical sources. The use of oxidative enzymes can increase the sustainability and performance of the currently available processes and enable the production of new lignin-based environmental-friendly products. Lignin, a highly branched, irregular three-dimensional organic polymer, is the most abundant polymer in Nature next to cellulose, and laccases shows the broadest substrate specificity, among the lignolytic enzymes, extended on substituted phenol, polyphenols, aromatic amines, and thiols requiring only atmospheric oxygen as cofactor. The substrate range of these biocatalysts is furthered enhance in the presence of low molecular weight redox mediators, a requisite for the oxidation of high-redox potential aromatic compounds. Nevertheless, the mechanism of lignin transformation in nature is complex and the role of laccases in this process is not fully understood. In the last years, we become interested in exploring the biochemistry and structure of laccases from prokaryotes for which, genetic tools and biotechnological processes are well established. Our research has focused on the CotA-laccase of *Bacillus subtilis* as a model bacterial laccase. The CotA is a thermoactive and thermostable protein with a  $T_{op} = 75^{\circ}\text{C}$  and  $T_m$  values around  $80^{\circ}\text{C}$ . Several X-ray crystal structure studies were undertaken revealing interesting features of the enzyme, namely insights on the nature of binding sites and role of oxidative mediators. Most of the mediators used so far, are synthetic compounds based on nitrogen heterocyclics. However, recently it has been found that some natural compounds derived from syringyl-type phenolics, could act as redox mediators in laccase catalytic transformations. In the present work syringaldehyde, acetosyringone and methyl syringate were used as mediators, and its performance was compared on the enzymatic oxidation of model non-phenolic compounds. Exploring the use of natural mediators would present environment and economical advantages, and might also contribute to understand the natural process of lignin degradation. The prediction and control of polymerization, grafting and delignification reactions in lignin is our final goal.

PS1: 16

**Key stress factors and parameters for batch production optimisation of silk-elastin-like proteins in *E. coli*.****Tony Collins, João Azevedo-Silva, André da Costa, Raul Machado, Margarida Casal**

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Silk-elastin-like proteins combining the physicochemical and biological properties of silk and elastin have a high potential for use in the pharmaceutical, regenerative medicine and materials fields. Their development for use is however restrained by their production levels. A series of novel silk-elastin-like proteins (SELPs) have recently been synthesised and here we describe the production optimisation for these with the pET-*E. coli* BL21(DE3) expression system. Both a comprehensive empirical approach examining all process variables (media, medium composition, inducer, induction time and period, temperature, pH, aeration, agitation) and a detailed characterisation of the bioprocess were carried out in an attempt to maximise production with this system. This study shows that maximum SELP production is achieved at 37°C using terrific broth at pH 6.8-7.6, a medium volume to shake flask volume ratio of 1:10 and an agitation speed of 200 rpm. Maximum induction is attained during the declining exponential phase with 0.5 mM IPTG and an induction period of at least 4 hours. We show that plasmid stability decreases dramatically on induction while acetate accumulates to greater than 5 g/l during the bioprocess. As little as 1 g/l acetate is shown to have a bacteriostatic effect while concentrations higher than 4 g/l appear to have a bactericidal effect under the conditions used. Using the optimised conditions, approximately 500 mg/l of purified SELP was obtained, representing a 2.5-fold increase on that previously reported. While this study is focused on SELPs, we believe that it could also be of general interest to any study where the pET-*E. coli* BL21(DE3) expression system is used. In particular, we show that induction time is critical in this system with, in contrast to that which is generally believed, optimal production being obtained by induction at the end of the declining exponential phase. Furthermore, we believe that we are at or near the maximum productivity for the system used, with plasmid instability on induction and high acetate production levels appearing to be the principal limiting factors for further improved production with shake flask cultivations.

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PS1: 17

**Laccase production by free and immobilized fungal mycelium of *Trametes versicolor*****Sara C. Silvério<sup>1</sup>, Sérgio Moreira<sup>2</sup>, Solange I. Mussatto<sup>1</sup>, Adriane M.F. Milagres<sup>2</sup>, Eugénia A. Macedo<sup>3</sup>, José A. Teixeira<sup>1</sup>**

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The production of laccase by free and immobilized mycelium of *Trametes versicolor* was evaluated. Fermentation experiments were carried out using a *Trametes* defined medium [1] supplemented with tween-80 (0.5%, w/v) and xyldine (30 µM) to stimulate and induce the secretion of extracellular enzyme. The support for the mycelium immobilization consisted in 0.3 cm<sup>3</sup> cubes of synthetic fiber (Scotch Brite, 3M Spain, SA), which were added to the culture system at 1 g/100 mL of medium. Assays were performed in 250-mL Erlenmeyer flasks and in a 2 L stirred tank bioreactor. For the flasks fermentations, three 7 mm diameter plugs from the fungal monoculture plate were inoculated into 50 mL culture medium in the presence or not of the immobilization support. For the assays in bioreactor, the volume of inoculum necessary to obtain an initial cell concentration of 70 mg/L was transferred to the reactor containing 1 L of culture medium with or without the immobilization support. In both cases, the fungus was incubated at 28 °C and 180 rpm. During the experiments, samples were periodically withdrawn for laccase and glucose determinations. Synthetic fiber was used as immobilization support since this material was demonstrated to be of great potential for fungi immobilization [2]. Additionally, many studies have demonstrated that fermentation systems with immobilized cells are able to increase the process productivity. However, the laccase production by *T. versicolor* (present study) did not show this performance. In both systems (Erlenmeyer flasks and bioreactor), the highest laccase production was obtained when using free mycelium. Additionally, the maximum laccase production obtained in bioreactor was lower than the maximum found in Erlenmeyer-flasks, suggesting that the conditions used in the bioreactor should be optimized to increase the laccase production results. Due to the great importance of the laccases in the industrial sector, more studies will be performed aiming to find a strategy to maximize the production of this enzyme by *T. versicolor*.

[1] Roy et al *Appl. Environ. Microbiol.* 59, 1855–1863, 1993. [2] Mussatto et al., *Carbohydr. Res.* 344, 795–800, 2009. Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and Fundação para a Ciência e a Tecnologia (FCT, Portugal).

PS1: 18

**Mass transfer determination in *Escherichia coli* biofilms formed under turbulent flow conditions****Joana Moreira, Joana Teodósio, Manuel Simões, Luis Melo, Filipe Mergulhão**

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Biofilm formation is common whenever water and nutrients are in contact with a surface. In most cases biofilms are deleterious but in some instances they can be beneficial. In the human body, biofilms are a common cause for disease and biofilm formation has been shown to protect bacteria from antibiotics. Part of this protection stems from the reduced penetration of the drugs into the biofilm, a phenomena that is controlled by mass transfer inside and outside the biofilm. Beneficial biofilms can be found, for instance, in wastewater treatment reactors. In this situation, biofilm growth should be stimulated and once more this depends on the mass transfer of nutrients into the biofilm. In order to assess the mass transfer during biofilm formation by *Escherichia coli*, we have used a flow cell system operated in turbulent flow conditions (Reynolds number of 6290). This system was previously validated as a simulation platform for biofilm formation on industrial conditions (Teodósio, Simões *et al.* 2011). Biofilm thickness and wet weight measurements were made and a maximum thickness of 550  $\mu\text{m}$  was attained at the end of the experiment, corresponding to a wet weight of 9.5 mg per  $\text{cm}^2$ . The growth of planktonic cells and the total glucose consumption were also evaluated. Glucose consumption increased over time, following the same trend obtained for biofilm thickness and wet weight. Finally, the external mass transfer coefficient was determined using correlations involving the Reynolds, Schmidt and Sherwood numbers. A value of  $1.167 \times 10^{-5} \text{ m}\cdot\text{s}^{-1}$  was obtained for this parameter which agrees well with published data (Vieira and Melo 1999). On that study Vieira and Melo demonstrated for similar hydrodynamic conditions and using *Pseudomonas fluorescens* that the mass transfer limitation occurred inside the biofilm. Having obtained in this work a similar value for the external mass transfer coefficient it is also likely that in our case the internal mass transfer is also controlling the access of the biofilm cells to glucose.

PS1: 19

**Prospective on the use of bacterial cellulose as an antimicrobial edible film****Jorge Padrão, Lígia Rodrigues, Fernando Dourado**

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Food and beverage packaging has been the target of an intensive conceptual revolution in the past twenty years, moving further away from the mere passive barriers highly dependent on petrochemical-based raw materials. The recent trends of the state of the art food packages consist of smart packages that are preferentially *green* manufactured due to environmental concerns, and edible, making it more practical to consume and simultaneously minimizing the resultant waste. Some of these smart packages are able to diagnose and inform "in real-time" the consumer/retailer of the encased food's quality (*intelligent packaging*). Other packages are responsible for an active interaction with the food or food's atmosphere increasing the products shelf life, improving its organoleptic and/or health properties (*active packaging*). The food and beverage packaging market is estimated to represent 1 trillion dollars by 2015 in the United States alone, making this field of research an interesting area to explore. The main goal of this work is to produce a novel edible packaging film with antimicrobial properties. The purpose of including a food grade antimicrobial compound is to delay the growth of microbial flora and thus increasing the food's safety and delaying its spoilage. For the packaging main raw material we have chosen the bacterial cellulose, which may represent an interesting alternative to the classic plastic casings, since this natural biopolymer possess a high toughness (Young's modulus of approx. 15 - 35 GPa), a low density ( $1.25 \text{ g cm}^{-3}$ ), a high crystallinity (95%), it is biocompatible, is highly pure (total absence of hemicelluloses and pectin's), provides a high surface area for modification ( $37 \text{ m}^2 \text{ g}^{-1}$ ), and finally, its low cost. As food-grade antimicrobial compound we selected the lactoferrin, a bilobar iron binding glycoprotein with a widely reported bactericidal effect. Different approaches are being used to covalently bind the protein onto bacterial cellulose. The preliminary antimicrobial effectiveness of the modified bacterial cellulose films is assessed by inhibition halo tests.

PS1: 20

**Purification and characterization of a chitinase from the marine archaea *Halobacterium salinarum*****Belén García-Fraga, Abigail F. da Silva, Jacobo López-Seijas, Carmen Sieiro**  
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Chitin, a linear  $\beta$ -1,4-linked homopolymer of N-acetylglucosamine (GlcNAc), is one of the most abundant polysaccharides in nature besides cellulose and starch. About  $10^{11}$  tonnes of chitin are produced annually in the aquatic medium alone. The main sources of chitin in the environment are the shells of crustaceans, insect exoskeletons and fungal cell walls. However, there is not accumulation of chitin in ocean sediments because a bioconversion process is carried out by very efficient chitinolytic marine microorganisms. Chitinases are glycosyl hydrolases, which can catalyze the degradation of chitin to its oligo and monomeric components. During the last years chitinases have received increased attention due to their broad range of applications. Although several microbial chitinases have been studied and characterized, the diversity of applications and conditions in which these enzymes must work also demand a large number of different enzymes capable of acting in such conditions. Exploration of microbial diversity must allow to find new enzymes with interesting properties. In the present work we characterized a novel chitinase from the marine archaea *H. salinarum*. The protein was expressed in *Escherichia coli*. After purification by affinity chromatography, 7.91 purification fold with a specific activity of  $0.514 \text{ U}\mu\text{g}^{-1}$  was achieved. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a molecular weight of approximately 60 kDa. The optimal pH, temperature and salinity for chitinase activity were 7.3, 40 °C and 1.5 M, respectively. Chitinase activity was increased by  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and strongly inhibited by  $\text{Mn}^{2+}$ . Analysis of the activity with 4-Nitrophenyl N-acetyl- $\beta$ -D-glucosamine substrate let us think this enzyme could be very interesting for obtaining chitin oligosaccharides with a determined size and GlcNAc with biological activity. These are products of great interest in medicine, food and agriculture where its chemical synthesis is expensive and generates toxicity. Throughout, and due to chitin is a highly abundant non-expensive natural biopolymer, enzymatic production of GlcNAc and chitoooligosaccharides can be a more cost-effective and environmental friendly approach.

PS1: 22

**Valorisation of wastes for single cell oil production by *Yarrowia lipolytica*****Marlene Lopes, João Melo, Isabel Belo**

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Various strains of oleaginous microorganisms, mainly fungi and yeast, have been widely used for the production of single cell oil (SCO) rich in polyunsaturated fatty acids or having an exceptional triacylglycerol structure. Difficulties for industrial scale production are related to the high cost of fermentation and oil extraction. The quantity of oil accumulated per unit of dry cellular mass is a critical factor that influences the final cost of SCO. The economics of these bioprocess become more favourable when zero or negative value waste substrates are utilized as carbon or nitrogen sources. Although utilisation of crude glycerol in the fermentation medium without prior purification offers a remarkable advantage against the traditional use of pure glycerol as substrate only few reports have appeared in the literature on the use of this substrate as sole carbon sources. The aim of current investigation was to assess the potentialities of valorisation of crude glycerol and lard, residues used as a carbon source by *Yarrowia lipolytica* strain in order to the production of SCO. Batch fermentations in 1-L Erlenmeyer flasks were performed using pure glycerol, crude glycerol and lard as carbon sources, with different concentrations (20 g/L, 50 g/L and 80 g/L). *Y. lipolytica* W29 was pre-grown overnight in YPD medium, centrifuged and resuspended in each carbon source medium, supplemented with yeast extract (0.5 g/L). The production of SCO was carried out during 168 h at 27 °C and 185 rpm. Yeast cells were able to grown on all carbon sources, although a slight inhibition with 80 g/L of pure and crude glycerol was observed. No significant differences on final cell dry weight were noted between the carbon sources, reaching approximately 5 g/L. The increase in carbon source concentration leads to an improvement in lipid accumulation inside the cells. The highest amount of reserve lipid was observed in medium with lard 80 g/L (21.3 % of cell dry weight). The strain showed the tendency to degrade its storage lipids when grown on 20 g/L of each carbon sources, probably due to the early consumption of substrate. Crude glycerol batch fermentation at 50 g/L in a 2-L bioreactor led to an accumulation of lipid content inside the cells of 37 % cell dry weight. The results of this study suggest that SCO could be produced by *Y. lipolytica* W29 using low-cost substrates, such as crude glycerol and lard.

PS1: 23

### **Antibacterial activity of semi-arid plant extracts against food-borne pathogen bacteria**

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This work describes the chemical characterization of extracts from seven plants (*Larrea tridentata*, *Flourensia cernua*, *Lippia graveolens*, *Agave lechuguilla*, *Yucca filifera*, *Opuntia ficus-indica*, and *Carya illinoensis*) which are acclimated to the Mexican semi-desert. The extracts were obtained using Soxhlet method by water, ethanol and an infusion method using alternative organic solvents (lanolin and cocoa butter); in addition, it was evaluated the antibacterial activity of semi-desert plant extracts against *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. Chemical characterization of plant extracts showed that they are rich in secondary metabolites; cocoa butter was the non conventional solvent which it was possible to obtain the highest content of total polyphenols. It was not possible to identify saponins in those extracts where non conventional solvents were used. While in extract where non conventional solvents were used, it was only possible to detect the presence of terpenes in creosote bush and prickly pear extracts. *S. aureus* was the bacterial strain that showed the highest growth inhibition as consequence of the plant extracts. The use of semi-desert plant extracts obtained using organic solvents are a good alternative for food-borne pathogen bacteria control because all the bacterial growth decreased with the tested extracts.



PS1: 24

### Antibiofilm effect of some main components of essential oils on *E. coli* biofilm

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The term biofilm was created to describe the sessile form of microbial life, characterized by adhesion of microorganisms to biotic or abiotic surfaces, with consequent production of extracellular polymeric substances. It has been noticed that the main chemical components of essential oils have promising antibacterial activity that can be explored as an effective alternative to control biofilms. The objective goal of this study was to assess the antibiofilm effect of eugenol, geraniol and terpine-4-ol on against *Escherichia coli*. Biofilms were developed within the 96-well microtiter plates in the presence of the main components of the essential oils from for the tests, we used eugenol (major component of cinnamon essential oil), geraniol (major component of essential oil of palmarosa) and terpin-4-ol (major component of essential oil of green tea). Cell suspensions (100  $\mu\text{L}$  of  $1 \times 10^8$  cells  $\text{ml}^{-1}$  in TSB) and composts components (100  $\mu\text{L}$  of solution of composts) were pipetted into each well and incubated for 24 h at 37°C in an orbital shaker at 120 rpm. The composts were dissolved in DMSO (2.0 %) and saline water (0.85 %) with tween 80 (0.5 %) in order to obtain final concentrations of eugenol 0,76%, geraniol 0,76% and terpine-4-ol 0,38%. Biofilms were characterized, before and after treatment, by total biomass, through crystal violet (CV), and number of cultivable bacterial cells, expressed as log CFU per  $\text{cm}^2$ . Terpine-4-ol essential oil did not have any effective antimicrobial action against culturable cells. Conversely, eugenol and geraniol showed a promising antimicrobial activity against *E.coli* biofilms as it was observed a significant reduction of the cultivable biofilm-growing cells. All composts showed high activity against biomass than in the reduction of the viable cells entrapped in biofilms. The results suggest the possibility of using these compounds as in antibiofilm surfaces such as in the paper used in food industries.

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PS1: 26

**Antimicrobial activity of seaweed extracts from the North Portuguese Coast****Marta Mendes<sup>1</sup>, Rui Pereira<sup>2</sup>, Isabel S. Pinto<sup>2</sup>, Ana P. Carvalho<sup>1</sup>, Ana M. Gomes<sup>1</sup>**<sup>1</sup>CBQF - Escola Superior de Biotecnologia - Universidade Católica Portuguesa, Portugal; <sup>2</sup>CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental

The utilization of marine algae as potential sources of pharmaceutical agents has been increasing recently, due to the realization that they contain bioactive compounds, which inhibit the growth of some pathogenic bacteria, as well as some yeasts. The aim of this work was to establish an improved procedure for obtaining extracts from marine algae, and to test the antimicrobial activity of these extracts against selected species of yeasts, Gram-negative and Gram-positive bacteria. Seaweeds used in this study were obtained in the North of Portugal, from integrated aquaculture. *Gracilaria vermiculophylla* was used for the preliminary assays of optimization of the extraction procedure, whereas *Gracilaria vermiculophylla*, *Porphyra dioica* and *Chondrus crispus* were used for antimicrobial assays. Optimization studies were focused on the definition of the pre-treatments of the algae (drying), and the temperature and type of solvents used during the extraction process. Results revealed that test organisms were more sensitive to extracts obtained with dried algae, continuously processed at higher temperatures. Concerning antimicrobial capacity evaluation, species tested were: Gram-negative - *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*; Gram-positive: *Listeria innocua*, *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus brevis*, *Staphylococcus aureus*, all from food origin and a strain of *Staphylococcus aureus* from clinical origin. The yeast *Candida* spp. was from clinical origin as well. Tests to assess the antimicrobial activity of the extracts were performed using the agar diffusion method, and results indicated a stronger antimicrobial activity of the ethyl acetate extracts, when compared with methanolic or diethyl ether extracts.

PS1: 27

**Antimicrobial and antioxidant activities of quercetin-chitosan based edible films****Marthyna Pessoa Souza<sup>1,2</sup>, Antônio Fernando Vaz<sup>1</sup>, Miguel Ângelo Cerqueira<sup>2</sup>, Antônio Augusto Vicente<sup>2</sup>, Maria das Graças Carneiro-da-Cunha<sup>1</sup>**<sup>1</sup>Departamento de Bioquímica, Universidade Federal de Pernambuco-UFPE, PE, Brazil; <sup>2</sup>Institute for Biotechnology and Bioengineering-IBB, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal

Contamination by pathogenic microorganisms and oxidative reactions are two of the main causes of deterioration and loss of freshness in perishable foods. Edible films can be used to maintain the quality and extend the shelf life of food products. Recently, there has been a renewed interest in food packages based on natural macromolecules due to the concerns about the environment and the need to reduce the amount of disposable packaging materials. Chitosan, as the most abundant naturally occurring amino-polysaccharide, is a very promising biopolymer because of its unique physiochemical characteristics, biodegradability and antimicrobial activities. Quercetin is one of the most potent antioxidant molecules of plant origin being its antioxidant activity higher than other well-known antioxidant molecules. The association of natural antioxidants and bioactive biopolymers as chitosan may be particularly useful to develop active food packaging with enhanced properties. In this context, the aim of this work was to develop an active chitosan-based film with quercetin incorporated. The film-forming solutions were prepared with chitosan concentrations of 1.0 % (w/v) in lactic acid solution (1.0 % (v/v)) without and with quercetin (200 µg mL<sup>-1</sup>). The antioxidant activity of quercetin-chitosan based films was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity method. The antimicrobial activity of quercetin, chitosan and quercetin-chitosan based film-forming solution was evaluated against, *Escherichia coli*, *Shigella sp*, *Proteus mirabilis*, *Morganella sp*, *Micrococcus luteus*, *Staphylococcus epidermidis* and *Bacillus subtilis* using the agar diffusion method. The quercetin-chitosan based films showed the strongest DPPH radical scavenging activity with an initial rate of DPPH consumption of 4.06±0.32 mol min<sup>-1</sup>. Agar diffusion assay revealed bioactivity against all tested bacteria, with zone of inhibition ranging from 21.00±1.00 to 25.33±1.52 mm for chitosan film-forming solutions; quercetin solution alone showed no inhibitory effect on the growth of the microorganisms tested. However, the antimicrobial effects against all gram-negative bacteria were increased ( $p<0.05$ ) in the association of chitosan and quercetin, allowing the elaboration of edible films with strong antioxidative and antimicrobial activities. This work suggests a significant potential in the application of chitosan-based edible films containing quercetin to enhance the safety of foods.

PS1: 28

**Biocompatibility of ionic liquids through *Candida antarctica* lipase B (CaLB) - Evaluation of its enzymatic activity****Luísa Daniela Ferreira Santos, Sónia Patrícia Marques Ventura, João Manuel da Costa e Araújo Pereira Coutinho**

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Enzymes have found great uses in several industries such as food, pharmaceutical, detergent, textile, pulp and paper, animal feed, leather and cosmetics. In nature, enzymes act as catalysts of living systems and are designed to function in aqueous solutions. However, anhydrous conditions are needed for synthetic transformations using these macromolecules. The use of organic solvents as non-aqueous environments offers the possibility of carrying out synthetic reactions by hydrolytic enzymes, increasing the solubility of molecular substrates. However, biocatalysts in non-aqueous media often suffer from reduction of activity, selectivity and/or stability. In addition, conventional organic solvents present high volatility and flammability being harmful to the environment. The use of ionic liquids (ILs) in biocatalytic processes has recently gained much attention as an environmentally attractive alternative to classical organic solvents. They have been seen as good solvents in a wide variety of biochemical processes, because they can improve enzyme stability, substrate and/or product selectivity and also are responsible for the suppression of some side reactions. The main idea of this work was to provide some information about the influence of several imidazolium- based ILs in the activity of *Candida antarctica* lipase B (CaLB). Parameters such as the IL concentration, the effect of different anions and distinct alkyl chain lengths were considered in this study. This cation core was used to study the effect of different anions and also the effect of the elongation of the alkyl chain (C<sub>2</sub> to C<sub>10</sub>). The studied anions were bromide Br, dicyanamide [N(CN)<sub>2</sub>], hydrogenosulphate [HSO<sub>4</sub>], methanesulfonate [CH<sub>3</sub>SO<sub>3</sub>], triflate [CF<sub>3</sub>SO<sub>3</sub>], acetate [CH<sub>3</sub>COO], trifluoroacetate [CF<sub>3</sub>COO] and chloride Cl. The enzyme activity was assayed spectrophotometrically. The main conclusion of the present work is that the enzymatic activity was affected by all the parameters studied. In terms of the effects of the different anions, these results indicate [HSO<sub>4</sub>] as the responsible for the major negative effects and Br as the responsible for the smaller decrease. The influence of different concentrations was also studied being proved that this parameter has a major role in the effects of alkyl chain in the enzymatic activity.

PS1: 29

**Biogenic amine quantification in a dry fermented blood sausage “Morcela” from Portalegre.****Carla Nóbrega Carneiro<sup>1</sup>, Cristina Alfaia<sup>1</sup>, José Prates<sup>1</sup>, Manuel Malfeito-Ferreira<sup>2</sup>, Virgílio Loureiro<sup>2</sup>**<sup>1</sup>Faculdade de Medicina Veterinária - UTL, Portugal; <sup>2</sup>Instituto Superior de Agronomia - UTL, Portugal

Dry fermented blood sausages are worldwide diffused fermented meat products which can be a source of biogenic amines (BA). BA can be naturally present in many foods but they can also be produced in high amounts by microorganisms through the activity of amino acid decarboxylases. Excessive consumption of these amines can be of health concern causing different degrees of diseases. Lactic acid bacteria are widely used in the meat industry not only as starter cultures for sausages fermentation but also to help decrease BA accumulation. The production of this blood sausage “Morcela” lacks a common critical point in production technology: the thermal treatment. To overcome this absence lactic acid bacteria are inoculated as starter cultures. Taking this into account this study was designed to achieve three main goals: i) to determine and quantify which BA were predominant throughout time; ii) to evaluate the influence of the packaging technology in BA accumulation; iii) to determine whether the addition of starter cultures could maintain BA levels below legal values. Two groups of “Morcelas”, packed with (modified atmosphere, n=18) and without protective atmosphere (n=18), were analyzed at three time points along half of its shelf life period (0, 30, 60 days). Six replicates from each group were analyzed for BA determination. All six non-volatile BA (putrescine, cadaverine, histamine, tyramine, spermine and spermidine) were determined as dansyl derivates by reversed-phase High Performance Liquid Chromatography. Putrescine, cadaverine and tyramine exhibited the highest concentration in both groups. However, “Morcelas” that were packed under modified atmosphere presented superior concentrations at all sample points. This observation occurred not only for the three BA mentioned above but for all six BA analyzed. On both groups, regardless of the packaging atmosphere, all BA tended to increase over time without ever surpassing legal accepted values. In our experimental conditions, histamine was never detected. Even though we could not determine whether starter cultures may influence BA accumulation on this blood sausage, its positive influence as competitive flora deserves further investigation. Hypothesis of naturally decarboxylating starter strains could not be discarded. Total BA concentration never exceeded legal accepted values which may lead us to conclude that, for the time period analyzed, this product presents no risks for its consumers.

PS1: 30

### Chemical composition and antimicrobial activity of essential oils of *Lavandula* spp.

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Lavenders belong to the family *Labiatae* and represent some of the most popular medicinal plants of great economic importance. Their essential oils are important for the perfume, cosmetic, flavouring and pharmaceutical industries. However, despite its popularity, and the long tradition of use, biological properties of the various *Lavandula* species are not yet been well sustained by scientific or clinical studies and some available data being inconclusive and controversial. Although *Lavandula* spp. have similar ethnobotanical properties, however, chemical composition and therapeutic uses differ from different species and main composition of essential oils showed differences with species and with the region were they grow. *L. stoechas* subsp. *luisieri* (Rozeira), *L. pedunculata* (Mill.) Cav. and *L. viridis* L'Hér are endemic to the Iberian Peninsula, widespread in the South of Portugal, namely in Alentejo and Algarve. In our work, essential oils from the stems or leaves from wild grown plants of *L. luisieri* (Alentejo), *L. pedunculata* (Alentejo) and *L. viridis* (Algarve), were extracted by hydrodistillation and analyzed by GC-FID. Antimicrobial activity was evaluated by solid diffusion disk assay and minimal inhibitory concentration (MIC) against pathogenic Gram-positive and Gram-negative bacteria and food spoilage fungi. Results showed some differences between chemical compositions of different analysed oils. Main components of essentials oils were 1,8-cineole, bornyl acetate and lavandulol for *L. stoechas* subsp. *luisieri*; camphor, bornyl acetate, fenchone,  $\alpha$ -pinene and 1,8-cineole for *L. pedunculata* and 1,8-cineole, camphor, linalool and  $\alpha$ -pinene for *L. viridis*. Essential oils showed a large spectrum of antimicrobial activity, and although this activity differed with the essential oil, all oils showed antibacterial activity against *Escherichia coli*, *Klebsiella pneumonia*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and antifungal activity against pathogenic yeast *Candida albicans*, *Candida krusei*, *Candida parapsilosis* and *Geothricum klebahnii*, and molds *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus japonicum* and *Fusarium oxysporum*. On the basis of our results, the essential oil of different *Lavandula* spp. of southern Portugal region showed interesting antimicrobiological properties, suggesting their potential use for pharmacological applications as nutraceutical and/or phytotherapeutic agent.

PS1: 31

**Cultivation of *Aspergillus niger* strains in a dairy by-product****Norberto Pinheiro de Freitas, Rosa Rodriguez Jasso, José Teixeira, Giuliano Dragone**

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Cheese whey, characterized as the watery part remaining after the precipitation and removal of milk casein during cheese processing, is one of the main by-products of the dairy industry. It represents an important environmental pollution, due to the high biochemical oxygen demand (30,000–50,000 mg/L) and chemical oxygen demand (60,000–80,000 mg/L). The most abundant nutrients present in cheese whey are lactose (45-50 g/L), soluble proteins (6-8 g/L), lipids (4-5 g/L) and minerals (8-10% of dried extract); therefore, it constitutes an inexpensive and nutritionally rich raw material for the production of valuable compounds through fermentation processes. *Aspergillus niger* strains are widely used in fermentations for the production of various products of commercial interest, such as organics acids and hydrolytic enzymes, among others. The objective of this study was to evaluate the ability of several *A. niger* strains to use lactose from cheese whey for cell growth. Fungal strains *A. niger* ESH, *A. niger* PSH and *A. niger* GH1 were isolated from the Mexican desert and *A. niger* AA20 was provided from a collection of the Mexican Autonomous National University. Cheese whey powder kindly supplied by Lactogal (Porto, Portugal), was used as carbon source, with 93% lactose content (quantified by HPLC). The culture medium, used in the submerged fermentation, contained (g/L): cheese whey, 71.43;  $(\text{NH}_4)_2\text{SO}_4$ , 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1; peptone, 7;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $0.1 \cdot 10^{-3}$ ;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $0.06 \cdot 10^{-3}$ ;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $0.1 \cdot 10^{-3}$ ; methanol, 3% (v/v), with and initial pH 4, adjusted with  $\text{H}_2\text{SO}_4$  1M. Fungal growths were performed in 250-ml Erlenmeyer flasks containing 100 ml of medium at 28 °C and 200 rpm. Results showed that all the tested strains of *A. niger* were able to grow on cheese whey media. The highest cell growth was obtained for *A. niger* PSH strain with a maximum biomass production of 18.37 g/L. On the other hand, *A. niger* AA20 showed the lowest biomass concentration at the end of the fermentation. Regarding lactose consumption by *A. niger* strains, AA20 and ESH species consumed all the lactose present in the media at the end of the fermentation. However, more than 20 g/L of lactose remained after 13 days for the *A. niger* PSH. It was concluded that cheese whey can be considered as a cost-effective carbon source for fermentation processes involving *A. niger* strains.

PS1: 32

### Ecology of malolactic bacteria associated with Albariño musts and wines from the Salnés region, in Northwestern Spain.

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University of Vigo, Spain

Malolactic fermentation (MLF) is one of the two main fermentations in winemaking. The process converts L-malate in L-lactate and carbon dioxide. Fermentation can be carried out by autochthonous lactic acid bacteria (LAB) present in must and wineries, although induction of malolactic fermentation by inoculation of commercial strains is becoming a frequent practice. Malolactic fermentation leads to the deacidification of wine, improves its microbiological stability and in addition is responsible of numerous transformations that improve the organoleptic characteristics of wine. Spontaneous FML is very difficult to achieve and induction of MLF with commercial starters is not always successful because wine is a very hostile environment for bacterial development. The possibility of having autochthonous starters adapted to the particular conditions of each type of wine would be very interesting in order to control successfully malolactic fermentation. This approach requires previous ecological studies of the bacterial diversity associated to the different spontaneous processes. In the recent years different studies have been done on the ecology of MLF in distinct oenological regions. The aim of this research was to study the bacterial diversity associated to malolactic fermentation in Albariño wines from the Val do Salnés region (NW Spain). Over two consecutive years we examined grape musts, different stages of alcoholic fermentation and malolactic fermentation from various fermentation tanks from *Adega Condes de Albarei* cellar, which receive grapes from different vineyards of the Salnés region. We isolate 35 presumptive malolactic bacteria in MRS and MLO media that were identified by biochemical and molecular methods (16S rDNA, ARDRA, ISR 16S/23S restriction analysis and *recA* multiplex PCR). The most of isolates (77%) were obtained from MLF. They fit into six species belonging to four genera: *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Oenococcus*. The most representative species were *Lactobacillus hilgardii* (34,3%) and *Lactobacillus paracasei* (20%) followed by *Pediococcus damnosus* (17,2%) and *Lactobacillus plantarum* (14,3%). *Oenococcus oeni*, the main responsible for MLF in many oenological areas only represented 8,6 % of the isolates. In a similar proportion (5,7%) was found *Lactococcus lactis*. Different strains for each species were found in a preliminary characterization.



PS1: 33

**Effect of encapsulation on survival for potential probiotic *Lactobacillus plantarum* ST16Pa isolated from papaya****Svetoslav Todorov**

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Capability to produce bacteriocins by lactic acid bacteria (LAB) can be explored by the food industry as a tool to increase the safety of foods. Furthermore, probiotic activity of bacteriogenic LAB brings extra advantages to these strains, as they can confer health benefits to the consumer. Beneficial effects depend on the ability of the probiotic strains to maintain viability in the food during shelf-life and to survive the natural defenses of the host and multiply in the gastrointestinal tract (GIT). Encapsulation of the probiotic LAB is a tool to increase their viability. This study evaluated the probiotic potential of a bacteriocinogenic *Lactobacillus plantarum* strain (*Lb. plantarum* ST16Pa) isolated from papaya fruit and studied the effect of encapsulation in alginate on survival in conditions simulating the human GIT. Good growth of *Lb. plantarum* ST16Pa was recorded in MRS broth with initial pH values between 5.0 and 9.0 and good capability to survive in pH 4.0, 11.0 and 13.0. *Lb. plantarum* ST16Pa grew well in the presence of oxbile at concentrations ranging from 0.2% to 3.0%. The level of auto-aggregation was 37%, and various degrees of co-aggregation were observed with different strains of *Lb. plantarum*, *Enterococcus* spp., *Lb. sakei* and *Listeria*, which are important features for probiotic activity. Growth was affected negatively by several medicaments used for human therapy, mainly anti-inflammatory drugs and antibiotics. Adhesion to Caco-2 cells was within the range reported for other probiotic strains, and PCR analysis indicated that the strain harbored the adhesion genes *mapA*, *mub* and *EF-Tu*. Encapsulation in 2%, 3% and 4% alginate protected the cells from exposure to 1% or 2% oxbile added to MRS broth. Studies in a model simulating the transit through the GIT indicated that encapsulated cells were protected from the acidic conditions in the stomach but were less resistant when in conditions simulating the duodenum, jejunum, ileum and first section of the colon. To our knowledge, this is the first report on a bacteriocinogenic LAB isolated from papaya that presents application in food biopreservation and may be beneficial to the consumer health due to its potential probiotic characteristics.

PS1: 34

## Effect of sesquiterpenes in the *Saccharomyces cerevisiae* growth and morphology

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*Saccharomyces cerevisiae* is perhaps the most useful yeast, since it is the microorganism behind the most common type of fermentation, the alcoholic fermentation. It is also one of the most intensively studied eukaryotic model organisms in molecular and cell biology due to its properties. Signaling mechanisms that govern physiological and morphological responses to changes in cell density is called quorum-sensing. This system is most studied in bacteria, but, in recent years, there have been several reports of quorum-sensing like phenomena in fungal species. In *S. cerevisiae*, phenylethanol and tryptophol were identified as quorum-sensing molecules for filamentous growth [1] and farnesol is a fungal quorum-sensing molecule with regulatory properties in *Candida albicans* [2]. The objective of this study was to evaluate the effect of the presence of two structurally different sesquiterpenes in the *S. cerevisiae* growth and morphology. For this purpose, farnesol, an acyclic sesquiterpene alcohol, and aromadendrene, a cyclic sesquiterpene, were used. *S. cerevisiae* was inoculated in the presence of two concentrations (50  $\mu\text{M}$  and 100  $\mu\text{M}$ ) of farnesol or aromadendrene and the cellular response to these compounds was evaluated at the level of cell density and morphology. Solid phase microextraction (SPME) combined with gas chromatography (GC) were used to determine the content of sesquiterpenes in the environment throughout the *S. cerevisiae* growth. The addition of 100  $\mu\text{M}$  farnesol promote an increase in cellular density in the exponential phase of *S. cerevisiae* growth. A morphological change was also stated, since cells were smaller in the media with farnesol. The decrease of farnesol concentration (50  $\mu\text{M}$ ) only promoted a slight increase on cell growth. The presence of aromadendrene revealed no significant changes in *S. cerevisiae* for both concentrations tested. The presence of farnesol and aromadendrene in the media were only detected after two hours of *S. cerevisiae* inoculation. This study reveals that the presence of farnesol in the culture media influences the cell density and morphology of *S. cerevisiae*. More studies are required in order to disclose if this is a quorum sensing molecule.

1. Chen & Fink, 2006. *Genes & Dev.* 20, 1150. 2. Hornby et al. 2001. *Appl. Environ. Microbiol.* 67, 2982. The authors thank to QREN (n<sup>o</sup>3462), FCT (PTDC/AGR-ALI/101251/2008), Research Unit 62/94 QOPNA (project PEst-C/QUI/UI0062/2011), and FCT grant (SFRH/BPD/46584/2008).

PS1: 35

**Effect of the support material and storage conditions of immobilized lactic acid bacteria on malolactic fermentation of white wine****Zlatina Azenova Genisheva, Solange I. Mussatto, José M. Oliveira, José A. Teixeira**

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In this work, the lactic acid bacterium *Oenococcus oeni* was immobilized on three different natural materials (namely corn cobs, grape skins and grape stems) and used to induce malolactic fermentation in white wine. Additionally, the biocatalyst reuse after different periods of storage in cold or in hot environments was also evaluated. The resistance of the immobilized lactic acid bacterium against inhibitors was determined by performing the MLF in presence of high SO<sub>2</sub> concentration. Immobilization occurred in situ during the fermentation, which was performed in 500 mL Erlenmeyer flasks containing 6 g of support material, 1 g/L cells of *O. oeni* and 200 mL of complex medium. Fermentations were carried out in duplicate, and samples were taken periodically for the estimation of glucose, fructose and malic acid consumption, and lactic acid production. At the end of the fermentation (16 h), since all the assays presented similar results, different strategies were adopted. One of them consisted on the recovery of the corn cobs with immobilized cells, and subsequent storage of this biocatalyst at 5 °C during 31 d. The flasks containing grape skins and stems were directly stored at 25 °C during 27 d and 37 d, respectively. After these periods, the support materials with immobilized cells were recovered, washed with sterilized distilled water and added to 200 mL of white wine for conducting MLF, which was performed during 18 d. Subsequently, the biocatalysts were recovered, washed and added to 200 mL of white wine for conducting MLF in presence of 32 mg/L of free SO<sub>2</sub>, which was maintained during 17 d. Malic acid consumption and lactic acid production was observed during all the MLF, independently of the used support material. However, fermentation runs with cells immobilized on grape skins gave the best results, providing the highest lactic acid concentration and also high conversion of malic acid. The presence of high SO<sub>2</sub> concentration (32 mg/L) did not affect the conversion of malic acid for cells immobilized in grape skins and stems, and gave similar results of produced lactic acid (3.60 g/L and 2.90 g/L, for grape skins and stems, respectively). The presence of high SO<sub>2</sub> concentration strongly affected the conversion of malic acid by cells immobilized on corn cobs. *Oenococcus oeni* immobilized on grape skins and grape stems can be successfully used on MLF even after long periods of storage at 25 °C, and in the presence of high SO<sub>2</sub> concentration.

PS1: 36

**Evaluation and characterization of antimicrobial and antigenotoxic properties of Portuguese propolis****Márcia Cruz<sup>1</sup>, Ana Margarida Ferreira<sup>2</sup>, Ana Cunha<sup>3</sup>, Cristina Aguiar<sup>4</sup>, Rui Oliveira<sup>4</sup>**

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Propolis is a substance produced by bees (*Apis mellifera* L.), after harvesting exsudates of plant buds and barks, and subsequent mixing with the salivary enzyme  $\beta$ -glucosidase. Bees use propolis in their combs as protection, to repair damage, to build aseptic locals for the eggs of the queen, and also as a thermal insulator. The composition of propolis varies geographically, with the available flora, the time of collection and the race of the bees. Different groups of compounds can be found in propolis such as polyphenols, terpenoids, steroids and amino acids. Some of these compounds have been associated with diverse biological activities: antimicrobial, antioxidant, antigenotoxic, genotoxic and antimutagenic. Portuguese propolis has recently attracted the interest of researchers because of the opportunity for its economic valorization and the need to scientifically support the biological properties commonly assigned to samples of different origins. Thus, our objective relates to the analysis and study of Portuguese propolis, particularly in what concerns its chemical characterization and the evaluation of biological activities. A propolis ethanol extract (PEE) was made from a sample provided from an apiary (Côa) in Beira Alta and tested in different assays, using *Saccharomyces cerevisiae* as biological model. We have performed viability assays in pre-, co- and post-incubation conditions using PEE and 5mM hydrogen peroxide ( $H_2O_2$ ) in order to investigate the effect of PEE as antioxidant and/or protective agent against oxidative stress. The comet assay was used in pre- and co-incubation to complement the previous method and to investigate the antigenotoxicity/genotoxicity of PEE under the same conditions. Chemical analysis of the extract was made to determine total polyphenol and flavonoid contents as well as antioxidant capacity of propolis. Results show that Portuguese propolis has antioxidant capacity when assessed by DPPH and ABTS assays. Accordingly, the viability of yeast cells shocked with the oxidant agent  $H_2O_2$  was improved in the presence of propolis, either in pre-incubation or in co-incubation conditions. However, when cells were analyzed by the comet assay, the antigenotoxicity of PEE was more evident in pre-incubation than in co-incubation assays. In addition, our results suggest that propolis has also a genotoxic effect in yeast cells, since cells treated only with PEE displayed more DNA damage than the untreated ones.

PS1: 39

**Garlic (*Allium sativum*) effect on yeasts isolated from fermented sausages****Márcia Inês Cunha Lima Reto, Amália Peito**

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Portugal has a long tradition in the production of traditional fermented sausages. In addition to lactic acid bacteria, yeast also proliferate during the fermentation and ripening of sausages, thereby they may contribute to the final quality of the product. *Debaryomyces hansenii* (teleomorph of *Candida famata*) was the dominant species on fermented sausages, being found at all stages of production. Several spices are used for the production of fermented sausages which include ground pepper, paprika and garlic. Garlic contains powerful antioxidants and also several antimicrobial components. Olesen *et al.* (2000) observed that dried garlic powder has at least a fungistatic potential against yeasts used as starter cultures in fermented sausages. The antifungal activity of several garlic presentations (powder, minced, puree and fresh) against yeasts isolated from fermented sausages was tested in order to study if besides enhance flavour garlic also acts on the microorganisms during fermentation. Primary screening was carried out by the agar plate technique. The sensitivity to garlic depended on garlic's concentration and presentation and on yeast strain. *Debaryomyces hansenii* strains were more sensitive followed by *Torulasporea delbruekii* and finally *Candida lipolytica* which were more resistant. The MIC's values also depended on the garlic presentation been powder and minced more effective on inhibiting yeasts growth. On the microplate assay fresh garlic showed a fungistatic effect. Garlic powder had a fungicide activity against *D. hansenii* and *T. delbruekii* and a fungistatic activity against *C. lipolytica*. The results suggest that use of garlic in fermented meat could influence the survival of the yeasts and consequently the fermentation process.

PS1: 41

### Histamine-forming bacteria in anchovies

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Anchovies, salted and cured are a traditional food product popular in Tunisia and other southern European countries. The final product can be consumed as a matured and non-packed product, most of the times exported in barrels for other countries, or more recently, preserved in a small sized can with olive oil. Histamine is a causative agent responsible for scombroid poisoning, a food-borne hazard related to the consumption of fish products, which may produce from mild to severe illness considering the ingested amount of this compound and the individual sensitivity to it. Its control is important in terms of public health (EC, 2007) as well as understanding the evolution upon this traditional product. Histamine concentrations in anchovies from anchovy (*Engraulis encrasicolus* L.) or sardine (*Sardina pilchardus*, Walb.) could be due to poor quality of the raw material, to inadequate handling or to other causes during its preparation and shelf life. The control of biogenic amines formation mainly focused on the controlling the growth of biogenic amines forming bacteria because histamine is heat stable and is not detectable organoleptically by even trained panelists (Chong *et al.*, 2011). In the present work the presence of histamine producing bacteria using modified Niven agar (mNv), as well as *Enterobacteriaceae*, sulphite-producers, faecal coliforms, halophilic and halotolerant bacteria, were investigated on several batches of anchovies (from both species) in different stages of maturation, collected in several industries from Tunisia. Total counts performed in Lyngby Iron agar were low in all the samples, and faecal coliforms were not detected. Most of the bacteria detected on mNv were gram positive cocci, *Streptococcus*, *Staphylococcus* genus and some *Bacillus*. The presence of histamine on the samples was also investigated using a C18 HPLC method (Agilent Chromatographer) with post-column derivatization and fluorescence detection.

PS1: 42

## Hygienic quality of raw sheep's milk used in cheese production in Idanha-a-Nova

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Soft cheese made with raw sheep's milk has been associated to several foodborne outbreaks. In Portugal, many traditional sheep milk cheeses used raw crude milk as raw material and in some cheeses had been detected the presence of foodborne pathogens. Therefore the quality of raw sheep's milk has to be known and monitored for the presence of foodborne pathogens. In this study, raw sheep's milk from dairy producers were monitored for a full year to six microbiological parameters to assess the hygienic status of raw sheep's milk used in cheese production in Idanha-a-Nova region. Raw sheep's bulk tank milk samples were collected from January to December 2010 from 13 dairy farms in Beira Baixa Region. The milk samples were examined for Total Bacterial Count, *Escherichia coli*, *Staphylococcus* coagulase +, *Listeria monocytogenes* and *Salmonella* spp. using routine methods (NP459:1985 Ed 2, ISO 16649-2:2001, ISO 6888-2:1999, ISO 11290-1:1996 and ISO 6785:2001 respectively). *Pseudomonas* spp. count was determined by surface plating in CFC *Pseudomonas* Agar plates incubated at 25°C for 48h. The results indicated that 90,2% of individual samples had total bacterial count < 500.000 cfu/ml with 71,2% <100.000 cfu/ml. *Pseudomonas* spp. were the most important contaminant microorganism followed by *Staphylococcus* coagulase +, *Listeria monocytogenes* and *Salmonella* spp. were not isolated from any of the samples tested. The results of this study indicate that the majority of raw sheep's milk used in cheese production in Idanha-a-Nova was of high microbiological quality with a low incidence of pathogens. High counts of *Pseudomonas* spp. were observed in sheep's milk particularly in Autumn, which should be included in food safety surveillance plans. These data will help dairy farmers and raw cheese plants to inform risk assessments associated with microbiological safety of raw sheep's milk and products.

PS1: 43

### Influence interfering substances in disinfection

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In dairy industry, the proliferation of microorganisms is common because of nutrient availability and high shear velocities. There is a risk associated with these microorganisms as they can be pathogens. Consequently, it is necessary to clean and disinfect the system. Clean-in-place is the most used process, which includes the cleaning of the plant without dismantling or opening the equipment, and consists on running cleaning solutions with increased hydrodynamics through the plant. Often, this process leaves behind some cell debris as well as inorganic and organic material from the food products that are recognized as potential interfering agents on the activity of the disinfectants. In this study, the influence of alginate, bovine serum albumin, yeast extract and humic acids on the antimicrobial activity of two quaternary ammonium compounds (QACs), cetyltrimethyl ammonium bromide and benzalkonium chloride was studied in low soil conditions. In order to assess the effect of each potential interfering substance, *Pseudomonas fluorescens*, *Bacillus cereus* and their consortium were exposed to QACs (single compound and their combination) in the presence of the interfering substances. The antimicrobial effect was calculated based on the respiratory activity measured by oxygen uptake rate due to glucose oxidation. Most of the interfering substances had no effect on the antimicrobial activity of the QACs. The only exception was the presence of humic acids, which completely quenched the antimicrobial action of QACs on *P. fluorescens*. For the tested conditions, the most efficient solution against both bacteria (single and consortium) was the combination of QACs.



PS1: 44

### **Influence of substrate quality on the microbial and enzymatic hydrolysis of tannic acid**

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Tannic acid is used in biotechnological processes as favorite substrate for induction of tannase enzyme under different culture conditions and systems, and for microbial or enzymatic production of gallic acid, an antioxidant relevant for food and pharmaceutical industries. However, for each indicated application there is a lot of serious inconsistency in the obtained results besides a strict control of parameters governing such bioprocesses. Present work reveals the substrate quality is highly responsible of those inconsistencies; in the hydrolysis patterns of tannic acid under enzymatic hydrolysis different yields of gallic acid production are obtained and different induction levels of tannase are generated during microbial hydrolysis. Several commercial tannic acids have important differences of quality and chemical profiles. The chemical profiles of several commercial brands of tannic acid were obtained by FT-IR and HPLC. A quantification of gallic acid produced during the enzymatic hydrolysis of tannic acid was made using a commercial tannase to determine the efficiency of degradation of the hydrolysable tannin. Microbial degradation of tannic acids was kinetically monitored at different initial concentrations evaluating the fungal growth and tannase activity produced by *Aspergillus niger* GH1. Different chemical profiles were detected in the several commercial brands affecting the enzymatic and microbial profiles of degradation.

PS1: 45

**Interactions between dairy proteins and rosmarinic acid: effects on the antioxidant activity****Ana Raquel Madureira<sup>1</sup>, Ana Maria Gomes<sup>1</sup>, Bruno Sarmento<sup>2</sup>, Maria Manuela Pintado<sup>1</sup>**<sup>1</sup>Escola Superior de Biotecnologia, Portugal; <sup>2</sup>CICS, Health Sciences Research Center, Instituto Superior de Ciências da Saúde-Norte, Gandra, Portugal

Antioxidants are defined as “any substance that delays, prevents or removes oxidative damage to a target molecule” (Halliwell and Gutteridge, 2007), and can be found in natural sources such as fruits or plants, in particular aromatic and medicinal plants. One such example is rosmarinic acid, which can be found in sage (common English name for *Salvia* sp.), a plant grown in poor soils of Mediterranean basin, and used in traditional medicine in Portugal. Besides their antioxidant activity, other biological activities have been associated to this plant, owing to their content in such compound, such as anti-diarrhea vector, and to help in digestion, contribute to heal wounds, play an anti-inflammatory role, fight insomnia and decrease blood pressure. Their inclusion in any food or being part of a nutraceutical ingredient will contribute to an increase of the nutritional and health benefits value, e.g. milk or yogurt. Unfortunately, several studies indicate that incorporation of polyphenols in protein rich matrices (e.g. milk) is not feasible, because of the interactions between matrices components (e.g. dairy proteins) (Kyle *et al.*, 2007; Arts *et al.*, 2002). These interactions can generate complexes protein-polyphenol which can affect the antioxidant activity and also the bioavailability of the polyphenol in the intestine. Therefore, the study of the effect of interactions between the main dairy proteins such as casein,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin and rosmarinic acid was studied. For this purpose, solutions of such dairy proteins at the concentrations which are normally found in raw milk were mixture with rosmarinic acid (0.1 mg/mL). To achieve the polyphenol percentage which precipitated with the proteins, the mixtures were centrifugated, and the supernatants were collected and submitted to analyses. Measurements using ABTS•+ method to assess the total antioxidant capacity *in vitro*, and Folin-Ciocalteu method, to assay for total phenolics were made in the initial solutions and in the supernatants obtained. In order to identify and quantify the complexes formed, the mixtures were subject to analysis by HPLC. The measurements of the antioxidant activity by ABTS showed different patterns, which were confirmed by the Folin-Ciocalteu and HPLC chromatograms. These results differ in what concerns the dairy proteins mixture.

PS1: 47

### **Isolation of new strains acid lactic bacteria with potential use with probiotics from traditional Mexican fermented drinks (agua miel, sotol y pozol)**

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Mexico is a country with big contrast, tradition and great cultural diversity and biological. It is possible to taste different foods like traditional fermented drinks. In these drinks the very important phase is the process on the microorganism growth and several activities (acid lactic production, acid acetic, alcohol production and mixes between others). In the literature exist different reports on the study of microorganism probiotics and effects on human, but in this project the aim was the isolation of novel strains probiotics using with samples new sources for the obtained for example Mexican fermented drinks (agua miel, pozol and sotol). In each sample was obtained the pH and Brix value, after were inoculated in liquid media MRS and incubated in room at 37°C. In the next step the probiotics strains were selected and purified through a characterization and identification microscopic, macroscopic and using biochemical test. Also were realize activity probiotic test, for example: pH acid tolerance, high temperature tolerance, milk clotting capacity, growth in hostile media, inhibition of pathogenic microorganism of strains selected and tested sensitivity of antibiotics. The strains isolated presented antimicrobial activity opposite with food pathogens microorganism (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp. and *Enterobacter aerogenes*). Also presented resistance versus antibiotics, benzilpenicillin, ampicillin, tetracycline and trimethoprim – sulfametoxazol. In the present research was obtain 5 probiotics strains isolated of new sources of fermented drinks made handmade (aguamiel, pozol black, pozol white, sotol filtered and sotol without filtered). This showed the possibility of using these strains isolated with probiotics based on the results *in vitro*.

PS1: 48

### Microbiological examination and antioxidant potential of ready-to-eat salads from organic and conventional production systems in Portugal

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In recent years, an increasing number of studies have linked the consumption of fresh vegetables and occurrence of gastrointestinal diseases. Some recent outbreaks have been associated with consumption of salads contaminated with pathogens. Vegetables can become contaminated while growing, during harvesting, postharvest handling or during distribution. Organic production has been considered to represent a potential risk to public health, due to the cultivation and processing methods. The aim of this study is to compare the microbiological quality and biological value of 6 different baby-leaf and ready-to-eat salads (green lettuce, red lettuce, watercress, rucola, chard and corn salad) produced in two different production systems, organic and conventional. A total of 60 samples (10 replicates per each) were used in the survey. The conventional production was purchased at supermarkets and the organics collected from local farmers. Microbiological analyses were done using the standard methodologies (ISO methods). The samples were analyzed for the presence of total aerobic bacteria, *Enterococcus* spp., *Escherichia coli*, *Pseudomonas* spp. and *Salmonella* spp. Simultaneously, total phenolics with Folin-Ciocalteu method and antioxidant activity, of respective methanolic extracts, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method were measured. The mean counts of total aerobic bacteria were between 8.0 and 9.2 log<sub>10</sub>cfu.g<sup>-1</sup> from conventional whilst from organic varied between 8.1 and 9.6 log<sub>10</sub>cfu.g<sup>-1</sup> respectively. For *Enterococcus* spp. the mean counts varied between 0.0 to 2.8 log<sub>10</sub>cfu.g<sup>-1</sup> from conventional production and between 0.0 to 3.0 log<sub>10</sub>cfu.g<sup>-1</sup> for organic production; for *E. coli* the mean counts varied between 0 and 8.0 log<sub>10</sub>cfu.g<sup>-1</sup> and 0 and 9.8 log<sub>10</sub>cfu.g<sup>-1</sup> from conventional and organic production respectively. The *Pseudomonas* spp. and *Salmonella* spp. were not detected. The bacteriological quality of the organic and conventional samples was acceptable and within the values recommended by the EC regulations, nevertheless the contamination of organic does occasionally occur. The total phenolics and antioxidant activity were in average higher in organic production for red lettuce (532.01±14.5 gallic acid equivalent\*100 g<sup>-1</sup> dry weight and 83.2 ±0.1 inhibition of DPPH radicals respectively), thus the red lettuce showed the highest biological potential.

PS1: 49

### Microbiological profile of sourdough throughout time

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This work was aimed at enumerating the viable microorganisms in sourdough for *broa* – a Portuguese traditional bread, in attempts to better characterize their viability and profile throughout fermentation of sourdough and ripening of mother-dough. Dough was prepared following the traditional manufacture protocol, and kept for several weeks under controlled temperature and relative humidity. The effect of aeration of sourdough upon the microbial viable counts was studied in parallel. Said sourdough was obtained from maize and rye flours, in the absence of any commercial starter culture – instead, a small amount of fermented dough from a previous batch (mother-dough) was used as inoculum. Viable counts of mesophilic vegetative forms, yeasts, molds, Gram<sup>-</sup> rods, endospore-forming and nonsporing Gram<sup>+</sup> rods, and catalase<sup>+</sup> and catalase<sup>-</sup> Gram<sup>+</sup> cocci were obtained following standard plate counting on 20 different culture media. Presumptive yeasts, *Lactobacillus* and *Bacillus* were the predominant microorganisms present by the end of regular ripening of sourdough. Fermentation proved to play an important role upon inhibition of undesired rods and *Staphylococcus*. By 24 h of fermentation, Lactic Acid Bacteria (LAB) belonging to Gram<sup>+</sup> cocci group accounted for an important fraction of the biodiversity found in sourdough. The pH value, arising especially from the metabolic activity of LAB, decreased significantly within the first 24 h. Moreover, aeration produced effects upon the microbiological count evolution within time. An increase in ripening time of sourdough for *broa* is advisable, so as to take full advantage of ecological competition against undesirable microflora, and thus eventually increase its shelf-life.

PS1: 50

**Molecular and physiological characterization of non-*Saccharomyces* wine yeasts and its potential use in winemaking****António Inês<sup>1,2</sup>, Arlete Mendes-Faia<sup>1,2</sup>, Alexandra Mendes-Ferreira<sup>1,2</sup>**<sup>1</sup>Universidade Trás-os-Montes e Alto Douro, Portugal; <sup>2</sup>Institute for Biotechnology and Bioengineering, Centre of Genomics and Biotechnology

Non-*Saccharomyces* yeasts include different species which comprise an ecologically and biochemically diverse group capable of altering the fermentation dynamics and wine composition and flavor. Formerly regarded as spoilage yeasts, are currently recognized as adjuncts to *S. cerevisiae* to exploit their flavor-complexing properties. Despite the increasing interest on their industrial application, only a few commercial preparations of single or blends of yeasts are available in the market. Therefore, work is still needed for better understanding their contribution and persistence during winemaking. In this study, sixty autochthonous non-*Saccharomyces* isolates from grape-must and wines were putatively identified as *Hanseniaspora* spp. and *Metschnikowia pulcherrima* based on their asexual reproduction and pulcherrimin production, respectively. Molecular characterization was performed by MSP-PCR with microsatellite oligonucleotide primer (GTG)<sub>5</sub> and csM13. The identification was achieved by comparing their genotype profiles with those of the reference strains, and confirmed by sequencing the D1/D2 domain and/or to PCR-RFLP analysis of ITS region. No correlation was found between the genotype affiliations and geographical origin of strains, indicating that musts or wines from a particular grape variety or winery are not preferentially colonized by a specific group. Genotypic and phenotypic variability was found within strains isolated from the same sample at different stages of fermentation. To study their potentially relevant features, all strains were surveyed for their ability to produce enzymes that can positive or negatively affect the wine quality, such as  $\beta$ -glycosidase, protease and sulfite-reductase activities and resistance to cerulenine, 5,5,5-trifluoro-*DL*-leucine (TFL), ethanol and SO<sub>2</sub>. Most of the *Hanseniaspora* isolates secreted  $\beta$ -glycosidase and were resistant to cerulenine, TFL and sulfite. Great divergence was found in proteolytic and sulfite reductase activities and on their tolerance to ethanol. High intra-species variability was found among *M. pulcherrima* strains regarding  $\beta$ -glycosidase activity and ethanol tolerance. The results indicate that these wine yeasts can play a major role during vinification, by introducing diversity and complexity into the final wine flavor. Therefore, selection of adequate strains to use as adjuncts of *S. cerevisiae* can be very useful for improving wine fermentation process.

PS1: 51

**Mycotoxins production by *Aspergillus* section *Flavi* isolated from harvested maize****Célia Soares, Armando Venâncio**

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Mycotoxins are toxic compounds produced by some species of filamentous fungi known to cause serious health problems in humans and animals. In cereals, the key mycotoxigenic fungi in partially dried grains are *Aspergillus flavus* (aflatoxins), *A. ochraceus* (ochratoxin A) and some *Fusarium* species (fumonisins, trichothecenes), being maize is one of the more susceptible commodities. Aflatoxins (AF) produced by *Aspergillus* section *Flavi* species and fumonisins produced by *Fusarium verticillioides* are prominent mycotoxins associated with maize economic losses. Cyclopiazonic acid (CPA) is produced predominantly by *A. flavus* strains and occurs naturally in a wide variety of crop products as a co-contaminant with AF. In Portugal, maize is one of the most important field crops. In 1999, maize occupied 163 497 ha, with a production of 933 800 tons. The aim of this work was to detect whether the isolated species of *Aspergillus* section *Flavi* were AF and CPA producers and whether these mycotoxins were present in post-harvested maize samples. In order to do so, ninety five maize samples were collected from different agroclimatic regions of Portugal. From these samples, 25 grains chosen randomly were plated in 5 Petri dishes with solidified agar, incubated and the *Aspergillus* section *Flavi* strains were isolated under stereomicroscope observation. All these strains were screened for AF and CPA production, as described elsewhere (Soares et al, 2010). Also, the maize samples were screened for AF and CPA. The extraction methodology was based on protocols provided by Vicam for aflatoxins (AF), with immunoaffinity clean-up and a chloroform extraction was used for cyclopiazonic acid (CPA). Both methods were validated by analysis of replicate spiked samples with 40 µg/Kg of AF and 4000 µg/Kg of CPA. A matrix blank was also analyzed to determine any residual mycotoxin levels. In conclusion, four hundred and seventeen strains of *Aspergillus* section *Flavi* isolated from maize grains from three Portuguese regions were evaluated for AF and CPA production on agar plates. These were found in seventy four samples (78%). CPA and AF were produced by 74% and 40% of the isolates, respectively. Mycotoxin detection of the 95 samples obtained was also performed revealing that 8% of the samples were positive for aflatoxins. CPA was not detected in any sample.

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PS1: 52

**New tools for enzyme production by the fungus *Bjerkandera adusta*****Rocío Reina<sup>1</sup>, Elisabet Aranda<sup>1</sup>, Patricia Godoy<sup>1</sup>, Martin Kluge<sup>2</sup>, René Ullrich<sup>2</sup>, Inmaculada García-Romera<sup>1</sup>, Christiane Liers<sup>2</sup>**<sup>1</sup>Spanish National Research Council (CSIC), Spain; <sup>2</sup>International Graduate School of Zittau (IHIZ), Germany

Fungal peroxidases (e.g. manganese peroxidase – MnP, EC 1.11.1.13) are involved in the biodegradation of lignocelluloses and lignin and participate in the conversion of recalcitrant pollutants as well as in natural turnover of humic substances. Contamination by olive mill wastes “alpeorujo” is becoming a crucial problem in the Mediterranean area, particularly for the main producers that release more than 4 million tons of “alpeorujo” per year. Due to its high concentration of aromatics (e.g. phenolics, terpenoids) olive mill waste comprises highly phytotoxicity effects, indeed these compounds seems to be appropriate as potential enzymatic inducers. The present study focused on the effect of “alpeorujo” for the enhanced production of peroxidases by *Bjerkandera adusta* and its effect on the main components. By *in-vivo* studies, the fungal growth and enzyme secretion was investigated using solid-state and liquid cultures with different growth media. Our results showed an increase of 700-fold ( $700 \text{ UL}^{-1}$ ) of MnP after 2 weeks in the presence of “alpeorujo” using barley as single carbon source. In liquid cultures using synthetic media supplemented with 10% of an aqueous extract of “alpeorujo” we detected an up to 3-fold higher MnP activity ( $2000 \text{ UL}^{-1}$ ) than in the respective control ( $600 \text{ U L}^{-1}$ ). This enzyme inducing effect was correlated in both cases with a strong reduction in phenolics (~85%) and with an increase of the molecular mass distribution of water-soluble aromatics to higher fragments (from 1.5 to 35 kDa), indicating a polymerization effect. By *in-vitro* reaction systems comprising of pure MnP and aqueous alpeorujo extract we were able to demonstrate this polymerising effect of the peroxidase. Our findings indicate that the phenol-rich “alpeorujo” directly stimulates the secretion of extracellular fungal biocatalysts, obviously as a detoxification process by the organism. The phenols were oxidized that leads finally to spontaneously polymerization of the phenoxy radicals to higher mass fragments, a process probably suitable for phenol removal of agricultural wastes.



PS1: 53

### Occurrence of antibiotic resistance in *Staphylococcus* spp. at dairy farms with conventional and organic system and its evaluation with GLMM

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The purpose of our study was to evaluate the occurrence of antibiotic resistance (ATBR) in *S. aureus* (SA), *S. haemolyticus* (SH), *S. chromogenes* (SC), at dairy farms in Czech Republic. Besides, we made comparison between conventional and organic production systems in dairy cattle. During statistical analysis we had to tackle with problems associated with log-transforming data and data from a field research: unbalanced data with combination of fixed (species, production system) and random (farm) effects. We found the package lme4 (Bates and Maechler 2010) in R software environment as the great tool for data diagnostics and fitting of generalized linear mixed regression models (GLMM) with the Poisson error distribution, that should be used when numbers of occurrence are evaluated as dependent variable. 502 milk samples from 6 farms with organic and 40 farms with conventional production system were tested on the occurrence of three *Staphylococcus* species and their resistance to 14 ATB. The mean occurrence of ATBR was lower at organic farms (0.19 per sample) than at conventional farms (0.49 per sample). The reason was because a few farms in the convention system had higher occurrence of ATBR, and moreover, the samples from these farms has been sent to the laboratory more often. This part of variability was represented as the significant random effect "farm" in the model (The AIC without "farm" got worse from 416.6 to 1116.4). Generally, the systematic fixed effect of production system on ATBR was not significant ( $p=0.766$ ). As concerns to species, the highest mean occurrence of ATBR was in SH (1.15 per sample,  $p<0.001$ ). Differences in occurrence of ATBR between SA (0.44) and SC (0.04) was not significant ( $p=0.213$ ). Besides, we did not find any SC at farms with organic production system at all. We can conclude that occurrence of resistance to ATB is not systematically affected by the production system but is caused by management at the very farm, as the significant random effect "farm" indicated. It was also obvious that there were 20 conventional farms with no ATBR. The lme4 package for fitting of regression GLMM seems to be very helpful equipment to test hypotheses and evaluate data by the efficient manner and should deserve more attention of scientists.

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PS1: 54

### Optimization of the expression of a chitinase gene from *Lactococcus lactis*

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Chitin, a homopolymer of N-acetyl-glucosamine connected together by  $\beta$ -1, 4 bonds, is the second most abundant polysaccharide in nature. Chitin is present in the exoskeleton components of arthropods and in the cell wall of higher fungi. Chitinases are chitin-degrading enzymes that hydrolyze the  $\beta$ -1, 4 linkages of chitin. Chitinolytic microorganisms and chitinolytic enzymes, able to degrade chitin, have received increased attention during the last years due to their broad range of applications in medicine, food and agriculture. Particularly, in agriculture, chitinases can play a very important role in the biological control of plant diseases because they affect fungal growth through lysis of the cell walls. Because of the significance of chitinases in the control of plant pathogens, there is an increasing interest in new more effective enzymes and good producer strains to be used in such applications. In the present study we report the optimization of the expression of a chitinase gene from *Lactococcus lactis* in *E. coli*. The chitinase was cloned and expressed, at different levels, in *E. coli* using different expression vectors, IPTG concentrations, induction time and culture conditions. The recombinant protein was active in an acidic range of pH and in a range of temperature between 25 °C and 55 °C, keeping a high activity under suboptimal conditions. Due to the characteristics and stability of the recombinant protein its potential antimicrobial activity was studied.

PS1: 55

**Photoinactivation of microorganisms under fluorescent light****Pedro Maçalhães<sup>1</sup>, Vera M. Sousa<sup>1</sup>, Fernanda Oliveira<sup>2</sup>, Adélio Mendes<sup>1</sup>, Olga C. Nunes<sup>1</sup>**<sup>1</sup>LEPAE - Department of Chemical Engineering, University of Porto - Faculty of Engineering, Porto, Portugal; <sup>2</sup>CIN, Corporação Industrial do Norte, S. A., Maia, Portugal

The increase of hospital-acquired infections with resistant microorganisms is an alarming issue in nowadays society. This worldwide problem drew the attention to the development of new antimicrobial cleaning materials in order to minimize the acquisition of antibiotic resistance and spreading. In this area, photoinactivation of microorganism is a promising methodology because it is believed that it does not promote resistance acquisition. The most commonly used photocatalyst is titanium dioxide (TiO<sub>2</sub>). Anatase, one of the three crystalline structures of TiO<sub>2</sub>, has a band gap of 3.2 eV; upon excitation by radiation with wavelength smaller than 385 nm, the photon energy causes electrons to jump from the valence to the conduction band, creating positive holes in the valence band. The holes, in the valence band, show a reduction potential of ≈3 eV and can react with H<sub>2</sub>O to produce OH<sup>•</sup> and H<sup>•</sup>; the excited electrons in the conduction band, have an oxidation potential of about 0.2 eV, and can reduce O<sub>2</sub> to O<sub>2</sub><sup>•-</sup>. Like in phagocytes, from these radicals a great number of highly reactive microbiocidal oxidants are formed. In this study, the photoinactivation performance of a commercial photocatalyst and the same photocatalyst decorated with copper and silver nanoparticles under radiation with higher wavelength (fluorescent light) is assessed. Suspensions containing 1 x 10<sup>6</sup> CFU ml<sup>-1</sup> *Escherichia coli* DSM 1103 were used to evaluate the antimicrobial activity of these photocatalysts. Irradiation of commercial TiO<sub>2</sub> particles with fluorescent light for 40 min resulted in a 0.46±0.03 log reduction of viable cells. The biocidal activity of the metals resulted in similar log reduction values (0.32± 0.02 and 0.36±0.03 log reduction of viable cells for Cu<sup>+</sup> and Ag<sup>+</sup> nanoparticles, respectively). However, the irradiation of the photocatalyst decorated with Cu<sup>+</sup> and Ag<sup>+</sup> nanoparticles with fluorescent light permitted to increase its activity (2.40±0.02 and 3.45±0.01 log reduction, respectively) to values closer to that obtained when the photocatalyst was irradiated with UV (2.88±0.04 log reduction). The high values of inactivation of *E. coli* cells obtained in this study suggest that the decorated photocatalyst may be used in future in interior decorative paints with antimicrobial properties.

PS1: 56

**Potential prebiotic effect of whey protein and spent brewer yeast hydrolysates by enzymes of *Cynara cardunculus* extract****Joana Odila Pereira, Maria Faria Amorim, Maria Manuela Pintado**

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Biologically active peptides are of particular interest to food science and health since they have demonstrated beneficial physiological roles upon ingestion. These biopeptides can be released and/or activated *in vivo* during gastrointestinal digestion or otherwise via enzyme-mediated proteolysis throughout food processing. The survival of many bacterial species inhabiting the large bowel depends essentially on the substrates made available to them, most of which come directly from the diet. Some of these substrates can be selectively considered as prebiotics - which are non-digestible food ingredients that can stimulate beneficial bacteria such as lactobacilli or bifidobacteria growth in the colon. Thus, the major objective of this research work was to study the potential prebiotic activity of peptide extracts obtained via hydrolysis of whey proteins from a mixture of milk (cow, ewe and goat) and spent brewer yeast extracts achieved by cardosins present in *Cynara cardunculus* aqueous extract. The strains tested were probiotic strains viz. *Lactobacillus acidophilus* Ki and *Bifidobacterium lactis*Bb12 and Fructooligosaccharides were used as positive control of prebiotic activity. From whey protein hydrolysates two fractions were tested, the ultrafiltration permeate (PM >1000 Da and < 20000 Da) and a nano-permeate (<1000 Da). In the yeast extract two fractions were tested after hydrolysis procedure, the nano-retentate (>1000 Da) and nano-permeate (<1000 Da), both obtained from ultrafiltration permeate. The fractions from the different extracts were studied at various concentrations with or without glucose addition. All the extracts analyzed showed good prebiotic activity through the enhancement of growth of both probiotic strains, when in presence of a sugar source (glucose). In most of the cases the growth stimulation was even higher than Fructooligosaccharides. *Lactobacillus acidophilus* Ki was the bacteria further stimulated by these extracts, particularly when added ultrafiltration permeate of whey hydrolyzate (>1000 and >20000 Da). Incorporation of the extracts in media without glucose did not produce such good results, but the better activity was obtained for the ultrafiltration permeate of whey hydrolyzate (>1000 and >20000 Da); this result was expected since both extracts stimulate mainly the bacterial growth through the nitrogen compounds: peptides and aminoacids.

PS1: 57

**Production and characterization of the extracellular chitinase from *Metarhizium anisopliae* strains 360 and 425****Cynthia Barbosa Rustique!, João Atilio Jorge, Luis Henrique Souza Guimarães.**

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There are about 2.5 million insect and 10% of this total can be pests. Entomopathogenic fungi such as *Metarhizium anisopliae* are able to control insect pests and for an efficient control, it is necessary to select the fungus correctly according to the biological and biochemical parameters, such as the production of enzymes. In this case, the production of chitinases is related to pathogenicity and virulence. The aim of this study was to compare the production of chitinases by the strains 360 and 425 of *M. anisopliae*. The strains were grown in different liquid cultures: yeast extract (1%), yeast extract (1%) + chrysalis (1%), yeast extract (1%) + glucose (1%), yeast extract (1%) + chrysalis (1%) + glucose (2%) and yeast extract (1%) + extract of *Diatraea saccharalis* (1%) for different periods (24 and 240 h) at 26 °C under agitation. The enzyme activity was determined using 1 mM 4-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide as substrate in 100 mM sodium acetate buffer, pH 5.0. Conidia was also observed by scanning electron microscopy (SEM). For strains 360 and 425 the best production of chitinase was obtained using medium constituted by yeast extract + chrysalis, for 96h and 144h, respectively. The DEAE-Cellulose chromatographic profile for the chitinases of both strains were similar. The optimum temperature and pH for chitinase activity was 5.0 and 60°C for both strains. The chitinase of isolate 360 was stable at pH range from 4.0 to 7.0, while the strain 425 was not. The enzyme produced by *M. anisopliae* 360 showed  $t_{50}$  of 5 minutes at 60 °C, while the enzyme of strain 425 was stable from 40 °C to 60 °C. The electrophoretic profiles (10% SDS - PAGE) of extracellular proteins of isolates 360 and 425 were different and the conidia showed small differences in the thickness. We conclude that the strain 425 produced higher levels of extracellular chitinase than that observed for strain 360, confirming its virulence potential on *D. saccharalis* as previously observed.

PS1: 58

**Production of alpha-amylase by *Bacillus licheniformis* (UCP 1010) based on economic medium applying factorial design**

**Daniel Paulo de Oliveira, Kaoru Okada, Galba Maria de Campos Takakai, Aline Elesbão do Nascimento, Clarissa Daisy da Costa Albuquerque, Carlos Alberto Alves da Silva**

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Alpha-amylases (E.C.3.2.1.1.) occur in animals, plants and microorganisms and breaks down the  $\alpha$ -1,4 glucosidic bonding of linear amylose and branching amylopectin which are the major building blocks of starch. This enzyme is of great significance in present day biotechnology with applications ranging from food, textile to paper industries. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. The production of economically important  $\alpha$ -amylase is essential for conversion of starches into oligosaccharides. As excretion of products of metabolism is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy. As excretion of products of metabolism is a part of survival strategy of microbes in certain environments, overproduction of enzymes by media manipulation may be considered a better strategy. The *Bacillus* genus is known to be good producers of thermostable alpha-amylase, and these have been widely used for commercial production of the enzyme for various applications. The production was carrying out to amylase production, replacing the soluble starch, by corn starch and potato starch, trough of factorial planning  $2^3$  with 4 levels of variance. The experiments were carrying out at 150 rpm, 37 °C, during 96 hours. The microbial growth was evaluated, pH determination, the glucose consumption by the DNS and determination of specific enzyme activity. The best results showed a specific activity of 0.756 U/mg, at pH of 8.82, and quantitative activity of amylase 0.99 U/dL.

PS1: 59

### **Production of biosurfactant by *Candida lipolytica* in semi defined medium using babassu oil as substrate**

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The composition of culture media for biosurfactant production in batch cultures of *Candida lipolytica* by a fractional factorial design has been studied. A set of 16 experiments with two central points in duplicate, utilizing five factors (yeast extract, urea, ammonium sulfate, and potassium phosphate and babassu oil) at two levels was statistically combined. The analysis of data from this experiment shows a negative effect of the variable ammonium sulfate. Two more factorial designs were performed to determine the effect of the lack of ammonium sulfate and urea. The results show that all the factors studied were found to influence biosurfactant production, and that best conditions for higher biosurfactant synthesis is obtained when yeast extract, urea, and potassium phosphate are at a high level +1 concentration. However, with ammonium sulfate at a lower level and babassu oil, the level is independent of the response (biosurfactant production). These results indicated the importance of this study in obtaining maximal production of biosurfactant from *Candida lipolytica* so that these compounds can be used for many purposes.

Supported by CAPES, CNPq, and UNICAP

PS1: 60

### Properties of protein hydrolysates from Cape hake by-products prepared by three different methodologies

**Carla Pires, Tereza Clemente, Irineu Batista**

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Fish protein hydrolysates (FPH) are high-valued products obtained from fish by-products or underutilized species. The usual method involves a fine grinding and of raw material a dispersion in water to allow a good enzyme access. Thus, the objective of this work was to study two alternative methods of FPH preparation in order to improve the yield achieved and their quality. Cape hake by-products were used as raw material. The protein hydrolysis was done by the following methods: directly on the minced raw material homogenized in water (A), solubilised proteins at pH 11.0 (B) and recovered proteins by the alkali-aided pH shift process (C). For the hydrolysates preparation the protein content was adjusted to 1.4 %, pH to 8.0, temperature to 50 °C and 1 % Alcalase 2.4L added. The functional and antioxidant properties of the hydrolysates obtained were evaluated. The degree of hydrolysis of the hydrolysates prepared by the three methods was 19-22 %. The yields achieved were 70.5 % for method A, 71.9 % for B and 46.0 % for C. The peptide profile of hydrolysates obtained by the three methods was very similar. All hydrolysates had a yellowish colour and contained 80-86% protein. Hydrolysates from method C had significantly higher ( $p < 0.05$ ) emulsifying activity index and fat absorption but lower foaming capacity (FC) than the others. The solubility of hydrolysates was in range of 71-76 % and increased the water holding capacity of minced fish of about 9 %. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of all hydrolysates was relatively low. However, the fractions of hydrolysates A and B (20 mg/ml) with MW > 1 kDa reduced DPPH 62 and 52.7 %, respectively. Hydrolysates A and B had similar reducing power which was almost the double of hydrolysate C. The reducing power of fractions of the hydrolysates A and B with MW < 1 kDa was significantly higher ( $p < 0.05$ ) than those with MW > 1 kDa. The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging activity of all hydrolysates was relatively high (ca. 2.5 mM) for 10 mg/ml. The fractions with MW < 1 kDa and > 1 kDa of all hydrolysates showed lower ABTS radical scavenging activity. The method C allowed obtaining considerably lower yield but all hydrolysates had similar characteristics.



PS1: 61

### **Strategies for increasing aroma production by *Yarrowia lipolytica*: hydrolysis of substrate and cell immobilization**

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γ-Decalactone is a peach-like aroma compound well known in several food and beverages, reason why the food industry has a high interest in its biotechnological production. One of the better known applications is the biotransformation of ricinoleic acid into γ-decalactone, catalysed by yeasts with GRAS status, like *Yarrowia lipolytica*, since in this case a natural label is conferred to the aroma, which is very important, considering the increasing health- and nutrition-conscious lifestyles. However, for the different processes of aroma production described in the literature, a low yield of γ-decalactone is obtained. An alternative production technique should be considered to improve aroma production. The immobilization of viable cells for use in the biotransformation process is an approach of great interest since, as compared with free cells, immobilized cells exhibit a higher tolerance to toxic compounds (such as the aroma itself) and higher productivity. Moreover, in order to increase the availability of the substrate (castor oil) to the cells for the γ-decalactone production, hydrolyzed castor oil can be used. This hydrolysis can be promoted by enzymatic action, more specifically by extracellular lipase (Lipozyme TL IM). The main purpose of this work is to study some strategies for increasing γ-decalactone production, namely the cell immobilization in calcium alginate and integrating an enzymatic hydrolysis of castor oil in the process, using a commercial lipase. Cells were immobilized in 3% (w/v) sodium alginate solution and the extracellular enzyme Lipozyme TL IM was used for castor oil hydrolysis. The use of hydrolyzed castor oil in the aroma production decreased the lag phase for γ-decalactone secretion and the immobilized cells exhibit more tolerance for toxic compounds than free cells and enhanced fermentation productivity.

PS1: 62

### Studies on the production of DHA, squalene and carotenoids by *Thraustochytrium* sp. ATCC 26185

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Strains of the Thraustochyrid genus have been used for the production of polyunsaturated fatty acids (PUFA), squalene and carotenoids. These microorganisms represent a valuable and sustainable source of those high added-value biomolecules and can be used as an alternative to fish and liver oil from some deep-sea sharks. The goal of this work was to study the effect of culture medium composition (salinity and glucose) on the production of such biomolecules by *Thraustochytrium* sp. ATCC 26185. This microorganism was cultured under heterotrophic conditions using a medium similar to that reported by Chen<sup>1</sup>. Two salt concentrations (1.5 and 3.9%) and two glucose grades (analytical and commercial) were used. Fatty acid profile and squalene content were determined by GC. Total carotenoids were measured by spectrophotometry and the main carotenoids were quantified by HPLC. Regarding the effect of salinity (S), any differences on the biomass production were observed. DHA was the major PUFA obtained, having been attained a maximum of 14.1% (1.5% S) and 12.8% (3.9% S) at 3-4 days. A maximum squalene production was obtained after 2-3 days with a considerably higher production (2.04%) in the medium with 3.9% S when compared with the level of 0.83% obtained in the lower salinity medium. The total carotenoids production was 8.29 mg/100g at 1.5% S after 120 hours, whereas it achieved 48.84 mg/100g at 3.9% S after 192 hours. Among the carotenoids,  $\beta$ -carotene production increased 4 times in the medium with higher salinity. No significant differences in synthesis of those targeted biomolecules were registered between the trials with analytical and commercial grade glucose. The average production of DHA and squalene after 7 days was 14% and 0.4%, respectively. Total carotenoids content amounted to 9.3 mg/100g. The main conclusions that can be drawn are: The glucose source has no influence on the synthesis of DHA, squalene and carotenoids. These results indicate that the commercial glucose could be advantageously used in culture mediums since its cost is 17 times lower than that of the analytical one. The increased salinity considerably stimulated the production of squalene and carotenoids, however there were no significant differences regarding DHA concentration. The increased synthesis of carotenoids may be related to a response to oxidative stress as reported for other microorganisms.

<sup>1</sup> Chen et al. 2010. New Biotechnology, 27, 4: 382-389.

PS1: 63

### Study of the effect of glucose concentration on the production of DHA by *Thraustochytrium* sp

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Polyunsaturated fatty acids (PUFAs) have gained great importance due to their health beneficial effect<sup>1</sup>. Some microorganisms of the genus *Thraustochytrium* are good alternative sources for the production of  $\omega$ 3 PUFA, particularly DHA<sup>2</sup>. However, it is very important to optimize the growth medium and growing conditions for obtaining the highest production of biomass and DHA. The objective of this work was to study the effect of different glucose levels on the production of DHA by *Thraustochytrium* sp. For the inoculum preparation, the microorganism *Thraustochytrium* sp (ATCC® 26185™) from PDA medium was inoculated in the growth medium 790 By+ ATCC, stirred at 150 rpm and incubated at 30°C in the darkness for 48 h. This starting culture was inoculated (10%, v/v) in a medium containing (g.L<sup>-1</sup>): KH<sub>2</sub>PO<sub>4</sub> (1.54), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (5.68), NaCl (0.71), MgSO<sub>4</sub>·7H<sub>2</sub>O (2.62), yeast extract (8), glucose 30 (G1 trial) or 60 (G2 trial) in salt water 1.2% (p/v) salinity. The cultures were performed in the bioreactor containing 3.5 L, at 23°C, 100 rpm, 2.5 vvm, pH 4-6, in the dark. Sample was done every 48 h for determination by of biomass (g.L<sup>-1</sup>), sugar content<sup>3</sup> and fatty acids profile<sup>4</sup>. In G1 trial, the maximum biomass obtained was 21.2 g.L<sup>-1</sup> after 144 h, whereas in G2 trial the maximum biomass was 7.0 g.L<sup>-1</sup> after 96 h. The maximum production of DHA was attained at 96 h (0.79 g.L<sup>-1</sup>) for G1 trial and 48 h (0.18 g.L<sup>-1</sup>) for G2 trial. Glucose in DHA conversion rate in G1 trial during the first 96 h was 0.045 (S<sub>DHA/Glucose</sub>) and, for G2 trial during the first 48 h, was 0.180 (S<sub>DHA/Glucose</sub>). It may be concluded that the highest production of DHA was achieved in the medium 30 g glucose.L<sup>-1</sup>. The highest glucose concentration seems to partially inhibit the growth of the microorganism, since, for 60 g.L<sup>-1</sup>, a lower biomass was attained.

<sup>1</sup> Ward et al 2005. Process Biochemistry, 40, 3627-3652. <sup>2</sup> Bajpai et al Applied Microbiology and Biotechnology, 35, 706-710. <sup>3</sup> Miller et al 1959. Analytical Chemistry, 31 (3), 426-428. <sup>4</sup> Lepage et al 1986. Journal of Lipid Research, 27, 114-120.

PS1: 64

**Surface hygiene assessment in a raw milk cheese plant: a case study****Patrícia Esteves Dinis<sup>1</sup>, Carlos Agostinho<sup>1,2</sup>, António José Mendonça<sup>3,4</sup>**

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Food contact surfaces in dairy industry are often a source of microbiological contamination of raw materials and dairy products. The presence of *Listeria monocytogenes* and *Salmonella* spp. in food contact surfaces can be a concern within dairy factories. Therefore, the efficacy of surface hygienic quality must be monitored to ensure that all surfaces are clean and free from pathogenic microorganisms. The aim of this study was to examine the hygienic quality of food contact surfaces in a raw sheep's and goat's milk cheese plant for six microbiological parameters and to identify critical points in the sanitation process. In this study 21 food contact surfaces were monitored during 75 days. Samples (sponge-swabs) were screened for Total Bacterial Counts (TBC), total coliforms, b-glucuronidase-positive *E.coli*, *Staphylococcus aureus* and *Pseudomonas* spp. counts and also for *Listeria monocytogenes* and *Salmonella* spp. detection. Our results show an average of 47 cfu/cm<sup>2</sup> TBC, 4 cfu/cm<sup>2</sup> total coliforms, 2 cfu/cm<sup>2</sup> b-glucuronidase-positive *E.coli*, 1 cfu/cm<sup>2</sup> *Staphylococcus aureus* and 6 cfu/cm<sup>2</sup> *Pseudomonas* spp. in food contact surfaces. Neither *Listeria monocytogenes* nor *Salmonella* spp. were detected from any of the 84 samples tested. This study showed that 86% of food contact surfaces were less than 100 cfu/cm<sup>2</sup> total bacterial counts, but 19% were more than 1000 cfu/cm<sup>2</sup> indicating the presence of biofilms in food contact surfaces. Cheese washing machines, cheese molds and cheese mold lips were the most contaminated surfaces. Total Coliforms and *Pseudomonas* spp. were the major contaminants. The Sanitation Plan implemented in the cheese plant were effective against *Listeria monocytogenes* and *Salmonella* spp., but needs improvement to control the contaminations in the cheese washing room, in particular in cheese washing machines. These data will help inform risk assessments associated with cleaning and disinfection of food contact surfaces in cheese industry.

PS1: 65

**Survival of a probiotic *Lactobacillus rhamnosus* strain on fruit smoothies as affected by pH modulation achieved with lemon juice****André Moreira<sup>1,2</sup>, Ana Pinto<sup>2</sup>, Domingos Almeida<sup>1,3</sup>**<sup>1</sup>Universidade de Aveiro, Portugal; <sup>2</sup>Frulact, SA; <sup>3</sup>Faculdade de Ciências, Universidade do Porto

Fruit-based food matrices can vehicle probiotics to lactose-intolerant consumers and provide diversification to the human diet. Fruit, however, are hostile environments for probiotic strains due to low pH and presence of antimicrobial compounds. The objective of this work was to evaluate the effect of lemon juice to alter pH of fruit smoothies on the survival of a probiotic strain. The use of lemon juice to adjust pH answers to the market demand for a clean label positioning (absence of food additives). Smoothies (100% fruit beverage) were prepared from mango and banana with final pH values of 3.2, 3.4, and 3.6. The pH adjustments were achieved via the addition of lemon juice concentrate ranging from 0.59 to 3.7% (mango smoothie) and from 0 to 4.1% (banana smoothie). The smoothies were pasteurized at 95 °C for 3-5 min, packaged into 200-mL glass bottles, cooled to 37 °C before inoculation with *Lactobacillus rhamnosus* R11 ( $10^9$  CFU/mL), and stored at 5 °C for 42 days. Viability was assessed throughout the storage period by plate counting in MRS agar. pH, soluble solid content, water activity, viscosity, and color were also characterized. Sensory analyses were used to assess the acceptability and preference of the probiotic smoothies. The initial  $a_w$  of 0.90 remained stable during storage. The average viscosity was 300 and 570 cP for the mango and banana smoothies, respectively. Soluble solid content was affected by the amount of lemon juice concentrate but remained unaltered during storage. The color of the smoothies remained stable. *L. rhamnosus* R11, without lemon juice concentrate added, remained within 0.2 log of the initial concentration during the 42 day storage period. However, significant acidification of the smoothies occurred. In the mango smoothie pH decreased from 3.8 (initial) to 3.2 (end of storage period), and in banana, from pH 4.0 to 3.4. Acidification had little effect on probiotic viability at pH 3.4 or 3.6, but significant decreases in viability were observed when pH was adjusted to 3.2. Under this pH value the reduction in cell count after 42 days was ca. 47%. Consumer panels revealed that acidification decreased the acceptability; however, a higher preference for the probiotic banana smoothie was observed. The results demonstrate that probiotic smoothies can be developed with *L. rhamnosus* R11. pH modulation via the addition of lemon juice concentrate can help the stabilization of the product, as long as the pH remains above 3.4.

PS1: 66

## The dual role of enterococci in food technology: bacteriocin production versus pathogenicity potential

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Enterococci are usually isolated from fermented foods, in which they contribute to the ripening/organoleptic characteristics, but nowadays are considered emerging pathogens, due to an increase of antibiotic resistances and production of virulence traits. Although enterococcal foodborne infections have not been described so far, this combination of factors raises special awareness regarding the food safety of products harboring enterococci. The present study analyzed Portuguese traditional meat fermented products namely, Catalão, Chouriço Preto, Linguiça, Paio and Salsichão, for the presence of *Enterococcus* spp., focusing on their pathogenicity potential and technological interest. After isolation the genetic diversity of the 154 meat enterococci was assessed by PCR-fingerprinting, leading to the selection of 75 strains representative of distinct groups, which were identified as *E. faecalis* (n=45), *E. faecium* (n=22) and *E. durans* (n=8) by multiplex-PCR. Antibiotic susceptibility testing revealed the following resistances: 1% for Ampicillin and Amoxicillin/Clavulanate, 100% for Cefalexin, 76% for Cephalexin, 3% for Chloramphenicol, 12% for Erythromycin, 71% for Gentamicin-10, 0% for Gentamicin-120, 100% for Nalidixic Acid, 36% for Penicillin G, 100% for Streptomycin, and Sulphamethoxazole/Trimethoprim, 60% for Tetracycline and 20% for Vancomycin. The presence of virulence factors was assessed by PCR-amplification and plate assays. The results were as follows: 5% for *ebpA*, 19% for *ebpB*, 28% for *ebpC*, 17% for *efaA<sub>IS</sub>*, 28% for *epa*, 25% for *fsrB* and 75% for *gelE*; the genes *agg*, *esp* and *cylA* were absent, 10 of the isolates produced gelatinase and none was  $\beta$ -hemolytic. Regarding the ability to produce biofilms: 8% were considered nonbiofilm-producers; 28% weak-biofilm-producers; 25.33% biofilm-producers and the majority, 38.67%, strong-biofilm-producers. Lastly, to evaluate their technological potential the meat-enterococci were tested for the production of bacteriocins and enzymes of technological relevance: 8% produced bacteriocins and several enzymatic activities were observed (e.g acid phosphatase, cystine arylamidase and esterase lipase). Overall, even though the meat enterococci present several antibiotic resistances and produce biofilms, due to a low number of virulence factors and to the absence of reports regarding foodborne infections, a low risk is probably associated with the presence of enterococci in these long-established traditional meat fermented products.

PS1: 67

### **The effects of phenolic acids on *Bacillus cereus* and *Pseudomonas fluorescens* single and dual biofilms**

**Madalena Lemos, Joana Teodósio, Paula Araújo, Filipe Mergulhão, Luís Melo, Manuel Simões**

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Biofilms constitute a concern to industry and biomedical areas. Their complex structures and the extremely resistant biofilm-forming bacteria indicate that new control strategies are necessary. Plant secondary metabolites have shown antimicrobial properties and are promising candidates for biofilm control. The use of these “green products” can significantly reduce the environmental burden caused by the use of conventional biocides. In this work, the effectiveness of two phenolic acids, ferulic and salicylic acid, was assessed on single and dual species biofilms formed by *Bacillus cereus* and *Pseudomonas fluorescens* on polystyrene microtiter plates. These bacteria are important contaminants on the food industry. The phenolic acids were tested as single compounds (ferulic acid-FA, salicylic acid-SA) and in combination (FSA). The effects of FA and SA were also assessed on swarming, swimming and twitching motilities. FA and SA only affected bacterial swimming, and had no effects on swarming and twitching motilities. The application of FA, SA and FSA caused low to moderate biofilm inactivation and removal. No clear relationship was obtained between the effects of phenolic acids on motility and biofilm control. The overall study demonstrates that the products tested were not effective on the control of *B. cereus* and *P. fluorescens* single and dual species biofilms. Additional work is in progress to assess the synergistic effects of green products with conventional biocides on biofilm control.

PS1: 68

**Use of high hydrostatic pressure extraction to enhance the flavonoids content of hops (*Humulus lupulus* L.) ethanolic extracts****Fátima L. Sousa, Mickael C. Santos, Jorge Manuel Alexandre Saraiva**

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Hops, the female flower clusters of *Humulus lupulus* is an essential ingredient in beer production, imparting beer with several organoleptic and sensorial properties. Moreover, some hops constituents are now known to have important health promoting properties and is the case of flavonoids, a group of phenolic compounds. Particularly important is the case of xanthohumol (XN), the main flavonoid of hops that has been shown to inhibit the development of tumors in early stages, to reduce the proliferation of pre-adipocytic cells, to have sedative and anti-inflammatory activities among several others [2]. At least some of these flavonoids are passed to wort beer during wort boiling and in smaller amounts appear on beer, contributing to increase the health benefits of beer consumption. Currently, for the sake of easier preservation and easiness of use, the beer industry uses hops extracts instead of hops itself. These extracts are usually produced by ethanol extraction, in a process that takes time and involves the use of temperature, what causes degradation of some of the flavonoid compounds showing health promoting characteristics. This work aimed at evaluate the effect of High (Hydrostatic) Pressure (HP) at room temperature on extraction of flavonoids from hops, by quantifying total flavonoids. Various experimental conditions were tested, such as concentration of ethanol (10-90%, v/v), as well as the ratio of hops to extraction solvent (from 20:1 to 200:1) at pressure treatments of 400 MPa during 5 min. Generally, extraction under pressure resulted in higher amounts of flavonoids. For instance, the pressurized samples with 50% of ethanol and ratio of 20:1 presented the higher amount of total flavonoids, being this amount 65% and 34% higher than the samples at atmospheric pressure with 50% and 90% of ethanol, respectively. Additionally, in the former case, much less extraction of chlorophylls was verified, resulting in an extract with a yellow color, while in the latter case a green color was observed. This is an important advantage since chlorophylls presence in hops extracts for beer production is undesirable. In terms of XN amounts the effect of pressure is more pronounced. The results obtained indicate that pressure treatment can increase the XN amount up to 560% higher, when compared with the same samples without pressure treatments. This work shows the potential to produce extracts from natural sources with new and improved properties.



PS1: 69

### Use of immobilized *Saccharomyces cerevisiae* cells to reduce volatile acidity of acidic wines

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Volatile acidity (VA) is one of the major issues of wine quality. Its legal limit is  $1.2 \text{ g L}^{-1}$  and at concentrations above  $0.8 \text{ g L}^{-1}$  (1) confers to wine an undesirable acidic taste and unpleasant vinegar aroma. Excessive acetic acid, frequently associated with *Botrytis-cinerea*-infected grapes, is formed by yeasts during fermentation but can also be formed by lactic acid bacteria during malolactic fermentation or as a product of acetic acid bacteria. The commercial strain *Saccharomyces cerevisiae* S26 is able to decrease volatile acidity of acidic wines with a volatile acidity higher than  $1.44 \text{ g L}^{-1}$  acetic acid (2), with no detrimental impact on wine aroma (3). Herein we assessed the efficiency of acetic acid removal from acidic wines by immobilized cells of strain S26. The results obtained show that immobilized cells of the *S. cerevisiae* strain S26 in double layer alginate-chitosan (DL) beads ( $4.0 \times 10^7 \text{ cell mL}^{-1}$  alginate) reduce 21.6% of the initial VA of an acidic wine ( $1.1 \text{ g L}^{-1}$ ) with 12.5% (v/v) ethanol and pH 3.5, after 72 h. The level of deacidification did not change after 168 h and was associated with a slight decrease in ethanol concentration (1.1% v/v) and cell leakage from the beads ( $3.5 \times 10^4 \text{ CFU mL}^{-1}$ ). Duplication of the initial cell concentration and decrease of the pH to 3.12, increased VA removal up to 61.8%, depending on the initial acetic acid concentration. Moreover, no cell leakage occurred whereas ethanol concentration slightly decreased (0.7%, v/v). Scanning Electron Microscopy analysis of immobilized cells in DL beads confirmed that the initial pH value is critical for beads integrity maintenance. Thus, immobilized *S. cerevisiae* S26 cells in DL beads appear as an efficient alternative to improve wine quality with excessive VA.

[1] Office Internationale de la Vigne et du Vin (2009) *Compendium of international methods of wine and must analysis. Vol1 OIV, Paris, p 419.* [2] Vilela-Moura et al Appl. Microbiol. Biotechnol., 80, 881-890. [3] Vilela-Moura et al (2010) Appl. Microbiol. Biotechnol., 87, 1317–1326.

PS1: 70

### Wine microorganisms inactivation with high hydrostatic pressure treatments

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During the last decade, the use of high hydrostatic pressure (HHP) as a non-thermal technology for food preservation and modification has increased substantially. Foods can be submitted to high pressures in order to destroy microorganisms and inactivate enzymes with minimal effects on their sensorial and nutritional properties [1]. The application of HHP to wine, 300 to 500 MPa for 2 min, has been shown to cause the inactivation of bacteria and yeasts [2]. These results suggest that HHP might be an alternative process for preservation of wine, which can lead to the production of a wine with reduced amounts of sulphur dioxide (SO<sub>2</sub>), which has been related to allergic reactions in a large number of consumers. The objective of this work was to evaluate the conservation of wine treated by HHP along storage. For this purpose, a red and a white wine (*Vitis vinifera* L.) were produced without the addition of SO<sub>2</sub>. At the end of the alcoholic fermentation, the wines were pressurized at 500 and 425 MPa for 5 min at 20°C. The effect of HHP treatment was evaluated along nine months of storage by quantification of microbial load. Antioxidant capacity, colour and sensorial analyses were also performed. The wine submitted to HHP showed no growth of yeasts and bacteria, contrary to the wine without treatment (0 ppm of SO<sub>2</sub>) and with 40 ppm of SO<sub>2</sub>. These results proved the efficiency of HHP on wine preservation at the microbiological level. Pressurized red wine showed, at the end of storage, an antioxidant activity and colour similar to the wine with 40 ppm of SO<sub>2</sub>. However, the white wine presented differences in colour after storage (higher a\* and b\* values of CIELAB parameters). In terms of sensorial aspects, the pressurized red wines showed a better global assessment when compared with the unpressurized ones. The pressurized white wines presented a worse global assessment, being evident a higher bitterness level and cooked fruit flavour. These results show that HHP is an efficient technology for wine conservation at a microbiological level. However, the use of HHP needs to be applied with care to minimize the impact on sensory quality.

1. Ramirez et al. 2009. *Food Eng. Rev.* 1, 16. 2. Mok et al. 2006. *J. Food Sci.* 71, M265. The authors thank to QREN (Project 3462), FCT (PTDC/AGR-ALI/101251/2008), and Research Unit 62/94 QOPNA (project PEst-C/QUI/UI0062/2011). Cláudia Nunes and Mickael Santos thank FCT for the grants (SFRH/BPD/46584/2008; SFRH/BD/70066/2010).

PS1: 71

### Use of a modified Gompertz equation to model synthetic dye decolourization by yeast in liquid media

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Forty six yeast strains isolated from two wastewater treatment stations along with other 81 cheese isolates were compared on their ability to decolorize five textile dyes in solid media. After a screening methodology that included liquid culture decolorisation ability evaluation, yeasts isolates, LIII S 36 and L III ST 7 presented the best performance in the decolourisation for the five dyes tested: Remazol Black B-A, Remazol Yellow RR, Levafix Blue CA, Remazol Brilliant Blue R and Levafix® Red CA). A modified Gompertz equation was used to model the decolourisation in liquid media; the estimated parameters, which all have biological attribution, allow us to assess quantitatively the decolourisation and a more accurate comparison between the different behaviours of the strains for each dye. Molecular biology methodologies also allowed the identification and the confirmation of the differences between the strains previous selected to liquid decolourisation based on classic methodologies. For the isolates from the wastewater treatment stations, we had a variety of different species identifications, such as *Candida ortopsilosis*, *Debaromyces hansenii* and for a small group of strains it should be necessary explore other methodologies of identification to obtain a correct identification. For the two strains with the best performance (L III S 36 and strain L III ST 7) were performed spectral scanning, is possible observe that, depending on the dye, the strains exhibit different behaviours in the decolourisation process, can achieve it through mechanisms of adsorption or due true degradation. Both strains produce extracellular manganese peroxidase. After 36 hours of incubation for the strain L III ST 7 and L III S 36 an average of 2.30 and 2.06 IU.  $\text{r}^{-1}$  of manganese peroxidase activity were detected, respectively. Due to the obvious morphological differences between filamentous fungi and yeasts, the enzymatic activities detected for the yeasts are interesting. Based on the results obtained it is possible to postulate that the decolourisation may be related with the MnP enzymatic activity.

PS1: 72

**Gills histopathology of *Solea solea* and *Solea senegalensis* comparative wild population and captivity population.**

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Histological changes in gills were used to compare the health of the *Solea solea* and *Solea senegalensis* sampled from natural environment and fish farms. Gills are a good bioindicator as they are in direct contact with water and thus also in direct contact with all types of contaminants, parasites and bacteria, reflecting the quality of the environment. The gills of 40 specimens, 10 for each place, were collected between January and March 2011 from two different fish harbours (Leixões and Aveiro) and 2 fish farms (Rio Alto and Aquacria) Portugal. The samples were fixed in ALFAC and processed following histological routine procedures. The gills were stained with Hematoxilin and Eosin; Alcian blue and Periodic Acid Schiff. The most frequent lesions were common to animals of both origins; however the lesions were more evident in gills from wild fishes then from fish farms. These lesions are basal fusion, medial fusion, total fusion, apical fusion, apical edema, lamellar edema and the number of mucous cells per tissue slice in the gills. Many factors can contribute for these differences between wild and captive fishes. Namely: sea water pollution, long periods of time between fishing and cooling, poor quality of the water used for cooling, increased stress in wild captures. Further studies assessing these factors should be done in order to identify the origin of the lesions. Histopathology showed to be a very suitable biomarker to determine environmental or capture techniques problems that may decrease the quality and value of the fish.

PS1: 73

### **A factorial design analysis of inorganic polyphosphate accumulation by *Cunninghamella elegans* strains**

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Microorganisms are highly dependent on the environmental conditions for their viability and metabolism, as well as for the functioning of their cell machinery. They have also the ability to store a number of important metabolites in the form of osmotically inert polymers. This compound was first identified more than 100 years ago as a polymers of phosphates linked by highly energetic phosphoanhydride bonds. They have also the ability to store a number of important metabolites in the form of osmotically inert polymers PolyP is a linear polymer of orthophosphates residues linked by high-energy phosphoanhydride bonds. The PolyP accumulations by ten strains of *C. elegans* were investigated using glucose 40 or 4 g/L, as carbon source, and the temperatures of 22 or 28 °C, respectively for cultivation, according to an appropriate experimental factorial design 22 without central point. The independents variables studied were glucose concentration and temperature of growth and the variable response was inorganic polyphosphate accumulation. The highest PolyP concentration was obtained with strain *C. elegans* (UCP 542), 119?g of polyphosphate/mg of dry biomass, which correspond to 11.9%. The results obtained with *C. elegans* showed an interrelation between the PolyP accumulation and the variation of glucose concentration, and temperature of cultivation. The highest yield was demonstrated by *C. elegans* (UCP 542) strain isolated from polluted area of mangrove sediment. The best results were obtained when all independents variables were set at their higher levels. The ten first order models proposed for PolyP production by *C. elegans* specimens showed excellent correlation with the experimental data.

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PS1: 74

### **Apian pollen: botanical study, nutritional and microbiological evaluation**

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Under European regulations, any food product claiming to have a health or nutritional benefit must be supported by science. As such, this work intends to characterize, for the first time in Portugal, the palynological origin, nutritional value and microbiological safety of bee pollen. This mixture of flower pollen, nectar and bee secretions, used to feed their larvae in the early stages of development, is recognized to be a valuable apitherapeutic product with potential for medical, health and nutritional applications. Its composition makes bee pollen a product of high nutritional value also for human consumption. In fact it contains proteins, the 22 basic amino acids, carbohydrates, lipids such as Omega-3 and Omega-6 fatty acids, vitamins and minerals. Moisture content, ash,  $a_w$ , pH, reducing sugars, carbohydrate, proteins, lipids, fatty acids and energy were the specific parameters analyzed in this study. Aerobic mesophiles, moulds and yeasts, fecal coliforms, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* and sulphite-reducing clostridia were the microorganisms studied. The most frequent plant families from a total of 10 taxa identified were *Boraginaceae* and *Ericaceae*. Portuguese bee pollens (that differ in composition and properties from the other locations' product) are nutritionally well-balanced and revealed high levels of moisture, proteins, fat, energy, ash, carbohydrates, reducing sugars, essential n-3 fatty acids and good ratios of PUFA/SFA. In fact, the polyunsaturated fatty acids (PUFA) represent 66% of the total fatty-acids. Microbiologically, the commercial quality was good. All samples showed negative results for toxigenic species. Although, to valorize the Portuguese bee pollen, it would be very important to establish quality requirements and to validate and then implement methods to analyze this product, whose nutritional properties and microbial quality were approached in this study.

PS1: 75

**Authentication of hams by molecular biology techniques****Graciete da S. Santos<sup>1,2</sup>, M. Beatriz P.P. Oliveira<sup>1,2</sup>, Isabel Mafra<sup>1,2</sup>**<sup>1</sup>REQUIMTE, Laboratório de Bromatologia e Hidrologia; <sup>2</sup>Faculdade de Farmácia, Universidade do Porto, Portugal

Authenticity of commercial products, particularly food, has a prominent importance especially by the need to protect the consumer health from the existence of potentially allergenic products. Since vegetable proteins are considerably cheaper than muscle proteins, they are frequently used as meat extenders in order to reduce the cost of the final product. Due to several interesting characteristics, soybean is reported to be the most widely used vegetable protein in the meat industry, including the production of ham. Soybean is included in the list of 14 groups of allergenic foods that are required to be declared, regardless of their amount (Directive 2007/68/EC). In fact, it has been described that amounts of soy below 0.1% and 1% (w/w) can lead to allergic reactions in sensitive consumers. Thus, sensitive and reliable methods are required for detecting the presence of this insidious potential allergen, and for checking for label compliance. The analytical methods used for soybean detection in foods rely mainly on protein and DNA analysis. However, it has been referred that protein-based methods can be significantly less sensitive in the evaluation of thermally processed foods because of protein denaturation. Recently, the analysis of DNA coupled with polymerase chain reaction (PCR) presents a fast, sensitive and highly specific alternative to protein-based methods. The aim of the present work was ascertain the authenticity of twenty-one commercial samples of ham and bologna, in conformity with the labeling statements, using species-specific PCR assays. Specific primers for soybean detection targeting the *lectin* gene were used. The methodology was optimized using reference binary samples with different known amounts of hydrated soybean protein in pork meat (0.1% to 50%). To identify animal species often found in this type food, namely pork (*Sus scrofa*), turkey (*Meleagris gallopavo*) and chicken (*Gallus gallus*) species-specific PCR primers targeting *cytb* gene were used. The results for soybean detection showed that from the 21 samples tested, three were not according the labelling since undeclared soybean was identified with strong PCR bands. The identification of meat species showed that for pork all the samples were in accordance with labeling. Regarding the poultry species, some samples showed the presence of undeclared chicken meat. The presented results emphasize the importance of assessing the authenticity of foods protecting the health of consumers.

PS1: 76

### **Eco-friendly production of biosurfactant from a new pigmented bacterium using cassava wastewater as renewable substrate**

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A novel bacterium pigmented isolate from semi-arid soil was characterized by biochemical and molecular assays, as well as, by rep-polymerase chain reaction (PCR) and 16S rDNA sequencing, and identified as *Serratia marcescens* based on 99% of similarity. The tensioactive and bioemulsifier production by *S. marcescens* was investigated using cassava (*Manihot esculenta* Crantz) wastewater, added lactose, and corn oil, according to full factorial design 23, at 28oC and static condition. The liquid metabolic of free cells reduced the surface tension of the water from 70 mN/m to 30.60mN/m-1, indicating the tensioactive agent was produced in the stationary phase (assay eight: 6.0% cassava wastewater, 1.0% lactose and 7.5% corn oil). The wild strain was able to produce at the same time emulsifier agent (4.012 UEA), assay four (6.0% cassava wastewater, 1.0% lactose and 5.0% of corn oil). This biosurfactant with improved physicochemical properties is suitable for a wide range of applications in industry and for environmental cleaning process.

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PS1: 77

## Effect of corn bread processing on DNA degradation of conventional and biotech maize

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Most methods for GMO detection and quantification are based on polymerase chain reaction (PCR), since protein-based assays are not suitable for highly processed food. It is well known that the efficiency of the PCR depends on DNA quality and purity [1]. Food manufacturing involves a number of processing steps that may negatively affect the performance of DNA-based GMO detection methods. Fragmentation of DNA may be initiated by grinding and milling owing to shear forces and hydrolysis by nucleases [2]. Maize plays a major role in nutrition in many countries all over the world and is the second most important GM crop with the highest number of authorized GM events (21) for food and feed in the EU [3]. The main goal of the present work was to assess the effect of processing in the detection and quantification of GM maize in maize bread along the production. Three different corn breads were prepared in a bakery according to the traditional method of preparation. DNA was extracted using the Wizard method. Yield and purity of DNA were assessed by spectrophotometry, while amplifiability was evaluated by targeting the endogenous maize gene *invertase*. Event and construction-specific PCR primers were used to detect MON810 and TC1507 events and two types of PCR assays were applied and adapted for each GM event tested: qualitative PCR for the specific detection and real-time PCR with TaqMan probes in order to quantify the GMO content. The results revealed that it was possible to detect and quantify the maize *invertase* (endogenous gene) and MON810 and TC1507 maize events along the production of maize bread and in all parts of the final cooked breads. However, as expected, some degradation of DNA was noticed, which might have contributed to the differences found between the estimated GM maize contents in two of the breads. These results represent a great achievement for the traceability of GMO in the food chain.

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PS1: 78

## Exploring the industrial value of the Portuguese continental shelf microorganisms

**Ana Rodrigues Martins, Cátia Rodrigues, Gonçalo Andrade, Marta Cerejo, Ricardo Pinheiro, Patrícia Calado, Helena Vieira**

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The interest in the usage of marine natural sources for bioactive discovery has increased tremendously in the last decade and BIOALVO is exploring this potential making use of Portuguese natural resources. BIOALVO presents itself as a one-stop-shop for natural product development, based on its extensive natural extract library and corresponding bioactivities, and also on expert and customizable assay development, which can be coupled to an in-house robotized screening facility, effectively speeding up the testing process and bringing a natural product solution closer and quicker to market. BIOALVO owns a unique collection of more than 100 prokaryotic extremophiles isolated from deep sea hydrothermal vents in the Mid Atlantic Rift, near the Azores Islands. The extreme conditions of temperature and chemical composition of those vents make these microorganisms potential sources for bioactive compounds. Based on this assumption, a collection of both aqueous and organic extracts produced from those microorganisms, entitled PharmaBUG collection, has been tested for both pharmaceutical and cosmetic usages in BIOALVO's internal research projects and also in collaboration with partners and clients. These assays have successfully shown the presence of bioactive compounds in this collection, with some showing new UV protective capacity. This will be shown as an example of how BIOALVO is building value from Portuguese marine live micro resources. With the aim of increasing its portfolio of bioactive marine natural products, BIOALVO has recently established partnerships with Portuguese institutions that are collecting marine microorganisms in various expeditions taking place along the Portuguese continental shelf. These new marine microorganisms will be characterized and various types of extracts will be produced and compose larger collections called LUSOEXTRACT and LUSOMAREXTRACT. These expanded collections are expected to reach 100.000 extracts, at the end of 2013, providing a unique tool to be vastly explored for presence of new bioactive compounds. BIOALVO's natural extracts collections, derived exclusively from Portuguese ecosystems, will allow the exploitation of the national heritage for biotechnological sectors as diverse as the cosmetic, pharmaceutical, nutraceutical and industrial, amongst others, leveraging Portugal's position within the worldwide natural product market.

PS1: 79

**Incorporation of probiotics into a fruit matrix****Ângela Rêgo, Joana Silva, Alcina Morais, Paula Teixeira**

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The consumption of probiotic functional foods, e.g. processed foods enriched with microorganisms that confer health benefits to the host, showed a progressive increase in the last decade due to changes in consumers' habits and trends who become more and more concerned with health. Several health benefits have been associated with the consumption of live probiotic bacteria, when administered in adequate amounts, such as: control of intestinal infections, reduction of serum cholesterol levels, synthesis of vitamin B, reduction of blood ammonia levels, beneficially influencing the immune system, improving lactose utilization, and providing anticarcinogenic activity. The development of novel probiotic foods has been focused mainly on dairy products including yogurts, cheese, drinks, and dietary supplements. Currently, the development of fruits and vegetables with probiotic content is a topic of high interest for the probiotic-food consumers as those are a popular item perceived as healthy by consumers, and issues related with lactose intolerance are overcome. The aim of this research work was to create a new functional dry food with a source of probiotic strains that could bring some benefits to the consumers of the product. Apple was selected as the food matrix and two different probiotic *Lactobacillus* species, *L. plantarum* and *L. kefir*, were tested separately. Fresh apple cubes were immersed in a concentrated solution (approximately  $1 \times 10^{10}$  CFU/mL) of the probiotic strain for one hour and then dried in a tray dryer for one day at approximately 40°C. Samples were taken immediately before and after the drying process in order to control the viability of bacteria adhered to the matrix. Dried apple cubes were stored in closed glass sterile containers at room temperature and at 4 °C. Bacterial enumeration was performed at 5, 10, 30, 60, 90 and 120 days of storage. For both probiotic strains, a decrease of approximately 2 log cycles in bacterial cell numbers was observed after drying. The bacterial numbers in apple cubes before storage were approximately  $1 \times 10^7$  CFU/mL. Both probiotic strains died after one month of storage at room temperature, while the cells remained viable after 3 months of storage at 4 °C, with bacterial numbers around  $1 \times 10^6$  CFU/mL.

PS1: 80

**Modeling dynamics of seawater disinfection with electrolyzed oxidizing water with applications in aquaculture****Antonio Rigueiro<sup>1</sup>, M.L. Cabo<sup>2</sup>, J.J.R. Herrera<sup>2</sup>, S.R Carrera<sup>2</sup>, E. Balsa-Canto<sup>1</sup>**<sup>1</sup>Bio)Process Engineering Group, IIM-CSIC, Vigo, Spain; <sup>2</sup>Microbiology and Technology of Marine Products, IIM-CSIC, Vigo, Spain

A critical part of the aquaculture is the assurance of microbiological safety. In this regard the depuration of shellfish is a critical step before commercialization. While traditional depuration process involves the use of oxidants such as hypochlorite, a modern alternative would be the use of the partial electrolysis of seawater. This technology (EOW, electrolyzed oxidizing water) has already shown in several applications to present benefits in cost, plant safety and environmental friendliness. The objective of this work is twofold: in one hand, to analyze the possibilities of the usage of EOW to the disinfection with applications in aquaculture and in the other hand, to develop mathematical models so as to describe and optimize the entire process. The capacity EOW for disinfection of the seawater of a mussel depuration plant has been studied. A scaled model of a depuration basin was constructed and disinfection experiments were carried out testing disinfection efficiency of *E. coli*. The experimental setup allowed following the dynamics of the remnant disinfectant and bacterial concentrations for several controlled inocula under an experimental design performed with different dosage conditions. The dynamics of seawater in the scaled plant were studied by means of computational fluid dynamics and its behavior was fitted to a series of chemical reactors. The disinfectant concentration along time was found to be best described by a model presented by Kastl. The evolution of *E. coli* concentration with time was found to be best represented by the classical Watson model. It should be remarked that kinetic parameters of the models were computed by means of an iterative identification procedure which involved data fitting and optimal experimental design. The experimental results confirmed the application of EOW is able to reduce the concentration of *E. coli* in seawater to the levels currently considered as safe. Therefore, EOW being suitable for its introduction in aquaculture related processes. In addition the mathematical models developed were able to satisfactorily predict the chlorine residuals and the surviving microorganisms of the continuous process within the range of industrial application, and offered an acceptable prediction of the transient dynamics at the process startup. Current work focus on the model based optimization of the dosage schemes.

PS1: 81

**Production of fibrinolytic protease by *Bacillus* sp. UFPEDA 485 using factorial design****Fabiana América S.D. Souza, Amanda E. Sales, Tatiana S. Porto, Ana Lucia Figueiredo Porto**

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Proteases belong to the class of the most important industrial enzymes, comprising about 30% of all commercial enzymes in the world. Among these enzymes are the fibrinolytic proteases, they degrade fibrin, the main protein component of blood clots. The accumulation of fibrin in the blood increases the possibility of thrombosis and other cardiovascular diseases. Proteases with fibrinolytic activity produced by bacteria of the genus *Bacillus* have been widely investigated. This study aimed to evaluate the variables that influence the production of fibrinolytic protease. The microorganism used was *Bacillus* sp. UFPEDA 485, previously selected from thirty bacteria of the same genus obtained from Bank of Cultures, Department of Antibiotics, Federal University of Pernambuco/Brazil. The inoculum was prepared in nutrient broth and standardized to a final concentration of  $10^6$  CFU/mL and added in the production medium MS (soybean flour medium) described by Porto et al. (1996). Production of fibrinolytic protease was performed on an orbital shaker (150 rpm) at 37°C for 48 hours. A  $2^2$  full factorial design plus four central points was performed in order to evaluate the effect of independent variables: substrate concentration (soybean flour 1%, 2% and 3%) and agitation speed (100, 150 and 200 rpm). The enzymatic extract was obtained by centrifugation at 10,000 g 15 min, and used for analytical determinations: total protein, proteolytic activity and fibrinolytic activity. In all experiments of the factorial design were produced halos of degradation of fibrin, values between 200,96 mm<sup>2</sup> and 252.16 mm<sup>2</sup> have been achieved, in this way, the best value achieved in fibrinolytic activity was  $452.16 \pm 0.01$  mm<sup>2</sup> obtained from the average of results from the central points (2% of soybean flour and 150 rpm) for these tests the average values obtained from proteolytic activity was  $3.12 \pm 0.02$  U/mL, and the protein content of  $244.62$  mg/mL  $\pm 0.04$ . Variables showed no statistically significant effect. This behavior was due the best condition for fibrinolytic activity was obtained in the central points. From these results we can conclude that *Bacillus* sp. UFPEDA 485 is a potential source of fibrinolytic proteases, and should be conducted to optimize the production, characterization and purification of the enzyme.

PS1: 82

**The antimicrobial effect of wine on *Bacillus cereus* in simulated gastrointestinal conditions****Miguel Vaz, Tim Hogg, José António Couto**

CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

Several studies demonstrate the antibacterial properties of wine against relevant food-borne pathogenic bacteria but none of them studied the effect on *Bacillus cereus*, a spore-forming bacterium that is a common contaminant in a wide variety of foods. Two distinct food-borne disease types are associated with *B. cereus*: the intoxication, or emetic syndrome (caused by a toxin produced in the food prior to consumption) and the toxic-infection, or diarrheal syndrome (caused by enterotoxins produced in the small intestine). This study aimed to evaluate the antimicrobial activity of wine against *B. cereus* vegetative cells and spores. The results clearly show that wine exerts a strong inactivation effect against vegetative cells of *B. cereus*. The red wine tested inactivated stationary phase cultures to undetectable numbers in less than 10 s. Thus, further inactivation assays were carried out with wine diluted with water (1:4 and 1:8). Diluted wine 1:4 caused a reduction of approximately 5 log cycles on viable cell counts in 20 s. On the other hand, *B. cereus* spores were found to be highly resistant to wine exposure. The influence of wine components (organic acids, ethanol and phenolic compounds) was investigated on vegetative cells. The wine organic acids tested exhibited a strong inactivation effect, and, when combined with ethanol, a slight synergistic effect was observed. The wine phenolic compounds assayed displayed no activity against the vegetative cells at the concentrations tested. At the simulated gastric conditions studied (in the presence of food), wine diminished considerably the number of *B. cereus* viable cells supplementing the effect of the synthetic gastric fluid. The behavior of *B. cereus* spores under gastrointestinal conditions was also evaluated. In a consumption-like scenario, the addition of wine led to lower total counts (vegetative cells + spores) of *B. cereus* in the simulated intestine conditions, showing that wine inhibits the proliferation of the vegetative cells obtained from the germination of spores. This work provides evidence that consumption of wine during a meal may diminish the number of viable cells of *B. cereus* and reduces the impact of the germination of spores that may occur in the small intestine.

# Environmental Microbiology and Biotechnology

*Poster session thematic symposium 2*

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Sousa | Freire | Albuquerque | Ventura | Reis |  
Fernandes | Gosmão | Rosado | Ribeiro | Alves | Mota  
| Crespo | Mata | Ferraz | Simões | Dias | Noronha |  
Alves | Morais | Antunes | Martins | Martins | Reis |  
Vaz-Moreira | Morales-Valle | Azevedo | Barros |  
Lopes | de Souza | Gudiña | Siqueira | Oliveira | Cruz |  
Godoy | Maciel | Carvalho | Alves | Lopes | Azevedo |  
França | Moreira | Franco | Mesquita | Aguilar | Ottoni |  
Henriques | Moreira | Cunha | Ventura | Varela |  
Sousa | Ferreira | Amaro | Choupina | dos Santos |  
Bekturova | Silva

## PS2: 1

**A rich and complex integron gene pool in wastewater environments**

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Antonio CM Correia<sup>1</sup>**

<sup>1</sup>Departamento de Biologia e CESAM, Universidade de Aveiro, Portugal; <sup>2</sup>Julius Kühn Institute – Institute for Epidemiology and Pathogens Diagnostics, Braunschweig, Germany

Integrans are genetic systems that enable bacteria to acquire and excise gene cassettes by site-specific recombination; often associated with plasmids and transposons, play a key role in acquisition of antibiotic resistance. Here, the role of wastewaters as reservoirs of integrans and conjugative plasmids was assessed by culture-dependent and independent methods. Samples were collected at two distinct treatment plants: one receiving urban wastewaters and other receiving wastes from a slaughterhouse. Culture-independent approaches consisted in analysis of phylogeny by 16S-DGGE, detection of conjugative plasmids and integrase genes (*intl*) by PCR and hybridization and construction of gene cassette libraries. Culture-dependent approaches targeted two bacterial groups: *Aeromonas* spp., widespread in aquatic environments and *Enterobacteriaceae*, common in polluted waters. Screening of *intl* was done by hybridization; positive strains were characterized in terms of phylogenetic affiliation, antibiotic resistance profile, integron's variable regions and conjugative plasmids. DGGE profiles revealed distinct bacterial communities in both effluents. IncP-1, IncN, IncQ and IncW plasmid-specific sequences and *intl* genes were present in both wastewaters. Gene cassettes recovered showed low homology with genes involved in cell wall synthesis, motility, gene regulation and secretion pathways. Additionally, the majority of clones possessed no homologues in public databases. For bacterial isolates, prevalence of *intl* was 3.7% and 35% for urban and slaughterhouse wastewaters, respectively. One third of integrase genes were in conjugative plasmids. Gene cassettes coded for antibiotic resistance to beta-lactams, aminoglycosides, trimethoprim and chloramphenicol. New cassettes coding for proteins that may contribute to biofilm formation were also found. In summary, wastewaters bring together different molecular elements that, in association, are major actors in bacterial adaptation and evolution. The presence of cassettes encoding proteins with potential biotechnological applications justifies further work and stresses the importance of wastewater as gene cassette reservoirs of functionally diverse proteins.

Moura et al. 2007. *J Antimicrob Chemother* 60:1243; Moura et al. 2009. *Microbiol Res* 164:560; Moura et al. 2010. *Res Microbiol* 161:58. Work financed by FCT project POCTI/BME/45881/2002 and grants SFRH/BD/19443/2004 and SFRH/BPD/21384/2005.



PS2: 2

**A strategy to isolate extended protein open ready frames from metagenomic DNA**

**Carla Botelho Machado, Simone Moraes Mantovani, Richard John Ward**

University of Sao Paulo, Brazil

The increased use of industrial enzymes is from the constant innovation that aims to increase performance and reduce costs. Cellulases are enzymes that catalyze the hydrolysis of cellulose. They are involved in numerous processes such as production of second generation bioethanol. With a view to applications in biomass biorefinery, the objective is to seek from environmental DNA samples new cellulases that are catalytically efficient at acidic pHs, and are thermostable. Bioprospection of these enzymes is by metagenomic analysis, through tracking the promoter region of genes to build ORF libraries. The microbial community used was from soil collected in Sao Carlos-SP. Using an innovative strategy, a library containing the coding regions of the enzyme is generated. Extraction of metagenomic DNA from soil was judged to be efficient since no contamination by humic acid was detected and DNA integrity was confirmed by amplification with primers specific for 16S RNA. Key steps during the selective PCR screening of cellulases were performed as standardization of the technique using the *Bacillus subtilis* organism, and the feasibility of the methodology was confirmed by DNA sequencing with identifies a 16S pseudouridylate synthase complete gene of 720nt. The strategy was validated using the endo- $\beta$ -1-4 glucanase from *Bacillus subtilis*, in which the detection of the enzyme is carried out on plates containing LB medium and carboxymethyl cellulose as substrate. So, the use of this innovative metagenomic strategy was able to identify gene a complete gene in frame. In soil samples, the application of the methodology identified enzymes such glucanases, beta glucosidases and xylanases by functionally screening. Thus, this strategy metagenomics screening the identifications of several enzymes of biotechnological interest through the construction of a metagenomic ORF libraries.

PS2: 3

**Oil degrading bacteria from the marine surface microlayer****Angela Cunha<sup>1</sup>, Francisco J.R.C. Coelho<sup>1</sup>, Sara Sousa<sup>2</sup>, Luísa Santos<sup>1</sup>, Ana Luísa Santos<sup>1</sup>, Adelaide Almeida<sup>1</sup>**<sup>1</sup>Department of Biology & CESAM, University of Aveiro, Portugal; <sup>2</sup>Department of Biology, University of Aveiro, Portugal

The surface microlayer (SML) is the uppermost millimetre of the water column and functionally represents the hydrosphere-atmosphere interface. Bacteria living in this compartment (bacterioneuston) face a challenging environment in terms of surface tension, exposure to solar irradiation and accumulation of pollutants. Although it is assumed that bacteria communities inhabiting this layer might develop specific resistance traits and hydrolytic capacities which differ from bacterial communities inhabiting the underlying waters (i.e., bacterioplankton), little is known on the diversity and ecology of estuarine bacterioneuston. The aim of this work was to provide a first insight on culturable oil-degrading bacteria from the surface microlayer by investigating the spatial variability of bacterioneuston in comparison to bacterioplankton along a gradient of oil hydrocarbon (OH) contamination in the estuarine system Ria de Aveiro (Portugal). Also, culturable representatives of the hydrocarbonoclastic bacterial populations were isolated and characterized. Culture-independent techniques (*Fluorescence In Situ Hybridization* and *Denaturing Gradient Gel Electrophoresis*) did not reveal significant differences between the structure of the overall bacterioneuston communities along the OH gradient. On the contrary, differences in terms of relative abundance and diversity of specific groups related with OH degradation were observed. *Pseudomonas* and *Klebsiella* were the dominant OH degrading groups and the phylogenetic analyses of polycyclic aromatic hydrocarbon (PAH) dioxygenase gene sequences revealed a high homology between genes retrieved from different bacterioneuston isolates. The ability to tolerate and even degrade toxic compounds associated with the capacity of surviving in conditions of high surface tension and UV irradiation make SML bacteria interesting to explore envisaging potential biotechnological applications. This study provided evidences that bacterioneuston communities can be a natural “seed bank” of hydrocarbonoclastic bacteria with both ecological and biotechnological relevance.

PS2: 4

### Synergistic action of azoreductase and laccase leads to maximal decolourisation and detoxification of model dye-containing wastewaters

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<sup>1</sup>Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Portugal; <sup>2</sup>Department of Bioengineering, Instituto Superior Técnico, Universidade Técnica de Lisboa; <sup>3</sup>IBB-Instituto de Biotecnologia e Bioengenharia, Centro de Engenharia Biológica e Química, IST

Many of the azo dyes used in the textile, paper, leather or plastic industries, and/or their breakdown products have been shown to be toxic, potentially carcinogenic and can lead to the formation of bladder cancer in humans, tumours, allergies, nuclear anomalies in experimental animals, and chromosomal aberrations in mammalian cells (1,2). Among the few redox-active enzymes showing azo dyes degradative activity, azoreductases are particularly effective since they act on the reduction of the azo linkage. However, azoreductases require the addition of expensive cofactors such as NAD(P)H as electron donors for the reductive reaction and the products released are aromatic amines which are potentially toxic. In contrast, laccases are oxidoreductases that have a great potential in various biotechnological processes mainly due to their high non-specific oxidation, the lack of a requirement for cofactors, and the use of readily available oxygen as an electron acceptor capacity. In the present study, the enzymatic degradation of an array of 18 azo dyes and of three model dye baths was tested with two enzymes with proven ability to degrade synthetic dyes, recombinant FMN-dependent NADPH azoreductase (PpAzoR) from *Pseudomonas putida* MET94 (3) and recombinant CotA-laccase from the bacterium *Bacillus subtilis* (4,5). The PpAzoR showed a broader specificity for decolourisation of azo dyes than CotA-laccase. However, the final products of PpAzoR activity exhibited in most cases a 2 to 3-fold higher toxicity than intact dyes themselves. We showed that addition of CotA-laccase to PpAzoR treated reaction mixtures lead to a significant drop in the final toxicity. An *Escherichia coli* strain co-expressing *ppAzoR* and *cotA* genes was constructed where the sequential action of PpAzoR and CotA enzymes could be tuned by aeration conditions. Whole-cell assays of recombinant strain for the treatment of model dye wastewater resulted in decolourisation levels above 80% and detoxification levels up to 50% (6). The high attributes of this strain, make it a promising candidate for the biological treatment of industrial dye containing effluents.

(1) Chen et al. 2006 *Curr. Protein Pept. Sci.* (2) Pinheiro et al. 2004 *Dyes and Pigments* (3) Mendes et al. 2011 *App. Microb. Biotechnol.* (4) Martins et al. 2002 *J. Biol. Chem.* (5) Pereira et al. 2009 *J. Biotechnol.* (6) Mendes et al. 2011 *Bioresour. Technol.*

PS2: 5

**Biodegradation of pharmaceutical compounds: who does it and how fast?****Bárbara Almeida<sup>1,2</sup>, Gilda Carvalho<sup>1,3</sup>, Maria Teresa Barreto Crespo<sup>1,2</sup>**<sup>1</sup>IBET, Portugal; <sup>2</sup>ITQB, Portugal; <sup>3</sup>REQUIMTE/CQFB, Portugal

Pharmaceutical compounds are commonly detected in wastewater treatment plant (WWTP) effluents and in natural environments suggesting that they are not completely removed by conventional WWTP. Many pharmaceuticals have potentially harmful effects for the environment and public health, thus there is growing concern regarding their discharge [1]. Ibuprofen and ketoprofen are two common non-steroidal anti-inflammatory drugs (NSAIDs) that are highly detected in WWTP influents [1]. Only two isolates were described to degrade ibuprofen, the *Sphingomonas* sp. strain Ibu-2 [2] and *Nocardia* sp. [3], and none for ketoprofen, and degradation tests using mixed cultures reported widely varying removal results [4-5]. Deeper knowledge is needed on the biodegradation kinetics, microbiology and metabolic pathways of these compounds in order to optimise WWTP operation for their removal. This study aimed at investigating the biodegradation kinetics of ibuprofen and ketoprofen by unacclimatised activated sludge and enriched communities, as well as to identify and characterize ibuprofen-/ketoprofen-degrading isolates. The mixed culture kinetic tests were carried out with activated sludge from a municipal WWTP, before and after enrichment with each NSAID, in presence or absence of another carbon source. Cultures fed with the NSAID as the sole carbon source had higher NSAID biodegradation rates than those fed with a combination of acetate and the NSAID, suggesting that the NSAIDs were not degraded by co-metabolism. NSAID-degrading isolates (bacteria and fungi), obtained by agar-plating with each compound, were identified as members of the Actinobacteria, Gammaproteobacteria, Alphaproteobacteria and Basidiomycota phylum. Isolates are currently being investigated for their degradation capacity and their role in the metabolic pathways of ibuprofen and ketoprofen biodegradation. Furthermore, proteomic analysis is ongoing to determine which enzymes are involved in NSAID degradation. These results will improve the knowledge on the biodegradation of pharmaceutical compounds in WWTP and in the environment.

[1] Kolpin et al 2002, ES&T 36(6), 1202-11 [2] Kagle et al 2009, Adv. Ap. Microbiol. 67, 65-108 [3] Chen et al 1994, AEM 60(4), 1292-96 [4] Zwiener et al 2003, Sci. Tot. Env 309(1-3), 201-211 [5] Quintana et al 2005, Wat. Res. 39(12), 2654-64 Acknowledgements: FCT through PTDC/AMB/65702/2006, PTDC/EBB-EBI/098862/2008, SFRH/BD/47748/2008 and SFRH/BPD/30800/2006.

PS2: 6

### **Biotechnology applied to heritage: characterisation and fungal biodegradation assessment of Renaissance frescoes**

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The ancient parish church of Santo Aleixo is located about eight kilometers northwest of Montemor-o-Novo, in Herdade do Raimundo, Portugal. It is a sixteenth-century building with simple structure, which has one of the most beautiful Renaissance Portuguese wall paintings. Many microorganisms produce serious damage in historic materials, which are decomposed from the impact of specific enzymes and organic acids. Fungi are particularly dangerous because they demonstrate a substantial tolerance to environmental conditions and their hyphae showed high level of proliferation in mortars. The aim of this work was the establishment of methodologies for material characterisation of mural paintings and biodegradation assessment, including the analysis of microbial growth and the effect of microbial proliferation, in view of their conservation. The methodology was applied to the study of the Renaissance frescoes from the church of Santo Aleixo. Microsampling of paint layers was performed on representative areas of the paintings. The characterization of the frescoes was carried out by microanalysis of cross sections by optical microscopy, scanning electron microscopy (SEM-EDS) and micro-XRD allowing the identification of pigments and stratigraphy. The painting palette is composed of red and yellow ochres, malachite, smalt, azurite, bone black and lead white. Fungal characterisation was made based on macro and microscopic characteristics, including texture and colour of fungal colonies, hyphae and reproductive structures (for sporulated isolates) and a M13-PCR approach was used in order to establish different genetic profiles, between different areas of the paintings. Microbiological assessment allow isolate 5 yeast and 53 moulds strains (mainly *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and sterile mycelia). The M13-PCR allow established different genetic *fingerprints* between the main fungal biodeteriogenic agents.

PS2: 7

**Finding novel biotechnological tools in the environment: characterization of *Pseudomonas putida* secretome.****Patrick Freire<sup>1</sup>, Inês Batista Guinote<sup>1</sup>, Gonçalo da Costa<sup>2</sup>, Ana Ribeiro Silva<sup>2</sup>, Romana Santos<sup>3</sup>, Cordeiro Carlos<sup>2</sup>**<sup>1</sup>Laboratório de Bacteriologia, INRB, IP/LNIV, Portugal; <sup>2</sup>Departamento de Química e Bioquímica, Centro de Química e Bioquímica, FCUL; <sup>3</sup>Unidade de Investigação em Ciências Orais e Biomédicas, Faculdade de Medicina dentária, FCUL

Environmental bio-prospection of novel active biological compounds is an attractive approach for the development of new biotechnological tools. Not only new bio-molecules can be discovered, but also new biochemically active proteins or proteic complexes. Bacteria and fungi are naturally producers of extra-cellular molecules that have in the past been successfully used in the most diverse areas (antibiotics, biochemical processes, food industry...). *Pseudomonas putida*, a Gram-negative bacterium generally found in soil, is considered a saprotrophic microorganism, capable of chemoheterotrophic extra-cellular digestion of decayed matter, often primarily associated with fungi. Attempts have been made to explore the potential of *P. putida* metabolism for bioremediation or biodegradation processes, without significant endpoints. An existing collection of environmental bacteria, essentially composed of *P. putida* distinct strains, was obtained during previous studies by selection through resistance to several antibiotics. That collection was screened for protein secreting strains and several interesting strains retained. Minimal growth media supernatant in stationary phase was collected and secreted protein profiles analyzed. One *P. putida* strain presented an exceptional production of extracellular proteins, even though no significant variation was observed in phenotypic behavior, like growth parameters. Screening of diverse potential biological activities was performed. The secretome of that specific strain, as the complete set of secreted biological substances, was shown to present a potent antimicrobial impact on bacteria, including other distinct *P. putida* strains. Peptide detection and 2D PAGE analysis showed the presence of a very complex secreted mixture. The analysis of enzymatic activities associated to the secretome also showed the presence of strong proteolytic capacity active from 35 to 45 °C, but still retaining 30% activity at 15°C. Furthermore, the protease or proteases secreted were shown to have a very low turnover after an overnight incubation at 37 °C. Additional preliminary data strongly suggests that this degradative activity has the potential to fragilize protein-based structures. It is therefore a source of novel bio-active molecules with concrete applications in biotechnological approaches to implement bio-controlled processes, in several distinct areas from health to industrial biochemical reactions.

PS2: 8

***Gaiella occulta* gen. nov., sp. nov., a novel representative of a deep branching phylogenetic lineage within the class Actinobacteria and proposal of *Gaiellaceae* fam. nov. and *Gaiellales* ord. nov.**

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Two isolates, with an optimum growth temperature of about 35–37 °C and an optimum pH for growth between 6.5 and 7.5, were recovered from a deep mineral water aquifer in Portugal. Strains form rod-shaped cells and were non-motile. These strains were non-pigmented, strictly aerobic, catalase and oxidase positive. Strains F2-233<sup>T</sup> and F2-223 assimilated carbohydrates, organic acids and amino acids. Major fatty acids were novel iso internally-branched such as 17:0 iso 10-methyl, 17:0 iso and 15:0 iso 8-methyl. The peptidoglycan contained *meso*-diaminopimelic acid and menaquinone MK-7 was the major respiratory quinone. Analysis of the 16S rRNA gene shows the strains to cluster with species of the genera *Thermoleophilum*, *Patulibacter*, *Conexibacter* and *Solirubrobacter* to which they have pairwise sequence similarity in the range 87 to 88%. Based on 16S rRNA gene sequence analysis, physiological and biochemical characteristics we describe a new species of a novel genus represented by strain F2-233<sup>T</sup> (=CECT 7815<sup>T</sup> =LMG 26412<sup>T</sup>) for which we propose the name *Gaiella occulta* gen. nov., sp. nov. We also propose that this organism represents a novel family named *Gaiellaceae* fam. nov. of a novel order named *Gaiellales* ord. nov.

PS2: 9

### **Toxicity evaluation - the enhancement of the Ionic Liquids hydrophobic nature with the decrease of their toxicity**

**Sónia Patrícia Marques Ventura<sup>1</sup>, Ana Marta Mendes Gonçalves<sup>2</sup>, Fernando Gonçalves<sup>1</sup>, João Manuel Araújo Pereira e Coutinho<sup>1</sup>**

<sup>1</sup>University of Aveiro, Portugal; <sup>2</sup>University of Coimbra, Portugal

Ionic liquids (ILs) are a novel promising class of solvents with interesting physicochemical properties. Many different applications have been described as alternatives to organic solvents in chemical processes. Despite their low vapor pressure, even the water immiscible ILs shown some solubility in water, that allows their dispersion into the aquatic systems resulting in water contamination. This work aims at enlarging the restricted knowledge about the ILs toxicity and inhibitory effects in aquatic ecosystems and to investigate the possibility of designing hydrophobic ionic liquids of low toxicity. It reports a set of toxicity results, which correspond to aromatic and non-aromatic immiscible ILs, through the use of different cations (pyridinium, piperidinium, pyrrolidinium and imidazolium) and hydrophobic anions (bis(trifluoromethylsulfonyl)imide [NTf<sub>2</sub>] and hexafluorophosphate [PF<sub>6</sub>]). In this context, biological assays were performed using organisms of different trophic levels, such as the decomposer *Vibrio fischeri*, the producer *Pseudokirchneriella subcapitata* and the first consumer *Daphnia magna*. Contrary to the common belief that the ILs toxicity increases with their hydrophobicity it is here shown that it is possible to design ILs with an enhanced hydrophobic character, expressed as water solubility, and lower toxicity, as determined by the tests here conducted, by elimination of their aromatic nature.



PS2: 10

**A new method for testing antimicrobial activity of filamentous fungi****Francisca Reis<sup>1</sup>, Ana Santos<sup>1</sup>, Eric Pereira<sup>2</sup>, Paula Baptista<sup>2</sup>, Rui Tavares<sup>1</sup>,  
Teresa Lino-Neto<sup>1</sup>, Cristina Aguiar<sup>3</sup>**

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Fungi have been studied as a potential source of antibiotics since 1941. Some of them have a strong antimicrobial effect against other saprophytic and phytopathogenic fungi, or even against yeast and bacteria. To detect the *in vivo* production of fungal antimicrobial compounds different strategies are currently used. The most common comprise dual cultures on solid culture medium, or growth in liquid media containing the substrate previously used by the fungal antagonist. However, these strategies can be compromised by the different growth rates of both microorganisms and/or by the low stability of antimicrobial compounds. In this work, a bioassay to detect the antimicrobial activity of filamentous fungi against single-cell microorganisms is presented. The developed assay was first established using as fungal model *Hypholoma fasciculare*, a basidiomycete found in chestnut grove soils of Trás-os-Montes (Bragança), in which it displays a significant antagonistic action against different filamentous fungi. The yeast *Saccharomyces cerevisiae* was selected to be tested as the sensitive indicator strain. Parameters such as the assay temperature and the culture medium assay, fungal growth and yeast inoculum concentration were optimized during assay design. Fungal antimicrobial activity was evaluated in PDA medium after 48h at 25 °C, using a 6 days grown fungal culture and 10<sup>6</sup> CFU/ml of the yeast indicator strain. The described assay can be easily used to test the *in vivo* fungal antibiotic activity against single-cell organisms such as yeasts or bacteria, allowing a faster and more reliable strategy than using, for example, fungal-grown liquid media. For confirming the method feasibility, a panel of different yeast species of industrial and clinical relevance was tested as sensitive strains. The obtained results confirm this new method as a rapid, effective and reproducible bioassay for testing antimicrobial activity of filamentous fungi against yeasts.

*This work was supported by a FCT project (PTDC/AGR-AAM/099556/2008).*

PS2: 11

## Antibiotic resistance in bacteria isolated from the floor of open air children playgrounds

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Antibiotic resistance is a serious problem of public health. Antibiotics are present in the environment and this leads to the emergence and dissemination of resistant genes among organisms. It is known that antibiotic resistant genes originated in the environment could be transferred to microorganisms including pathogenic bacteria. Children air playgrounds are places where children and adults go to play, but also dogs, birds and other animals share the environment. So, children could be exposed to a great number of microorganisms, some of them potentially pathogenic carrying antibiotic resistance. The objective of this work was to identify microorganisms potentially pathogenic in the floor of open air children playgrounds and to characterize their antimicrobial resistance. Samples from the floor of six playgrounds located in Lisbon, were collected bimonthly for a period of eight consecutive months. The playgrounds vary in geographical location, number of users, social status of the target population, surroundings and cleaning conditions. The samples consist of 5mm pebblestone, which were collected near the surface of playground toys and the trees. The isolated bacteria were scattered in culture media with ampicillin and the ones that grow in this culture media were tested for antimicrobial susceptibility with a panel of antibiotics in order to characterize possible mechanisms of resistance. A great variety of microorganisms resistant to ampicillin were isolated, some of them that can be pathogenic to humans, such as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and others. The most isolated bacteria with resistance to ampicillin was *E. coli* (21 isolates), followed by *Enterobacter cloacae* (9 isolates) and *Escherichia hermannii* (7 isolates). In all the six playgrounds bacteria showing resistance to ampicillin were detected, being the one where more samples presented resistance (12/16) a place with a lot of trees, no vigilant and a high frequency of people with dogs. The playground where the number of samples with detectable resistance was lower (5/16) is located in a large public garden, that is supervised by township employees and it has a very good clean status. Resistance to other antibiotics like cefotaxime, ceftaxime, gentamicin and amoxicillin plus clavulanic acid was also detected. These results demonstrate the importance of a continuous microbial control of these facilities.

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PS2: 12

**Biodegradation of mixed microbial consortium for kerosene**

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Bioremediation is a technique that stands out in that it aims at restoring areas which has undergone chemical impacts, be it toxic or recalcitrant in nature. The process is based on the pollutant degradation through the action of organisms such as bacteria and fungi, for a large proportion of these organisms uses oil hydrocarbons as a source of carbon in their life processes. The objective of this study was to assess kerosene degradation through a mixed microbial consortium. The microorganisms used in this and belong to the Collection of Microorganisms, at the Department of Antibiotics (UFPE). These were previously selected by the technique of Hanson et al. (1993) and following, an acclimatization of the microbial cultures was carried out (*A. tamarii*, *R. aurantiaca* and *B. cereus*) at the oil source (10%). Once the strains were acclimated, a factorial design (CCD-Central Composite Design), involving three variables ( $2^3$  plus 3 center points). The variables used were: the concentration of the nitrogen source ( $\text{NH}_4\text{NO}_3$ ), of the glycerol and inoculum in three levels (-1.0 and +1), totaling 11 trials. The simultaneous variation of these factors allowed for the verification of the consortium performance in kerosene biodegradation, when subjected to different nutritional stimuli. The experiments were undertaken at a temperature of  $30 \pm 1$  ° C for 10 days. At the end of tests degradation was analyzed through gas chromatography, and next, evaluate the effect toxicity according to the methodology by Tiquia et al. (1996). After ten days of experiments the chromatography trials from the experimental design were analyzed and were done so through the Statistical 6.0. It can be observed that there has been some degradation of kerosene hydrocarbons in all trials. But it was ascertained that the best condition in which to attain further degradation was the test 3. In this condition, the lower inoculum, the highest glycerol concentration and the lowest C: N ratio was chosen. None of the variables were significant for the process. However, two effects were found: the concentration of glycerol and the interaction between the C: N ratio and inoculum. The results suggest that test 3 metabolized kerosene and yielded low toxicity to the bean seeds, what makes it the ideal condition to promote efficient degradation. Thus, the mixed microbial consortium has the potential to degrade petroleum derivatives, and is also recommended for the bioremediation process.

PS2: 13

### Biodeterioration assessment of the 16th century Renaissance frescoes from Casas Pintadas Garden in Évora

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The microbial biodeterioration of art objects is an issue that has arisen during the last two decades. Microorganisms can be responsible for the destruction of cultural heritage assets, including the decay of mural paintings, statues and many other art objects. Mural paintings represent good media for a wide range of heterotrophic microorganisms due to their porous nature. However, biological attack occurs only when there are favorable humidity and temperature environmental conditions. The identification of microorganisms involved in biodeterioration is necessary to understand the effects of microorganisms on cultural assets, as well as to elucidate the functional properties of these microorganisms and their role in biodeterioration. The crucial step in this area is to use the accumulated information to create an efficient strategy based on the biodeterioration processes to conserve and protect monuments and works of art from microbial colonization. The aim of this study was to identify and isolate the microorganisms involved in the biodegradation of the 16<sup>th</sup> century renaissance frescoes from the garden of the Inquisition Palace in Évora, classified as UNESCO World Heritage, in order to find the “key microbial players” that are most active in the biodeterioration process. Furthermore, to assist and develop an efficient intervention, different biocides were investigated to control the propagation of the microbial communities responsible for the biodegradation. For microbiologic assays the samples were aseptically collected from areas of the paintings with significant contamination. The microbial population was isolated and identified. The microbiological study allowed the identification of several bacterial strains (eg Gram+ cocci/bacilli, *Actinomyces* sp.), yeast strains and filamentous fungi of the genera *Penicillium*, *Cladosporium*, *Aspergillus* and sterile micelia were also isolated. The effect of Preventol, Panacide and Linquad biocides on the growth of fungi was carried out in the presence of different biocide concentrations. The tested biocides showed satisfactory inhibition results. However, when the application was carried out by combining two biocides, the action spectrum is noticeably enlarged.

*The support of the conservators Alice Cotovio and José Pestana from Mural da História and Fundação Eugénio de Almeida, owner of the building, and the Portuguese Science and Technology Foundation for T. Rosado PhD Grant (SFRH/BD/65747/2009).*

PS2: 14

### Enantioselective quantification of fluoxetine by a chiral HPLC-FD method in biodegradation assays

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Chiral pharmaceuticals and the fate and effects of their enantiomers in the environment are still largely unknown [1]. Enantiomers have different interactions with enzymes, receptors and any chiral molecules leading to different biological activities and affecting organisms in a different manner. Thus, biodegradation tends to be enantioselective in contrast to abiotic degradation. Fluoxetine (FX), a selective serotonin uptake inhibitor antidepressant, is one of the most prescribed fluorinated pharmaceuticals and has been detected in surface waters. The methods developed to quantify the enantiomeric fraction in the environment and to follow biodegradation are scarce [2]. Thus, in this work we describe the quantification of FX during biodegradation assays by a developed and validated HPLC method, that allow the enantiomeric separation of FX. The macrocyclic antibiotic vancomycin CSP (ASTEC Chirobiotic V 5µm) was used under polar ionic mode and fluorescence detection for enantiomeric fraction quantification. The developed method was established using a minimal medium inoculated with activated sludge. The ability to degrade FX was tested by using two consortia: a consortium of bacteria (FP1, F11, S2) isolated at ESB and able to degrade different fluorinated compounds and activated sludge collected from a municipal WWTP. Concerning to CF, the enantiomer ratio did not change during degradation, so the consortium seems to be equally able to degrading (*S*)- and (*R*)-fluoxetine. Over half of the compound was degraded in both cases, slightly more in the enrichments without acetate than with acetate as a supplemental carbon source. Activated sludge consortium was able to degrade partially FX, with and without an additional carbon source. The enrichment with acetate led to a slightly higher degradation, contrary to the CF enrichments. This study shows wastewater treatment aimed at fluorinated compounds can be more effective than currently, using a consortium of specific bacterial strains.

[1] Stanley et al, *Integr Environ Assess Manag* 5: 3, 364–373 (2009). [2] Hashim et al *Environ Technol* 31:12, 1349-1370 (2010). A.R. Ribeiro acknowledges FCT, Portugal for the grant (SFRH/BD/64999/2009), QREN-POPH, European Social Fund and MCTES. This work was also financially supported by CESPUP (09-GCQF-CICS-09) and Fluoropharma -PTDC/EBBEBI/111699/2009.

PS2: 15

**Enterococci in common cockles from Ria de Aveiro: diversity and antibiotic resistance****Sofia Leite, António Correia, Artur Alves**

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Enterococci are important members of the gut microbiota of humans and animals and can also be found in plants, soil, water, and food. Interest in enterococci increased in the last few decades due to their use as probiotics and starter cultures and their natural occurrence in various fermented foods. In addition, enterococci have emerged as clinically relevant bacteria frequently causing nosocomial infections with a high mortality rate. Their intrinsic and acquired resistance to antibiotics is an important aspect that contributes to this phenomenon. This study focused on the characterization of *Enterococcus* isolated from samples of common cockles taken from Ria de Aveiro. A total of 200 isolates obtained from common cockle samples were subjected to genotyping by ERIC-PCR followed by identification to the species level by 16S rDNA sequencing and evaluation of the susceptibility to 15 antimicrobials. Identification was performed according to the species groups: *E. faecalis* group, *E. faecium* group and *E. casseliflavus* group. In the 200 isolates the *E. faecium* group was predominant (60%), followed by *E. faecalis* (32.5%) group and finally the less representative was the *E. casseliflavus* group (7.5%). Antibiotics susceptibility testing was performed by the disc diffusion method according to the CLSI standards. All isolates showed high levels of resistance to the antibiotics tested. In fact, 99.5% were multiresistant. The lower and intermediate resistance percentages corresponded to gentamycin (9.5%), streptomycin (21%), fosfomicin (9%), teicoplanin (20.5%) and penicillin (24.5%). In contrast, the highest resistance percentages were detected for erythromycin (98%), linezolid (91.5%) and ciprofloxacin (87%). High levels of resistance to the glycopeptide vancomycin were also detected (64.5%). Comparing the 3 species groups, the group *E. faecium* revealed the highest percentage of resistant isolates for the majority of antibiotics. These results suggest that common cockle grown in coastal waters, namely in Ria de Aveiro, can be potential reservoirs of multiresistant enterococci. The risk of dissemination to the community by re-entering the food chain exists since these bivalves are usually consumed poorly cooked or uncooked. The high levels of resistance to antibiotics commonly used to treat human infections raise concern and the need to monitor this aquatic environment.

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PS2: 16

### Extracellular polymeric substances (EPS) from *Cyanothece* sp. CCY 0110: conditions influencing their production and EPS characterization

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Many cyanobacterial strains are able to synthesize and secrete extracellular polymeric substances (EPS) that can remain associated to the cell or be released into the surrounding environment (RPS - released polysaccharides). The unique characteristics of these EPS, such as the presence of two different uronic acids, sulphate groups and higher number of different monomers, makes them promising for biotechnological applications such as the removal of heavy metal from polluted waters. For the successful implementation of these systems, it is necessary to identify physiological/environmental factors that influence the synthesis and/or characteristics of the polymer [1]. Several *Cyanothece* strains are reported as strong EPS producers and efficient in the removal of metal ions from aqueous solutions. The major aims of the present work are the identification of the conditions that promote EPS production and the characterization of the produced polymer, using as a model organism the marine N<sub>2</sub>-fixing unicellular cyanobacterium *Cyanothece* sp. CCY 0110. *Cyanothece*'s growth and EPS production were evaluated in different physiological and environmental conditions. The results obtained showed that the higher amount of EPS is mainly related to the number of cells and not to the amount of EPS produced by each cell. Therefore, conditions that promote growth (e.g. aeration, presence of nitrate, continuous light) also increase the total amount of EPS produced per liter of culture. Moving from these results, a scale up is being performed taking into account the conditions that favor EPS production. Differential scanning calorimetry (DSC) analysis depicted the EPS amorphous nature, with a single glass transition temperature at 103°C. No crystallization or fusion peaks were observed. Termogravimetry (TGA) analysis showed that the degradation of EPS takes place in three well-differentiated steps: first step at 67°C, mainly due to water loss; second step at 283°C, a degradation process with a weight loss of about 47%; third step at 758°C, leads to 25% of residual mass highlighting the remarkable thermal stability of the EPS. X-ray powder diffraction reveals a partial degree of crystallinity which is in agreement with X-ray diffraction analysis of EPS obtained from other sources. FT-IR spectroscopy suggests the presence of uronic acids, sulphate groups and proteins in the EPS.

[1] Pereira et al (2009) *FEMS Microbiol Rev* 33: 917-941

PS2: 17

**Fungi and the aquatic environment: occurrence and disinfection effectiveness****Vanessa J Pereira<sup>1,2</sup>, Rosalina Marques<sup>1,2</sup>, Marta Marques<sup>1,2</sup>, Maria Vitória San Romão<sup>1,2,3</sup>, Maria Teresa Barreto Crespo<sup>1,2</sup>**<sup>1</sup>IBET, Oeiras, Portugal; <sup>2</sup>ITQB, Oeiras, Portugal; <sup>3</sup>L-INIA, INRB, Dois Portos, Portugal

Despite their wide occurrence, little attention has been given to the presence of fungi in aquatic environments. Fifty three fungi isolates were recently isolated from different source waters, most of which have never been described to occur in water sources (Pereira et al, 2009). These results showed that fungi occur widely in drinking water sources and that further studies should be conducted to address if the drinking water treatment processes currently used are effective to remove these microorganisms from our drinking water supplies. Final disinfection of drinking water using free chlorine is widely used worldwide and has saved millions of lives from waterborne diseases. This study reports the effectiveness of free chlorine to inactivate different species of filamentous fungi that were found to occur in different water matrices (Pereira et al, 2009). Free chlorine inactivation rate constants of *Cladosporium tenuissimum*, *Phoma glomerata*, *Aspergillus terreus*, *Aspergillus fumigatus* and *Penicillium citrinum* were determined in laboratory grade water and surface water using different disinfectant concentrations, temperatures, and pH levels. The sensitivity degree of different fungi isolates to UV treatment varied among different genera with some species showing a higher resistance to disinfection and others expected to be more prone to protection from inactivation by the water matrix components. When the disinfection efficiency measured in terms of the chlorine and contact time (Ct) values needed to achieve 99% inactivation were compared with the Ct values needed to achieve the same degree of inactivation of other microorganisms, fungi were found to be more resistant to chlorine inactivation than bacteria and viruses and less resistant than *Cryptosporidium* oocysts. Overall, final chlorine disinfection was found to be extremely efficient to achieve fungi inactivation.

Pereira et al *Water Research*, 43, 3813–3819. Financial support from Fundação para a Ciência e a Tecnologia (PTDC/AAC-AMB/108303/2008 and BPD/26990/2006) is gratefully acknowledged.



PS2: 18

**Granulation of activated sludge in a laboratory SBR****Ana M. Tavares Mata<sup>1,2</sup>, Nídia Dana Lourenço<sup>1</sup>, Helena Maria Pinheiro<sup>1</sup>**

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Granular activated sludge is a new and promising biological process for wastewater treatment, with the advantages of quick biomass settling and the possibility of creating aerobic and anoxic/anaerobic zones within the same granule. The latter can improve the biodegradation of several types of compounds namely xenobiotics. Generating the granules is the first step. The aim of this study was thus to produce granular sludge in a sequential batch reactor (SBR) seeded with flocs harvested in a full-scale, conventional activated sludge reactor, to examine the parameters influencing the process and to characterize the obtained granules. The SBR had a height/diameter ratio of 2.5, differing from the tubular reactors usually described, and closer to full-scale SBR units. The SBR was operated with an exchange volume ratio of 50% and a 6-h cycle time, i.e., 5h of aeration, and 1 hour for feeding, settling, discharge and idle. The settling time was gradually reduced from 40 to 2 minutes. The feed solution contained macro and micronutrients, and a starch derivate, delivered at 1.5-2.0 kgCOD.m<sup>-3</sup>.d<sup>-1</sup>. The system was operated for 95 days. After 25 days of SBR operation, with 3 minutes of settling time, granules larger than 1mm were dominant. The biomass settling properties were markedly improved, corresponding to a drop in Sludge Volume Index (SVI) from 250 to 28mL/g. The COD removal yield averaged 66% after 1h of reaction time, reaching 88% at the end of the cycle. After a further reduction of the settling time to 2 min, settling properties deteriorated and no biomass accumulation could be achieved during 40 days, with TSS remaining around 2.0g/L. Returning to the 3-min settling regime restored the SVI values and enabled biomass accumulation from 1.9 to 6.9 g/L in TSS during the following 30 days. Granules were observed under Gram stain (ViaGram™ Red+ Bacterial Gram Stain Viability Kit), and were dominated by Gram negative bacteria as expected. This work demonstrated the successful generation of granular activated sludge from a flocculent inoculum in a laboratory SBR, with good COD removal yields from a starch-based feed. The settling time was a critical parameter in the process. With the introduction of an anaerobic phase, the system will be further studied and tested for the biodegradation of xenobiotic compounds associated to textile industry wastewaters.

PS2: 19

**Kinetic study of chromium (III) biosorption onto brewery spent grains****Ana Isabel Ferraz<sup>1,2</sup>, Teresa Tavares<sup>1</sup>, José António Teixeira<sup>1</sup>**<sup>1</sup>Centro de Engenharia Biológica, Instituto de Biotecnologia e Bioengenharia, Portugal; <sup>2</sup>Escola Superior Agrária, Instituto Politécnico de Viana do Castelo

Heavy metals, present in several industrial wastewaters, are considered major inorganic pollutants due to their recalcitrance and persistence, high mobility in aquatic systems, toxicity and ability to accumulate through the food chain. Thus, it is unanimously considered that their uncontrolled discharge into natural water streams poses serious environmental and public health risks. In fact, environmental management guidelines and regulations concerning metal discharges and waste disposal are becoming progressively stricter, pointing to strategies that include wastewater and residues minimization, recycling and valorization. Hence it arises the urgent need to develop new treatment technologies, more efficient and economical. Biosorption, currently assumed as a promising technology to decontaminate high volumes of dilute solutions, may enhance its contribution to pursuit eco-efficiency goals when using residual biomass from industrial or agronomic activities that enables endogenous products valorization in environmental biotechnology applications. Spent grains residual from a Portuguese brewing industry were tested as potential biosorbents to decontaminate Cr(III) solutions. Kinetic studies involved the use of unmodified spent grain (NTSG) and spent grain treated with NaOH (TSG). Experimental essays were performed in batch systems with metal initial concentration ranging from 25 to 400 mg Cr(III).L<sup>-1</sup>. Metal solutions pH was adjusted to 5.0, biomass was added (5 g.L<sup>-1</sup>) and flasks were incubated at 30 °C with orbital shaking (150 rpm). Experimental data revealed that metal uptake follows a rapid initial step, well described by the pseudo-second order kinetic model up to 2-7 h of incubation, indicating metal chemisorption, with ionic change and covalent bonding between Cr(III) and biomass binding sites, to be the rate limiting step. Fast kinetics is highly relevant for biosorption, turning feasible the use of lower volume bioreactors ensuring process economy and efficiency. During this rapid uptake phase, both biosorbents have similar pseudo-second order constants ( $k_2$ ):  $3,47 \times 10^{-3}$  to  $2,76 \times 10^{-2}$  g.mg<sup>-1</sup>.min<sup>-1</sup> for NTSG, and  $3,97 \times 10^{-3}$  to  $7,16 \times 10^{-2}$  g.mg<sup>-1</sup>.min<sup>-1</sup> for TSG, considering increasing metal initial concentration within the range in study. Fitting experimental results to the intraparticle diffusion kinetic model shows that this phenomenon is not the limiting step, but assumes an important role in Cr(III) overall sorption rate, specially for high contact times.

PS2: 20

### **Micoteca da Universidade do Minho (MUM): implementation of a quality management system based on ISO 9001:2008**

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Micoteca da Universidade do Minho (MUM) is a filamentous fungi culture collection established in May 1996, hosted by the Biological Engineering Centre, which is a centre of excellence integrated in the Institute for Biotechnology and Bioengineering (IBB). The mission of MUM is to provide the highest quality services to our customers, collecting, maintaining and supplying fungal strains and their associated information for teaching and research in biotechnology and life sciences. MUM intends to be a centre of knowledge, information and training in mycology, operating at a global level and under national and international regulations. In order to keep its high standards MUM has implemented a Quality Management System (QMS) based on the normative reference ISO 9001:2008. MUM has defined three processes, those being: Material Reception Process, Material Preservation Process and Material Supply Process; and has also developed a documental base for all the QMS including proceedings, standard operating procedures, forms, as well as quality objectives and goals for a continuous improvement. The implementation of the QMS occurred for almost 1 year during which 3 main steps were developed. The first step was the planning of the QMS, followed by the design of the system and documental development and the third step was the implementation of the QMS with the achievement of the certification in May 2011. From this date on MUM has kept on performing QMS revision and pursuing a continuous improvement. MUM QMS team attained all the necessary conditions for the Certification of the implemented QMS and MUM has obtained the certificate of ISO 9001:2008 for the Deposit, Preservation and Supply of Filamentous Fungi from the independent assessment agency (Portuguese Association for Certification - APCER with the International Certification Network - IQNet). Of the 590 culture collections from 68 countries registered in the WFFC, MUM is the 1<sup>st</sup> Portuguese and the 23<sup>rd</sup> culture collection in the world to obtain this important qualification. The new certificate will enhance further the reputation of MUM as a culture collection that pursues the goals of excellence and leadership implicit in these new and exacting standards. Additionally, MUM will become an even more relevant partner of the GBRCN and EMbaRC projects.

PS2: 21

**Novel strategy for separation and identification of extracellular proteins in wastewater treatment systems****Ana Filipa Silva<sup>1,2</sup>, Renata Soares<sup>2</sup>, Ana Varela Coelho<sup>2</sup>, Gilda Carvalho<sup>1,3</sup>, Maria Teresa Barreto Crespo<sup>1,2</sup>**<sup>1</sup>IBET, Portugal; <sup>2</sup>ITQB, Portugal; <sup>3</sup>REQUIMTE, Portugal

Extracellular polymeric substances (EPS) play an important role in mixed cultures. EPS promote cell aggregation, host extracellular reactions (substrate-degradation related, quorum sensing, etc), and retain by-products and excess cellular macromolecules. In wastewater treatment plants (WWTP), the EPS molecules responsible for aggregation are important for floc settle ability, and biofilm and granule formation, promoting the retention of the cells in the system. However, EPS molecules can also be cumbersome in membrane bioreactors, where they contribute for fouling, resulting in decreased process performance. Therefore, to better understand cell aggregation and membrane fouling, it is important not only to quantify, but also identify the proteins present in EPS. Besides proteins, EPS are composed of polysaccharides, humic acids, nucleic acids and lipids. Regarding the low protein concentration and the level of contamination in this type of samples, a clean-up/concentration method is necessary before protein identification. Few previous studies investigated this problem, and only a very low number of extracellular bacterial proteins were retrieved. Thus, the currently available protocols for EPS protein identification in WWTP samples need further improvement, which was the motivation for this study. Samples used in this study were obtained in a membrane bioreactor fed with domestic wastewater (0.2- $\mu$ m membrane pore diameter and biomass concentration of 4.9 g/L). A previously developed method for EPS separation from cells was used. The protein clean-up/concentration methods tested in this study were: ammonium sulphate precipitation, dialysis, and ultrafiltration centrifugation. Proteins were further precipitated with acetone, trichloroacetic acid (TCA), perchloric acid or a protein precipitation kit, and then separated through SDS-PAGE. The gel electrophoresis process was also optimized in this study, where homogeneous and gradient gels were compared. Protein bands were then excised and trypsin-digested prior to mass spectrometry analysis (MALDI-TOF-TOF). Protein identification was achieved using MASCOT search engine and the NCBI and SwissProt databases with no taxonomy restrictions. As a conclusion from this work, a step-by-step optimized method was established for maximal retrieval of EPS proteins from WWTP samples.

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PS2: 22

**Soil nitrifying community: how does it change in response to nitrogen additions in a Mediterranean ecosystem?****Teresa Dias<sup>1</sup>, Sandra Chaves<sup>2</sup>, Rogério Tenreiro<sup>2</sup>, Ana Tenreiro<sup>2</sup>, Manuela Carolino<sup>1</sup>, Maria Amélia Martins-Loução<sup>1</sup>, Lucy Sheppard<sup>3</sup>, Cristina Cruz<sup>1</sup>**<sup>1</sup>FCUL, CBA, Portugal; <sup>2</sup>FCUL, BioFig, Portugal; <sup>3</sup>CEH, United Kingdom

Man is profoundly altering nitrogen availability in ecological systems, through increases in the deposition of nitrogen-containing compounds. Belowground processes, often mediated by soil microorganisms, are central to the response of ecological systems. Nitrification is a unique process since it mediates the transformation of reduced into oxidized inorganic nitrogen. Until recently it was believed that nitrification was restricted to bacteria in soils with near neutral pH. However evidence has arisen showing nitrification in acidic soils. At first this was attributed to soil heterogeneity, since even in acidic soils there are microsites with neutral pH. More recently it has been ascribed to heterotrophic microorganisms, such as fungi, bacteria and archaea. The aim of this work is to understand how increased availability of nitrogen, as nitrate or ammonium, may affect nitrification in a nitrogen-limited ecosystem with slightly acid soils. The experiment was performed in a Mediterranean-type ecosystem, with low phosphorus and nitrogen availabilities, and soil pH between 5 and 6. Nitrogen was added as ammonium or as ammonium nitrate three times per year ( $40 \text{ kg ha}^{-1} \text{ y}^{-1}$ ). Soil nitrate and ammonium concentrations were assessed over three years (2007-2010). Microbial community structure was assessed, in Spring, by Temperature Gradient Gel Electrophoresis (TGGE) fingerprinting following DNA extraction: total DNA was extracted from the soil samples and universal primers defined for bacteria, archaea; and ammonia oxidizing bacteria (AOB) and archaea (AOA) genes were used for PCR amplification. The resulting amplification products were separated by TGGE. Water content, pH, total soil carbon and nitrogen, and nitrification potential were also determined. It was observed that nitrification potential and the structure of the nitrifying community responded not only to ammonium addition, but also to multifactorial changes, including water availability and temperature. The structure of the AOB and AOA communities responded to nitrogen addition, but time (due to cumulative nitrogen additions, or other factors) had the strongest effect. No direct correlation was found between the changes in the AOB and AOA communities and the nitrification potential.

PS2: 24

### **The effect of heat and salicylic acid on the DAHPS expression and activity in grape cells**

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High temperatures affect grape berry set and development, alter the normal sugar content of the fruit, and reduce the accumulation of anthocyanins. Also, peaks of high temperature may stop the ripening progress. Despite its impact in fruit and wine quality, the mechanisms contributing to the reduction of anthocyanin levels under high temperature are still elusive. Some studies indicate that this reduction is caused by a downregulation of the genes responsible for anthocyanin synthesis, while others propose that heat upregulates anthocyanin degradation. The enzyme 3-deoxy-D-arabino-heptulo-sonate 7-phosphate synthase (DAHPS), which as two isoforms in plants (DAHPS-01 cytosolic and  $\text{Co}^{2+}$  activated; DAHPS-02 plastidial and  $\text{Mn}^{2+}$  activated), catalyzes the first step of the Shikimate pathway by condensing phosphoenolpyruvate and D-erythrose 4-phosphate to form 3-deoxy-D-arabino-heptulo-sonate 7-phosphate. The final product of this pathway is phenylalanine that fuels the Flavonoid pathway responsible for the biosynthesis of anthocyanins and proanthocyanidins (condensed tannins). Despite its recognized importance, very little is known about the role of DAHPS in the synthesis and accumulation of important secondary metabolites under heat stress. In this study, we aim at the elucidation of the genetic and biochemical steps that regulate anthocyanin accumulation in grape cells under heat. Results on the effect of high temperature and salicylic acid (SA), a plant hormone involved in abiotic stress response, on the expression and biochemical activity of DAHPS in grape cell suspensions are presented and discussed.

PS2: 25

**Are there N<sub>2</sub>-fixing bacteria associated to *Lolium multiflorum*?****Paula Isabel Loução Lopes Alves<sup>1,2</sup>, Ana Catarina Dourado<sup>1,2</sup>, Nádia Castanheira<sup>2,3</sup>, Ana Isabel Delgado-Rodríguez<sup>3</sup>, Paula Fareira<sup>3</sup>, Maria Teresa Barreto Crespo<sup>1,2</sup>**<sup>1</sup>IBET, Oeiras Portugal; <sup>2</sup>ITQB-UNL, Oeiras Portugal; <sup>3</sup>INIA/INRB,I.P., Oeiras Portugal

The ability of legume plants to obtain nitrogen from the activity of symbiotically associated N<sub>2</sub>-fixing microorganisms has long been recognized. These associations have acquired enormous importance, since the increased use of nitrogenous fertilizers in agriculture has resulted in unacceptable levels of environmental pollution. Some non-legume crops (rice, maize or wheat) were also recognized as having the ability to establish associative but non-symbiotic interactions with either free-living or endophyte N<sub>2</sub>-fixing microorganisms, and diazotroph-based inoculants for these crops start to be envisaged as a plausible way of supplying a significant part of their nitrogen requirements. Here we describe the search for N<sub>2</sub>-fixing microorganisms associated to annual ryegrass, a forage crop that is extensively used in poor-productive areas in southern Portugal. Ryegrass requires moderate to high levels of N-fertilizer to achieve maximum production yields, but these needs can be considerably lowered by the combined use with legumes, mainly due to their N<sub>2</sub>-fixing contribution. The objective of the work is to investigate whether the same might be achieved throughout association with diazotrophic microorganisms that could directly supply the plant with biologically fixed nitrogen. The initial strategy was to trap native ryegrass-associated diazotrophs in "montado" ecosystems. Samples of surface soils were collected and seeded with a commercial variety of annual ryegrass. After plant growth, isolation of diazotrophic bacteria proceeded from both the root external environment and plant tissues, using a N-free semi-solid medium. More than 200 diazotrophic isolates were recovered, presenting several colony morphotypes. ERIC-PCR genomic fingerprinting revealed a high degree of genetic diversity. In order to identify the most promising strains for subsequent plant inoculation assays, diazotrophic isolates were additionally tested for other biochemical activities related with the promotion of plant growth. Most isolates, either originated from rhizospheric environments and plant tissues, were able to produce phytohormones, to solubilize mineral phosphate, and/or to hydrolyze plant polymers, thus presenting increased interest for further utilization. Restriction analysis of the 16S rRNA gene is currently being performed and will allow the taxonomic identification of these isolates.

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PS2: 26

### **Bacteria isolated associated with the nematode *Bursaphelenchus xylophilus* showed nematocidal activity and may have a role in Pine Wilt Disease**

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*Bursaphelenchus xylophilus*, the pine wood nematode, is one of the most interesting nematodes since it is a pathogen of trees and is the causal agent of Pine Wilt Disease. More recently a potential role for bacterial symbionts in the disease process has been proposed. A series of studies have shown that bacteria from various genera could be isolated associated with *B. xylophilus*. Structural studies have not shown bacteria within *B. xylophilus* but the presence of bacteria on the nematode surface has been described by some authors. The work published on bacteria associated to the nematode showed that in Portuguese recently invaded areas by *B. xylophilus*, healthy pine trees of the species *Pinus pinaster* had a diverse endophytic microbial community. From these areas, nematodes isolated from infected trees showed associated bacteria that were from different species according to the area where the nematodes were isolated. In consequence, the possibility of a protective role to the tree, by the endophytic microbial community, against the nematode was an hypothesis. In order to test it, the bacteria isolated associated to the nematodes infecting *P. pinaster* were screened for their potential in killing *B. xylophilus* in vitro. The bacterial strains more active in killing nematodes were selected and the bacterial products, produced during growth, studied in order to determine their nematocidal activity. This nematocidal ability was analyzed for 46 strains. Only 7 bacterial strains did not showed toxicity against *B. xylophilus* and the only genus with all the strains non-toxic was *Burkholderia*. All strains of the genus *Pseudomonas*, except one strain of *P. putida*, showed toxicity against the nematodes. The genus *Serratia* included the strains more toxic to the nematodes: all except one strain showed the highest toxicity level. *Serratia* strains were screened for the different products that could interact with the nematode. Major products identified with potential biotechnological use were Serrawettin and proteases.



PS2: 27

### **Biological treatment of olive oil mill wastes using edible mushroom *Pleurotus ostreatus***

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Olive oil mill wastes (OMW) constitute an important environmental problem due to the large amount of phenolic compounds and the high toxicity they present. Additionally when canned olives were produced, a large amount of brine water difficult the treatment of wastes. Further, the variability and complexity of the constituents of these wastes makes almost indispensable their characterization. In the present work a biological approach was applied using edible mushroom *Pleurotus ostreatus*. The aim of this work was to characterize the working OMW and to develop a biological treatment that promotes a new approach of OMW management. The OMW was acquired from a two phase olive oil mill system which produces a combined waste composed by the olive oil production wastewater and brine water from canned olives production. The characterization of OMW was carried out analyzing the parameters, chemical oxygen demand, pH, total nitrogen, total phosphorus, total phenol, alkalinity, conductivity and metal content. The biological treatment was conducted using batch cultures of *P. ostreatus* performed in a liquid medium with 100%, 75%, 50% and 25% OMW content. The total phenolic content, biomass increase and OMW discoloration was monitored periodically, during 21 days. *P. ostreatus* cultures performed with OMW showed a fungal biomass enhancement as well as medium clarification. A 50%-74% total phenol reduction was observed. Furthermore study is needed in order to achieve the proper management of OMW. However, the biological treatment is an ecological alternative for this management, promoting further valorization of these residues.

PS2: 28

**Characterisation by molecular biology and MALDI-TOF ICMS of zygomycete *Gongronella* sp. isolated from an Alentejo vineyard soil****M. Rosário Martins<sup>1</sup>, Cledir Santos<sup>2</sup>, Pablo Pereira<sup>3</sup>, Fernanda Simões<sup>4</sup>, Júlio Cruz-Morais<sup>1</sup>, Nelson Lima<sup>2</sup>**

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Zygomycete *Gongronella* sp. CCMI 1101 (MUM 10.263) was isolated from an Alentejo vineyard soil submitted to repeated treatments with the fungicide metalaxyl. This fungicide is widely used against Oomycetes species that causing downy mildew. After selective enrichment, adapted *Gongronella* sp. CCMI 1100 (MUM 10.262) showed high tolerance to metalaxyl with  $EC_{50}$  of  $36.1 \text{ mgL}^{-1}$ . Additionally, this adapted fungus was able to use metalaxyl as main carbon and energy source in liquid cultures containing 10 and  $50 \text{ mgL}^{-1}$  with a degradation rate of  $0.119 \pm 0.001$  and  $0.386 \pm 0.002 \text{ mgL}^{-1} \text{ day}^{-1}$ , respectively. Afterwards, both *Gongronella* strains (non-adapted and metalaxyl adapted strains) were submitted to a polyphasic approach identification and characterisation. To perform this, morphology characterisation, ITS sequence region and Matrix Assisted Laser Desorption Ionization Time of Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) were carried out. In order to differentiate soil strains *Gongronella* sp. from adapted strains M13-PCR typing and proteomic using MALDI-TOF ICMS were used. The ITS phylogeny tree and MALDI-TOF ICMS dendrogram both corroborate that *Gongronella* sp. grouped with related species *G. butleri* and *G. lacrispora*. However, it seems to be a putative new species. Additionally, no differences of non-adapted and adapted *Gongronella* sp. strains were observed by M13-PCR typing. In contrast, proteomic analysis showed spectra with substantial quantitatively phenotypic differences between both strains that could be a consequence of a strain adaptation to metalaxyl. In conclusion, *Gongronella* sp. CCMI 1101 is now under further taxonomic studies and the current results suggest that the adapted strain CCMI 1100 can be explored in soil bioremediation for metalaxyl-degradation.

PS2: 29

**Copper impacts on grape berry cells: uptake and detoxification****Viviana Martins<sup>1,2</sup>, Mohsen Hanana<sup>3</sup>, Eduardo Blumwald<sup>4</sup>, Hernâni Gerós<sup>1,2</sup>**

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Copper has been extensively used as the active principle of fungicides, since the late 1800s when the “Bordeaux mixture” was developed and its spectacular efficiency proved against fungal pathogens such as downy mildew, which is a large threat to winegrowers. Although initially it seemed to improve plant growth in unproductive lands, repeated use of copper-based fungicides has led to the accumulation of large concentrations of this metal ion in vineyard soils and raised concerns regarding phytotoxicity. As major targets for heavy metal stress, plants have developed a number of mechanisms to withstand the elevated metal levels. Such responses include exclusion, chelation and compartmentation of metal ions. Both the mitochondria and plastids are copper sinks, and the vacuole is believed to constitute a copper delivery pathway within the cell, and not just a sequestration compartment, due to the proximity of the tonoplast to the other organelles of the plant cell. In the present study, grape berry cells (cv. Cabernet Sauvignon) were used as a model system to study the effect of copper on cell growth and viability. In the concentration range of 0 (+ the copper chelator BCS) to 100  $\mu\text{M}$   $\text{CuSO}_4$  growth was virtually unaffected. However, concentrations from 100 to 500  $\mu\text{M}$  caused a sharp decrease in cell growth. The viability of grown cells decreased with the increase in copper concentration in a dose-dependent manner. Studies with the copper-sensitive fluorescent probe PhenGreen™ SK allowed for the identification of copper sinks in grape berry cells. Furthermore, transport studies were performed in isolated intact protoplasts loaded with this probe. The initial velocities of fluorescence quenching upon addition of copper followed a Michaelis-Menten kinetics, suggesting the involvement of mediated transport with a  $K_m = 0.7$  mM. Isolated vacuoles labeled with the pH-dependent fluorescent dye ACMA were used to study copper compartmentation as a mean of metal tolerance. Results showed that  $\text{CuCl}_2$  dissipates a pre-established pH gradient across the tonoplast suggesting the involvement of a  $\text{Cu}^{2+}/\text{H}^+$  antiport system. Eight putative *VvCTR* (*Vitis vinifera* Copper Transporter) genes were identified, among which *VvCTR1* was isolated and cloned and its expression is currently being studied.

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PS2: 30

**Development of treatment methods for the degradation of antimicrobial compounds present in wastewaters****Ana C Reis<sup>1</sup>, João H Pereira<sup>2</sup>, Vítor P Vilar<sup>2</sup>, Rui R Boaventura<sup>2</sup>, Célia M Manaia<sup>3</sup>, Olga C Nunes<sup>1</sup>**<sup>1</sup>Laboratório de Engenharia de Processo, Ambiente e Energia (LEPAE), Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto (FEUP), Porto, Portugal;<sup>2</sup>Laboratório de Processos de Separação e Reacção (LSRE), Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto (FEUP), Porto, Portugal; <sup>3</sup>Centro de Biotecnologia e Química Fina (CBQF), Escola Superior de Biotecnologia, Universidade Católica Portuguesa (ESB – UCP), Porto, Portugal

The intensive use of antibiotics and disinfectants has been leading to environmental contamination with these micropollutants or with their degradation products. The widespread distribution of these contaminants may have harmful effects, such as the reduction of microbial diversity and the spreading of antimicrobial resistance (AR). The increase of AR leads to a generalized claim that micropollutants with antimicrobial activity must be removed from industrial, hospital and domestic wastewaters. The implementation of degradation methods is, thus, urgently needed. The present study aims at developing low cost efficient treatment systems to remove some of the most used antimicrobials (beta-lactams, sulfonamides, tetracyclines, quinolones and triclosan) from wastewaters. Two complementary degradation approaches, biological and photochemical oxidation, are being considered, and the kinetics of degradation, the formation of possible degradation products, as well as importance of biodegraders in their natural environment is being investigated. Biodegraders have been enriched from samples collected from sites exposed to antimicrobial compounds for several years, using antibiotic supplemented culture media. Mixed cultures were able to transform approximately 90, 60 and 40 % of 10 mg L<sup>-1</sup> amoxicillin, 10 mg L<sup>-1</sup> tetracycline and 320 mg L<sup>-1</sup> sulfamethoxazole, respectively, and were obtained and characterized. Until now, no quinolone biodegraders were enriched from the same samples. For this class of antibiotics, the solar photocatalytic degradation approach proved to be advantageous, as the phototreatment of a 40 mg L<sup>-1</sup> oxolinic acid solution led to a progressive decrease of its antibacterial activity, and reduced approximately 90 % of its TOC content.

PS2: 31

**Diversity and antibiotic resistance of *Aeromonas* spp. in aquatic environments****Vânia Figueira, Ivone Vaz-Moreira, Márcia Silva, Célia M. Manaia**

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*Aeromonas* are ubiquitous bacteria, considered indigenous to aquatic environments. Some *Aeromonas* species are recognized as opportunistic pathogens in humans and fishes, and quinolones are frequently used to treat such infections. Given the facility to develop antibiotic resistance, *aeromonads* may be a potential reservoir for quinolone resistance. This study aimed at tracking the bacterial populations of *aeromonads* and quinolone resistance determinants in surface and wastewater samples. Samples were collected from drinking and waste water treatment plants (surface, ground and disinfected water in a drinking water treatment plant, and raw and treated waste water), and tap water. Taxonomic diversity and antibiotic resistance phenotypes of *Aeromonas* spp. were examined. Bacteria identification and intra-species variation were determined based on the analysis of the 16S rRNA, *gyrB* and *cpn60* gene sequences. Resistance phenotypes were determined using the disc diffusion method. The presence of the resistance determinants *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qepA* and *aac(6')-Ib-cr* and the *gyrA* and *parC* mutations were surveyed. No *aeromonads* were detected in ground water, after the chlorination tank or in tap water. Eleven species of *Aeromonas* were identified. *Aeromonas veronii* prevailed in raw surface water, *A. hydrophyla* in ozonated water, and *A. media* and *A. punctata* in waste water. Ceftazidime and meropenem resistance was detected in the drinking water treatment plant and nalidixic acid resistance was intrinsic to waste water. Quinolone resistance was associated most of the times with the *gyrA* mutation in serine 83. The gene *qnrS*, but not the *qnrA*, B, C, D or *qepA*, was detected in both surface and waste water isolates. The gene *aac(6')-Ib-cr* was detected in different waste water strains isolated in the presence of ciprofloxacin. Both quinolone resistance genes were detected only in the species *A. media*. *Aeromonads* were confirmed as relevant environmental reservoirs of quinolone resistance, and this is the first study tracking antimicrobial resistance in *aeromonads* in drinking, tap and waste water.

PS2: 32

### **Diversity of *Botrytis cinerea* from vineyards in the north west Iberian Peninsula**

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*Botrytis cinerea* is associated with a fungal gray rot in the concomitant regions of northwest Spain and northern Portugal, where it is the most damaging pathogen and results in severe economic losses. Also, the physiological interactions of *B. cinerea* with *Penicillium expansum* are responsible for the production of geosmin, a volatile metabolite that transmit undesirable earthy odours to must and thus to wine. *B. cinerea* is not a homogeneous species and may be divided into several sub-species groupings. Some previous studies indicated two groups or cryptic species (I and II). Other work revealed that spore size and vegetative compatibility were characteristic features of these groups. In the present work a survey was taken of the population of *B. cinerea* from the above mentioned regions. The spore size and compatibility tests allowed characterisation of most isolates of *B. cinerea* into Group I or Group II taxa. Interestingly, some isolates could not be characterised according to their spore size and also presented ambiguous vegetative compatibility features. Furthermore, the influence of other factors on the spore size and grouping were studied. Grape variety, vineyard, country, sanitary state of the bunch and whether the isolates were obtained from the exterior or the interior of the bunch were compared with spore size and vegetative compatibility. Characterization of strains was affected by whether isolates were obtained from a particular grape variety. Also, isolates from Group II were obtained exclusively from a particular vineyard. The results suggest that there is considerable genetic diversity within the species which may explain patterns of gray rot within grapes.

PS2: 33

**Diversity of the cold-adapted genus *Psychrobacter* in a temperate estuary****Juliana Nina Azevedo, Susana Araujo, Antonio Correia, Isabel Henriques**

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Cold-adapted bacteria are considered endemic of extremely cold environments. However, recently, the occurrence of psychrophilic bacteria has been detected also at temperate and tropical environments. Until now, the cold-adapted genus *Psychrobacter* was reported to be only marginally successful and less diverse outside cold environments. Previously, two water layers from a temperate estuary (*Ria de Aveiro*, Portugal) were characterized in terms of phylogenetic composition based on culture-dependent methods. Unexpectedly, the genus *Psychrobacter* emerged as the most frequently retrieved from both water layers. Since culture-dependent methods may give a limited overview of bacterial diversity, culture-independent methods specific for the 16S rRNA gene of *Psychrobacter*, namely DGGE and clone libraries, were applied to unveil *Psychrobacter* diversity during the warm season between May to July at three sites in *Ria de Aveiro*. In this work, we developed and optimized a DGGE-based method that was found reliable and specific for the analysis of *Psychrobacter* populations in aquatic systems. The obtained DGGE profiles allowed inferring that *Psychrobacter* populations were very stable in the estuary, a strong indication for the presence of well-adapted phylotypes. Clone libraries results show that cultivable *Psychrobacter* represents only a minor fraction of the diversity found. Interestingly, albeit a part of our sequences grouped with *Psychrobacter* species already described from extremely cold environments, some clusters were clearly distinct from previously described species and might represent phylotypes specific from this temperate estuary. Phylogenetic analysis indicates that, also among cultivable phylotypes, new *Psychrobacter* species are still to be characterised. The characterization of novel species is currently on-going. Our results revealed a surprisingly high diversity among *Psychrobacter* in *Ria de Aveiro* suggesting that this genus is well-adapted to this environment.

PS2: 34

### **Dyeing of wool using an enzymatic system**

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This study reports the dyeing of wool using an enzymatic system comprising a laccase, a dye precursor and a dye modifier. Enzymatic dyeing was performed in a batch process at the temperature and pH of maximum enzyme activity. The variables of the process (precursor, modifier and laccase concentration, as well as temperature and dyeing time) were studied, according to an appropriate experimental design. Different hues and depths of shades could be achieved by varying the concentration of the modifiers and the time of laccase treatment. The duration of the enzymatic reaction appeared to be the most important factor in the dyeing process. Additionally, it was seen that the enzymatic dyeing solution could be reused up to 5 times without a significant loss in colour intensity, which stands as an important advantage in terms of reduction of cost and environmental impact. This study shows the development of an innovative enzymatic coloring process, with important reductions in effluents load and in water and energy consumption, when comparing with the traditional wool dyeing process. It is also important to state that this coloration technique, due to the soft conditions at which is performed, imparts less damage to the fiber structure, which in turns increases the final product quality.



PS2: 35

### Effect of rice crop rotation stage on the composition and activity of microbial populations of bulk soil from organically farmed rice fields

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This study aimed at comparing the composition and activity of microbial communities of bulk soil of two adjacent organically farmed rice paddies (A and B) differing on the stage of the rice crop rotation. The crop rotation was: alfafa (2 years) and rice (2 years) and the paddies studied were in the first (A) and second year (B) of rice crop. Triplicate composite soil samples were collected before seeding (April), at maximum tillering (July), and after harvesting (September). Soil samples were characterized physicochemically (pH, moisture; redox potential, total C and N; labile C, organic and inorganic labile P, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N), biochemically (enzymatic activity, N mineralization capacity) and microbiologically (MPN of (an)aerobic heterotrophs, diazotrophs, ammonifiers, denitrifiers; and anaerobic ammonia oxidizers, Community Level Physiological Profiles (CLPP), DGGE profiling and 454 pyrosequencing of total bacterial DNA). Canonical Correspondence Analysis was carried out in order to plot the variance of the microbial community composition and functional activity (CLPP data). In both soils, the composition of microbial community (analysed using culture dependent and independent methods) and its functional activity were different at each sampling date, evidencing a pattern of variation over the rice cycle. Additionally, there were differences in the composition and functional activity of microbial communities of soils A and B. Variations on the aerobic heterotrophic and anaerobic ammonia oxidizing populations allowed the distinction of the microbial communities of these soils in April and in July, while variations in anaerobic diazotrophs abundance differentiated the microbial communities in July and in September. Interestingly all these populations were more abundant in soil A than in B. These variations were related to variations in labile inorganic P, casein-protease activity and redox potential of the soils. The most important differences in the functional activity of soils A and B were observed in July. The abundance of organisms growing on putrescine, *p*-hydroxybenzoic acid, xylose, cyclodextrin, tween 60 and malic acid were responsible for the differentiation of both paddies and the soil inorganic N and moisture contents and the pH values were the variables explaining the differences. This study showed that rice crop rotation stage influences the composition of microbial communities and the functional activity of bulk rice paddy soil.

PS2: 36

### Effect of surfactants on the kinetics of anthracene biodegradation by *P. putida* ATCC

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Polycyclic aromatic hydrocarbons (PAH) are organic contaminants that resemble a certain threat to human health and natural ecosystems due to their known carcinogenic and mutagenic properties. The low water solubility of PAHs limits their availability to microorganisms, which is a potential problem for bioremediation of contaminated sites. Nonionic surfactants at concentrations above the critical micelle concentration (CMC) may contribute to increase the rate of biodegradation of PAH by solubilization or emulsification (Edwards *et al.*, 1991). On the other hand, surfactants may reduce the adhesion of cells to the hydrocarbon-water interface (Rosenberg and Rosenberg, 1995). The ability of surfactants to enhance the bioavailability of PAH will depend on the mechanisms used by bacteria to get access to such hydrophobic substrates. Therefore, the present study aimed to investigate the effect of surfactants on the kinetics of PAH biodegradation by *P. putida* ATCC 17514 and addressed the specific issue of the effect of the surfactant on bacterial adhesion to PAH, which is believed to be an important mechanism for the uptake of hydrophobic compounds (Rodrigues *et al.*, 2005). For that purpose, anthracene biodegradation assays were carried out with three surfactants, namely, the nonionic Tween 20, at a concentration of 0.08 mM, slightly higher than its CMC, the anionic SDS (Sodium dodecyl sulfate) and the cationic surfactant CTAB (Cetyltrimethyl ammonium bromide), both at a concentration lower than the CMC, 0.35 mM and 0.27 mM, respectively. Measurements of zeta potential and bacterial surface hydrophobicity were also performed. The results obtained showed that the surfactants caused different effects on the biodegradation of anthracene, as sole carbon and energy source for growth, which is considered one of the most hydrophobic PAH. In fact, the presence of the non-ionic surfactant Tween 20, at a concentration of 0.08 mM doubled the maximum specific biodegradation rate of anthracene. Moreover, the presence of SDS (0.35 mM) also doubled the removal rate of anthracene, reaching  $0.35 \text{ mg L}^{-1} \text{ h}^{-1}$ . On the other hand, the cationic surfactant CTAB (0.27 mM) had a negative effect on the biodegradation of such PAH, leading to an abrupt decrease on the biomass growth curve. The results also demonstrated that in the presence of Tween 20 or SDS (100 to  $1000 \text{ mg L}^{-1}$ ) an increase in the oxygen uptake rate was observed during the utilization of anthracene by *P. putida*.

PS2: 37

### Effect of temperature, inoculums concentration and nitrogen in the degradation of diesel oil by microbial consortium isolated from the port area of suape, Pernambuco - Brazil

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The Diesel oil is a potential environmental pollutant, which justifies the need to select degrading microorganisms. The objective of this study was to investigate the degradation of the constituents of diesel oil by a mixed microbial consortium isolated from the port area of Suape, in the Brazilian state of Pernambuco, against different conditions of temperature, inoculum concentration and nitrogen. *Selection of the consortium* - In this study we used microorganisms which are stored in the Culture Collection of the Department of Antibiotico, Universidade Federal de Pernambuco. The strains were previously selected by the technique of the redox indicator 2,6-dichlorophenol indophenol (DCPIP). For the composition of the consortium has conducted a test of microbial antagonism. *Acclimation* - Acclimatization was carried out in Erlenmeyer flasks, containing the mineral medium of Bushnell Haas (BH), the selected consortium and increasing concentrations (1%, 4%, 7% and 10%) of Diesel oil, which were kept under agitation of 150 rpm and temperature 30±1°C for 48 hours. *Experimental design* - It was used the complete factorial design 2<sup>3</sup> where it was verified the effect of adding nitrogen (NH<sub>4</sub>NO<sub>3</sub>) through the relation C:N de 10:1, 50:1 e 90:1, amount of inoculum (1, 2 e 3 blocks of agar) and temperature (25°C, 30°C e 35°C). The tests were conducted for seven days. At the end of the tests, chromatographic analysis was performed using gas chromatograph coupled to mass spectrometry (GC-MS). The results were analyzed by software Statistica 7.0. Microorganisms investigated, those with less time to reduce indicator DCPIP (24h, 48h, 16h and 26h) were selected and subsequently identified as *Serratia marcescens* (UFPEDA 839), *Staphylococcus saprophyticus* (UFPEDA 800), *Rhodotorula aurantiaca* (UFPEDA 845) and *Candida ernobii* (UFPEDA 862). There were no zones of inhibition of microbial growth in the antagonism test in the selection of the consortium, suggesting that the four selected microorganisms showed no antagonistic activity. The matrix analysis of the complete factorial design 2<sup>3</sup> showed different percentages of biodegradation ranging from 10.98% to 54.53%. Among the factors studied, increasing the amount of inoculum and the decrease of temperature and ammonium nitrate had a positive effect on degradation of diesel oil hydrocarbons, however only the last factor showed at that stage a significant effect on the ability of biodegradation by the consortium.

PS2: 38

**Enhanced oil recovery under laboratory conditions using biosurfactant-producing microorganisms****Eduardo J. Gudiña<sup>1</sup>, Jorge F. B. Pereira<sup>2</sup>, Lúcia R. Rodrigues<sup>1</sup>, João A. P. Coutinho<sup>2</sup>, José A. Teixeira<sup>1</sup>**<sup>1</sup>IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal; <sup>2</sup>CICECO – Chemistry Department, University of Aveiro, Portugal

Oil recovery comprises a primary phase, which produces oil using the natural pressure drive of the reservoir, and a secondary phase, which includes the injection of water to improve the flow of oil to the wellhead [1,2]. While primary recovery produces 5-10% of the original oil in place, recovery efficiencies in the secondary stage vary from 10% to 40% [1]. Most of the unrecovered oil (up to two-thirds of the total oil reserves) is trapped in the reservoir pores by high capillary forces [2]. Microbial Enhanced Oil Recovery (MEOR) is a tertiary oil recovery process where microorganisms and their metabolites are used to retrieve unrecoverable oil from mature reservoirs. Stimulation of biosurfactant production by indigenous or injected microorganisms can reduce the capillary forces that retain the oil into the reservoir. In this work, a sand pack column model was designed to simulate the oil recovery operations in oil reservoirs and evaluate the mobilization of residual oil by microorganisms. Three *Bacillus subtilis* strains (309, 311 and 573), previously isolated from crude oil samples, were used in this study. They grow and produce extracellular biosurfactants at 40°C under anaerobic conditions in medium supplemented with hydrocarbons. Biosurfactants produced by those isolates reduce the surface tension of water from 72 to 30 mN/m, exhibit emulsifying activity and are not affected by exposure to high temperatures (121°C) which makes them good candidates for application in biosurfactant mediated MEOR. Acrylic columns (250 ml) packed with acid washed sand were first flooded with water, after that saturated with paraffin, and then washed with water to remove the excess of paraffin. Afterwards, the isolates were injected into the columns with the optimized medium and incubated at 40°C. After 14 days, the columns were flooded with water and the additional oil recovery (AOR) was calculated as the percentage of paraffin recovered. AOR using *B. subtilis* 309, 311 and 573 was  $35.0 \pm 1.0 \%$ ,  $23.5 \pm 1.2 \%$  and  $19.8 \pm 1.9 \%$ , respectively. The results obtained suggest that stimulation of biosurfactant production by these strains in the oil reservoir can contribute to mobilize entrapped oil.

[1] Sen R. 2008. *Progress in Energy and Combustion Science* 34: 714-724.[2] Brown LR. 2010.. *Current Opinion in Microbiology* 13: 1-5.

PS2: 39

### **Fungal biofilms in a Brazilian water distribution system including bacteria and algae**

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Filamentous fungi in drinking water are associated with blockage of water pipes, organoleptic biodeterioration, and contributing pathogenic and/or mycotoxigenic species. Consequently, there is loss of water quality. The contribution of filamentous fungi to biofilms is underreported, despite an increasing numbers of studies concerning the organisms from potable water. This study presents a sampling device that can be inserted directly into pipes within water distribution systems (WDS), hence exposing biofilms to conditions experienced *in situ*. Calcofluor White M2R staining and fluorescent *in situ* hybridization with morphological analyses using epifluorescence microscopy, was used for fungal biofilm analysis. DAPI was also applied for bacterial observation. Filamentous fungal in biofilms were detected predominantly after 6 months on coupons exposed to raw, decanted water and at the entrance of the water distribution system. Algae, yeast and bacteria were also observed representing a high biodiversity. The use of samplers which allowed analyses of coupons *in situ* is a useful innovation when studying biofilms. CW was a rapid and efficient stain to detect fungi and filamentous fungi which could be differentiated by well-known morphologies. FISH allowed the detection of specific group of eukaryotic microorganism and fungi, and hence confirming their presence. Fungi are likely to play an important role in microbial interactions within water biofilms and consequently in microbial water quality.

PS2: 40

**Fungi and the aquatic environment: are mycotoxins produced?****Beatriz Reis Oliveira<sup>1</sup>, João Pedro Ferreira<sup>3</sup>, Maria Rosário Bronze<sup>3</sup>, Vanessa Jorge Pereira<sup>1,2</sup>, Maria Teresa Barreto Crespo<sup>1,2</sup>, Maria Vitória San Romão<sup>1,2</sup>**<sup>1</sup>IBET, Oeiras, Portugal; <sup>2</sup>ITQB - UNL, Oeiras Portugal; <sup>3</sup>iMED, Faculdade de Farmácia - UL, Lisboa, Portugal

To protect consumers from waterborne diseases, drinking water utilities should ensure that the distributed water is completely free of pathogenic organisms. However, many drinking water utilities rely solely on monitoring indicator bacteria to verify the microbiological quality of drinking water. Hageskal *et al* (2006) reported a wide occurrence of fungi in Norwegian drinking water and concluded that the mycobiota of water should be considered when the microbiological safety and quality of drinking water are assessed. In a recent study, hundreds of isolates were stored after two years of monthly routine sampling of source waters with very different matrix compositions (Pereira *et al.*, 2009). When present in certain matrices, fungi may produce mycotoxins (naturally occurring metabolites of fungal species) that can display overlapping toxicities to invertebrates, plants, and microorganisms (Bennett *et al.*, 2003). EU legislation already exists to limit the levels of mycotoxins (aflatoxins, ochratoxin A, deoxynivalenol, zearalenone, patulin, and fumonisins) in food (Commission Regulation N1881/2006), but little attention has been given to their presence in water (e.g. Hartmann *et al*, 2008; Paterson *et al* 1997). In this study, eight species of fungi (*Aspergillus fumigatus*, *Aspergillus terreus*, *Cladosporium cladosporioides*, *Cladosporium tenuissimum*, *Fusarium begonia*, *Penicillium citrinum*, *Penicillium melanoconidium* and *Phoma glomerata*) detected in water were cultured in rich media and untreated surface water. Sensitive and selective HPLC-MS/MS methods were optimized and validated using multiple reaction monitoring mode for quantification of zearalenone, a-zearalenol, b-zearalenol, a-zearalanol, b-zearalanol, nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, aflatoxins (B1, B2, G1, G2 and M1) and ochratoxins. The limits of detection of the methods varied between 0,3 and 10 ug/L. Sample concentration procedures using solid phase extraction were optimized in order to achieve lower limits of detection and for some compounds, values under 1 ng/L were obtained. Sample analysis were performed in order to target the selected mycotoxins as well as in full scan mode to determine other compounds produced by fungi and to answer the question: are mycotoxins produced in drinking water sources?

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PS2: 41

### Herbicidal properties of Portuguese propolis samples

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Propolis is a natural product made by honeybees (*Apis mellifera* L.) with important physical and biological protective functions, being used to maintain hive integrity, as well as adequate thermal and sanitary conditions. It is a complex mixture made essentially from collected plant exudates, being composed of waxes, resins, balsams, pollen, essential oils, and other organic and mineral compounds. It has a high plant source-dependent chemical variability and more than 300 constituents were already identified. Over the last decades, several biological and pharmacological properties have been identified in propolis, namely antiviral, antibacterial, antifungal, antiprotozoan, anti-inflammatory, antitumor, cytostatic, antioxidant but, so far, no phytotoxic properties were reported. The principal compounds responsible for propolis bioactivities are flavonoids and phenolic compounds, but very often different propolis types have distinct main bioactive compounds. Propolis value is still poorly acknowledged by the Portuguese beekeepers, generally constituting a by-product that is discarded during hive management, and only recently Portuguese propolis has started to be investigated. It is our goal to contribute to the evaluation of its chemical diversity and biological activities. The study here reported concerns specifically with the evaluation of propolis phytotoxic effects envisaging its potential economic valorization as a bioherbicide. Phytotoxic effects of *n*-hexane (HE) and ethanol (EE) extracts obtained from propolis samples collected in two apiaries (Côa – C, Pereiro - P) in the east-central region of Portugal, were tested in plantlets of flax (*Linum usitatissimum* L.) grown *in vitro*. Results shown that both samples and types of extracts impaired plant growth but specific differences were detected: all treatments inhibited root growth but P samples, in particular HE.P, were much more effective than C ones, and while HE did not affect epicotyl and hypocotyl growth, EE, in particular EE.P, strongly inhibited their growth. Total polyphenols and flavonoids quantification revealed that EE.P has higher contents than EE.C, but, due to the low content of such compounds in H extracts, the effect on root growth must be caused by other type of compound. The present work allowed concluding that propolis present strong phytotoxic effects and that there are significant differences among samples even from close geographical regions.

PS2: 42

**High throughput screening of hydrolytic enzymes of *Aspergillus* section *Nigri* strains****Marta Filipa Simões<sup>1</sup>, David Navarro<sup>2</sup>, Laurence Lesage-Meessen<sup>2</sup>, Cleidir Santos<sup>1</sup>, Nelson Lima<sup>1</sup>**

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Biological Resource Centres (BRCs) and culture collections are essential components of the infrastructures for scientific research and industry and their establishment and maintenance depends on the implementation of reliable preservation techniques and appropriate quality assurance to allow them to become effective and efficient. Fungal strains identification and characterisation are important tasks within the context of BRC. Using a polyphasic approach for identification, allows the increment of associated information for every fungal strain. With this in mind and to harness the experimental based knowledge for scientific research on filamentous fungi, specifically the *Aspergillus* section *Nigri*, the enzymatic activity of this section was assessed by screening the activity of a set of enzymes, using several methods, in which one is based upon miniaturized cell cultures and automated expression screening in microwell plates. *Aspergillus* use mainly polysaccharides as a carbon source, which they need to degrade before using as substrates. Some of these polysaccharides can be split in three major groups: cellulose, hemicellulose (xylan, galactomannan and xyloglucan) and pectin. For the degradation of the referred plant polysaccharides, fungi produce a broad range of hydrolytic enzymes with different and complementary catalytic activities that can be screened for each fungal strain. Some of these enzymes have a large industrial potential and studying enzymatic profiles, exploring the biopotential of fungal strains supports research on their application. The chosen medium for the growth of the targeted strains was adequate to determine, evaluate and screen the enzymatic profiles for the four targeted enzymes: carboxymethyl cellulase, xylanase, pectinase and mannanase. The enzymes analyzed in this screening assay were present in most of the *Aspergillus* strains tested. The obtained results allow to better differentiate between the studied fungi and to complement the information about each fungal enzymatic profile of interest for the MUM - Micoteca da Universidade do Minho – culture collection and also to add information for research on the different preservation protocols as a mean of analysing the post-preservation characteristics of the fungi.

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PS2: 43

***Humicola brevis*: The first evidence for anthracene biodegradation.**

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Anthracene is a non-carcinogen polycyclic aromatic hydrocarbon (PAH) found frequently in PAH-contaminated sediments. Anthracene is metabolized efficiently to a dead-end metabolite, anthraquinone, by different fungal strains of the genera *Bjerkandera*, *Phanerochaete*, *Trametes*, and *Coprinellus*, among others. However, no studies regarding PAH metabolism have been reported in *Humicola* strains. Although these thermophilic fungi have long been known to be involved in composting and humification processes, previous and recent studies show their potential in biotechnology industry due to the secretion of high levels of extracellular enzymes, such as lipases, used in laundry detergents, or cellulases and xylanases, with increasing application in biobleaching of pulp in the paper industry. We show now the first study of anthracene biodegradation by a *Humicola brevis* strain. *H. brevis* was cultured in soy flour liquid medium supplied with 100  $\mu$ M anthracene along 6 weeks. Further analysis of supernatants by HPLC revealed that this strain was able to degrade up to 74% anthracene. This decrease in anthracene level was followed by increased levels of more polar intermediate compounds, as a result of anthracene biodegradation. The discovering of this new role of a *Humicola* strain regarding PAH biodegradation in alkaline environments, which differs notably from the typical habitats of white rot fungi, leaves an open door to further research in bioremediation of polluted soils.

PS2: 44

### Identification of black aspergilli group based on a polyphasic approach, including MALDI-TOF ICMS

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Black aspergilli is a group of filamentous fungi comprised by species of *Aspergillus* section *Nigri*. They can be isolated from different environments however the main habitat of these species is the soil. According to Samson *et al.* (2007) there are 19 species of *Aspergillus* section *Nigri* accepted. The species identification must be delineated based on a polyphasic approach, including morphology, physiology, profile of secondary metabolites and molecular biology (Samson and Varga, 2009). Additionally, according to Santos *et al.* (2010a, 2010b) it is clear that spectral analyses add value to the polyphasic approach. Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) is a spectral technique that analyses the chemical molecular mass of the microbial cellular composition providing rapid and discriminatory fingerprints for identification. This work aimed to perform a polyphasic approach based on morphological, biochemical and spectral analysis by MALDI-TOF ICMS for the characterisation and identification of the section *Nigri*. Seventy-four isolates of the section *Nigri* deposited at University of Recife Mycology (URM) Culture Collection were analysed. Additionally, 12 type strains of the section *Nigri* deposited at Micoteca da Universidade do Minho (MUM) Culture Collection were used as reference for MALDI-TOF ICMS studies. The data obtained from the polyphasic approach indicates that MALDI-TOF ICMS results corroborate with those data obtained using classical taxonomy and biochemical analyses. Overall, from the 74 cultures, 75% were finally identified as *A. niger*, 15% as *A. japonicus*, 5% as *A. carbonarius*, 4% as *A. aculeatus* and 1% as *A. foetidus*. Moreover, the biochemical analyses showed that from the whole population of *A. niger* 20% and 13% were characterised as ochratoxin A (OTA) and fumonisin B<sub>2</sub> producers, respectively. *A. carbonarius* and *A. foetidus* were in total OTA producers. The 74 isolates belonging to section *Nigri* deposited at URM were deeply studied and their associated information update and requalified.

Samson *et al.* (2007). *Stud. Mycol.* 59: 129-145. Samson *et al.* (2009). *Med. Mycol.* 1-8. Santos *et al.* (2010a). *J. App. Microbiol.* 108: 375-385. Santos *et al.* (2010b). *Res. Microbiol.* 161: 168-175.

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PS2: 45

### Impact of experimental factors on polyhydroxyalkanoate (PHA) analysis in microbial systems using multivariate statistics

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Polyhydroxyalkanoates (PHA) are polymers composed of various polyester monomers. PHA production by microbial cultures has gained interest as a biodegradable and biocompatible alternative to common plastics such as polystyrene. Additionally, PHA are a fundamental part of the metabolism of several bacteria. Therefore, an accurate and reliable analysis of PHA is critical for studies focused on PHA production and many environmental biotechnology systems. Braunegg (1978) proposed a method for poly-3-hydroxybutyrate (PHB) analysis, where the PHA monomers are hydrolyzed to their methyl-ester form (methanolysis) and then analyzed by gas chromatography. Since then, modifications were introduced to adapt the method to activated sludge samples (Apostolides, 1981) and to extend it to other monomers such as poly-3-hydroxyvalerate (PHV) and poly-3-hydroxy-2-methylvalerate (PH2MV) or poly-3-hydroxy-2-methyl butyrate (PH2MB) (e.g. Comeau, 1988; Oehmen, 2005). These and other modifications resulted in a large number of variations of the method. This study investigated the optimal conditions for PHA quantification (PHB, PHV, PH2MV and PH2MB monomers), in six different systems using a design of experiments strategy. The six microbial cultures were collected from a full-scale wastewater treatment plant and 5 lab-scale reactors (two for PHA production, one photosynthetic, one for enhanced biological phosphorus removal and one for biological nutrient removal using real wastewater). The factors tested included sulfuric acid concentration in the methanolysis reaction (3-20% v/v), hydrolysis reaction time at 100°C (3-20h) and concentration of biomass (2-8 g/L). Results indicated that high acid concentration has a negative effect on PHB extraction. PHB and PHV are fully hydrolyzed after 3h at 3% acid, but PH2MB and PH2MV need longer hydrolysis, inversely proportional to the acid concentration (5h, 20% acid). The biomass concentration does not play a very significant role in the analysis, but it is recommended using 5-8 g/L, especially for low-content PHA cultures. Overall, this study advances our knowledge on the appropriate conditions needed to implement this important analytical method.

Apostolides (1981) *AMB*, 13(1), 62–63 Braunegg (1978) *EJAMB*, 6(1), 29-37 Comeau (1988) *AEM*, 54(9), 2325-2327 Oehmen (2005) *JChr A*, 1070(1-2), 131-136. Acknowledgements: FCT for PTDC/EBB-EBI/103147/2008, SFRH/BD/25275/2005, 38763/2007, 42085/2007, 65113/2009, 29477/2006 and BDP/30800/2006.

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PS2: 46

### Inter- and intra-specific molecular and ecological variations among *Aphanizomenon* (Nostocales, Cyanobacteria) strains isolated from a eutrophic shallow lake

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The occurrence of cyanobacterial blooms of *Aphanizomenon* spp. (Nostocales, Cyanobacteria) is recurrently reported worldwide, with deleterious effects on aquatic communities. However, the diversity within the genus *Aphanizomenon* is still controversial. The present work gathers information retrieved from molecular, ecological and ecotoxicological data. *Aphanizomenon* bloom-forming strains isolated from a eutrophic shallow lake (Vela Lake, Portugal) were, in a first stage, characterized by a combination of phenotypic and molecular approaches. The molecular affiliation of the strains was established through sequences of *16S rRNA*, *nifH* and *hetR* genes, and inter-specific genetic variability was checked through repetitive sequences fingerprinting. Both morphological and molecular data showed that strains belonged to *Aphanizomenon issatschenkoi* (*Cuspidothrix issatschenkoi*), *Aph. gracile* and *Aph. aphanizomenoides*. Fingerprinting showed a persistent occurrence of *Aph. gracile* and *Aph. aphanizomenoides* strains over the years but also indicated that *Aph. aphanizomenoides* strains were not clonal within the same bloom. In a second phase, we investigated the effects of different levels of phosphorus and nitrogen on the growth of these strains. Phosphorus had a significant effect on the growth of all the strains tested. The growth of *Aph. gracile* strains was unaffected by variation in nitrate levels, but strains of *Aph. issatschenkoi* (UADFA1) and *Aph. aphanizomenoides* (UADFA6, UADFA7 and UADFA13) were moderately to extremely sensitive to nitrate depletion. In a third step, algal growth inhibition tests were performed to assess the potential allelopathic effects of the strains' filtrates on the growth of four green algae (*Pseudokirchneriella subcapitata*, *Chlorella vulgaris*, *Pandorina morum* and *Coelastrum astroideum*). Results showed a significant inhibition of microalgal growth by filtrates of strains from the three species, but also demonstrated intra-specific differences in allelopathic activity. In general, the results from the present work strengthen the idea that intra-specific variation may play an important role in bloom dynamics.

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PS2: 47

### Is the microbial population and functional activity of bulk paddy soil influenced by rice plants (*Oryza sativa*)?

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To study the effect of rice plants on different soil parameters over the rice cycle, triplicate composite samples of bulk soil, from plots seeded (AS) and non-seeded (ANS), were collected in an organically farmed paddy field. Sampling was carried out at distinct steps of the rice cycle: before seeding (April), at maximum tillering (July), and after harvesting (September). Physico-chemical (pH, moisture, redox potential, total C and N, total and inorganic labile P;  $\text{NH}_4^+\text{-N}$ ;  $\text{NO}_3^-\text{-N}$ ; and labile C), biochemical (enzymatic activities and N mineralization capacity) and microbiological (total cells, microbial biomass-C, MPN of (an)aerobic heterotrophs, diazotrophs, ammonifiers, denitrifiers; and anaerobic ammonia oxidizer, Community Level Physiological Profiles (CLPP), and DGGE profiling) parameters were determined and Canonical Correspondence Analysis (CCA) was carried out. The composition of soil microbial community (analysed using culture dependent and independent methods) and its functional activity varied over the rice cycle, in both plots (AS and ANS). The variables that mainly explained the variations were the total and inorganic labile P and  $\text{NO}_3^-$  concentration. Differences between the microbial communities of plots AS and ANS could be explained by pH KCl variation and occurred mainly in September. Indeed, at this sampling date the anaerobic diazotrophs were more abundant in plot AS than in ANS. The presence of rice plants influenced also the functional activity of the soil communities, but only in July. Major variations corresponded to microorganisms growing on *o*-hydroxybenzoic acid, D-glucose-1-phosphate, asparagine and cellobiose and were influenced by the content of labile C, redox potential and moisture content. In summary, rice plants influenced the microbial communities of bulk paddy soil after harvest, as well as the microbial functional activity during rice growth towards maturation.

PS2: 49

### Marine fungal communities of driftwood collected on Portuguese sandy beaches

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This communication aims to report the occurrence, diversity and similarity of fungal communities associated to driftwood from 4 sandy beaches: Vagueira, Cascais, Meco and Vila Nova Mil Fontes, located on the west coast of Portugal. At each beach 6 collections were performed at the intertidal zone and the total number of samples ranged from 40 to 60. In the laboratory, samples were examined under a dissecting microscope for the detection of marine fungal fruit bodies and spores. During this period of time between collection and observation the samples were incubated in moist chambers, at 18° C. The intervals of incubations ranged between 4 to 6 weeks. Fruit bodies and spores of marine fungi were observed under a light microscope and the identification was based on dichotomous illustrated keys. We calculated the percentage of occurrence of each taxon, the average number of fungi per sample, the percentage colonization and the diversity indices. The taxa were classified as "very frequent" (>10 %), "common" (5-10%), "rare" (1% ≤ x <5%) and "sporadic" (<1%). To compare fungal communities and determine species contributions we used ANOSIM and SIMPER procedures (PRIMER version 5.2.4, PRIMER-E Ltd, Plymouth, UK). The fungal communities contained members of Halosphaerales, Pleosporales and Lulworthiales and anamorphic fungi, comprising a total of 37 taxa. Fungal richness achieved the same highest value (20 taxa) on the samples from the sandy beaches in the southern locations of Meco and Vila Nova de Mil Fontes and the lowest value (15 taxa) on driftwood from the northernmost beach, Vagueira. There were no statistically significant differences on fungal diversity between the sandy beaches. An ANOSIM analysis showed that only driftwood from Meco supported fungal communities different from the other beaches. The geographic location, the good quality of sea water and the high preservation of Meco sandy beach might explain this fact. A SIMPER analysis showed that *Corollospora maritima*, *Ceriosporopsis halima* and *Lignicola laevis* were the species that contributed the most for the differences between the driftwood mycota from Meco and the other beaches. We also performed a MDS analysis to compare driftwood fungal communities from our data with results published from other surveys on temperate and tropical locations. As expected, these comparisons showed higher similarities with the other temperate locations.

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PS2: 50

**Microbial diversity of groundwaters and bottled natural mineral waters****Luís França<sup>1</sup>, Milton S. da Costa<sup>2,3</sup>**<sup>1</sup>Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; <sup>2</sup>Department of Life Sciences, University of Coimbra, Coimbra, Portugal; <sup>3</sup>Microbiology Unit, BIOCANT Biotechnological Park, Cantanhede, Portugal

Natural mineral waters are defined as microbiologically wholesome waters, characterized by constancy of chemical composition. Modification of their biological components is prohibited by European law. The lack of knowledge about the flora of the aquifers is limited to our ability to assess microorganisms in subterranean environments from which the microorganisms in bottled water may originate. So far, the majority microbiological data has been obtained using classic culture methods. However, the widespread use of molecular phylogenetic studies, revealed the inadequacy of these methods in providing a reliable picture of the existing biodiversity. Our major goal is to assess the microbial community diversity of subterranean water and understand the microbial population dynamics after bottling and storage of natural mineral water. The comparison of data from molecular phylogenetic analysis and simultaneous parallel isolation studies will give us a detailed profile on the microbial biodiversity existing in the aquifers, as well as their stability. Samples were collected from borehole head from two aquifers and from the bottles, immediately after bottling and after 6, 15, 30, 60 and 180 days of storage. These sample collections were made in duplicate. Samples were concentrated by filtration, through variable pore size filters. These were placed on R2A and on diluted R2A agar plates, and incubated at 22 and 37 °C for up to 21 days under aerobic conditions. Strains were grouped by typing and the 16S rRNA gene of selected representatives was sequenced. The most frequently isolated microorganisms from the aquifers were heterotrophs belonging mainly to the phyla Actinobacteria and Proteobacteria but also to Bacteroidetes, *Deinococcus-Termus* and Firmicutes. The bottling procedure induced changes in the autochthonous bacterial structure. After bottling were retrieved isolates shown to belong to the Alpha, Gamma and mainly to the Beta class of proteobacteria with predominance of *Ralstonia*. Storage time has induced a shift in the community composition, since the cultivable community isolated after 60 days of storage was identified as Betaproteobacteria, with predominance of *Variovorax* and *Ralstonia*. Further analysis, with culture-independent techniques, namely DGGE to fingerprint the bacterial populations and massively parallel tag sequencing, will provide us with new data that will allow an overview of the environments biodiversity.

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PS2: 51

### Microbial population dynamics in aerobic and anaerobic/aerobic sequencing batch reactors

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Textile dyes are designed for color stability and often for high water solubility, rendering color removal difficult in conventional aerobic biotreatment units. The use of staged anaerobic/aerobic regimes has been used to overcome this difficulty, with notable success in the decolorization of azo dyes. However, the effect of introducing an anaerobic phase on the microbial diversity and population dynamics has scarcely been reported in this context. The main goal of this work was to investigate this effect through biomass characterization through optical and fluorescence microscopy techniques. The system included three 1-liter sequencing batch reactors (SBR) operating in 8-h cycles. SBR1 had a 6-h aerobic reaction phase whereas SBR2 and SBR3 had a 4-h anaerobic phase followed by a 2-h aerobic phase. An azo dye was added to SBR1 and SBR2 while SBR3 was operated without dye, as a control. Cell viability and Gram status were evaluated through fluorescence microscopy. The Sludge Biotic Index (SBI) was determined by microscopic protozoa identification and counting, according to Madoni (1994). Initially there was a biomass depletion effect as the inoculum adapted to the conditions described above, also evidenced by the low viability levels obtained through fluorescence staining. Gram-negative bacteria (GNB) were generally dominant. The SBI kept an average level of well colonized and stable sludge with sub-optimal biological activity, corresponding to good treatment efficiency for all SBR. Thus, the onset of an anaerobic phase did not significantly change the protozoan population, as compared to the aerobic cycle. As expected, color was not removed in SBR1 while SBR2 reached a maximum of 92% dye removal. After 225 cycles a decline in dye removal yield was noted in SBR2, coincident with decreased Chemical Oxygen Demand (COD) removal yields in all reactors. In SBR2, this was accompanied by an increase in observed extracellular polymeric substances (EPS) production without viability loss, suggesting high cell stress levels. Before this occurrence, SBR2 was very similar to SBR3 in EPS, floc size and composition, scarce in gram-positive bacteria (GPB), differing from SBR1 in all aspects. Afterwards, SBR3 grew to an almost balanced population in GPB and GNB. To shed light on the COD (all reactors) and color (SBR2) removal yield decline occurrence, population composition is currently under characterization by Fluorescence *in situ* Hybridization (FISH).

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PS2: 52

### **Plant growth bacteria as promoters of *Betula pubescens* establishment in anthropogenic sediments**

**Albina R. Franco, Nadine R. Sousa, Miguel A. Ramos, Paula M.L. Castro**

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Over the last decades, industrial sediment deposition has increased, contaminating not only soil and their surrounding areas but also other natural resources. The potential use of these sites for agriculture and forestry practices is jeopardised and their remediation is critical and expensive. The utilization of biotechnological tools, such as plant growth promoting bacteria (PGPB), could help remediation of industrial contaminated soil as they can be used as plant facilitators for land recovery. The aim of this study was to assess the potential of PGPB to enhance the growth of *Betula pubescens* in high pH anthropogenic sediments and forest soil. *Betula* seedlings were inoculated with *Bacillus pumilus* (B1), *Mesorhizobium* sp. (B2) and *Streptomyces* sp. (B3) and harvested after 6 month growth under control conditions. Results show that B2 and B3 significantly increased seedling growth performance (dry weight of shoot and root) and height in forest soil whereas in industrial sediment, only B2 positively affected seedling growth and performance. B1 had no significant effect in the tested soil. The effect of inoculation on the bacterial community in seedling roots and rhizosphere was also analysed by PCR-DGGE Differences arose between inoculated and uninoculated soil, suggesting PGPB may significantly influence the rhizosphere bacterial community established over a period of time. The study shows that PGPB may be used as a biotechnology tool contributing to the successful plant establishment in contaminated environments.

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PS2: 53

**Polyphosphates and poly- $\beta$ -hydroxybutyrate granules identification through quantitative image analysis in enhanced biological phosphorus removal systems****Daniela P. Mesquita<sup>1</sup>, Cristiano Leal<sup>2</sup>, Adrian Oehmen<sup>3</sup>, António L. Amaral<sup>1,2</sup>, Maria A. M. Reis<sup>2</sup>, Eugénio C. Ferreira<sup>1</sup>**<sup>1</sup>Universidade do Minho, Portugal; <sup>2</sup>Instituto Politécnico de Coimbra, Portugal; <sup>3</sup>Universidade Nova de Lisboa, Portugal

Enhanced biological phosphorus removal (EBPR) is a widely implemented technique to remove phosphate from wastewater treatment processes, being cost effective and more reliable than traditional chemical methods. EBPR is performed by operating the system sequentially with anaerobic and aerobic conditions. Several studies have already been performed ranging from different strategies for the competition between polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) to modeling both types of bacterial activities. Until now, little attention has been given to the development of newer, faster, simpler, and better suited monitoring techniques for this type of system. This work is focused on the development of fluorescence based image analysis techniques for polyphosphates and poly- $\beta$ -hydroxybutyrate granules detection in EBPR systems since off-line analyses are labor intensive and difficult to apply in full-scale plants. A lab-scale sequencing batch reactor fed with synthetic wastewater containing volatile fatty acids (VFAs) and orthophosphate was used. The reactor had a working volume of 4 L and was operated with a cycle time of 6 h consisting of 2 h anaerobic, 3 h aerobic, 50 min settling and decanting, and 5 min wasting. In each cycle, 2 L of synthetic wastewater was fed to the reactor in the first 5 min of the anaerobic period, resulting in a hydraulic retention time (HRT) of 12 h. The pH was controlled during both the anaerobic and aerobic periods around 7, and the temperature was controlled at 30 °C in order to provide selective advantages to GAOs over PAOs. The ratio between chemical oxygen demand (COD) and P in the feed was kept at 10 (g COD/g P). Biomass samples were collected at the end of the anaerobic and aerobic phases and fixed with phosphate buffer saline solution (PBS) and ethanol. Two fluorescence staining methods were used: (1) DAPI for poly-P identification; and (2) Nile blue for poly- $\beta$ -hydroxybutyrate granules. So far, promising results were achieved regarding the quality of images obtained by these fluorescence staining methods which are later treated by image analysis procedures.

PS2: 54

**Polyurethane biodegradation by filamentous fungi: influence of culture medium.****Andres Arguello-Nuñez, Araceli Loredo-Treviño, Raul Rodriguez, Cristobal N Aguilar**

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The aim of this work was to design a culture media for biodegradation of a Polyurethane (PU) using filamentous fungi. 2 carbon sources and 2 nitrogen sources were tested in 2 different ratios according to PU amount in a bifactorial 2<sup>2</sup> experimental design. Three microorganisms were tested, *Trichoderma* sp., (DIA-T), *Paecilomyces* sp (NK12) and *Aspergillus niger* (PSS). At the end of incubation time, cultures were filtered and protein production, esterase, urease, laccase and protease activities were determined and total phenol content were determined in the supernatant. Biomass was determined measuring glucosamine. Structural modifications to residual PU were detected using FTIR and thermogravimetric analysis (TGA) analysis. No protease and laccase activity were detected. Urease was detected in all microbial treatments. No differences in the amount of extracellular protein were found at the end of culture time for any of the treatments. However, it can be seen that DIA-T strain shows, in general, the biggest production of protein out of the three strains tested. Also, it can be observed, that a major amount of protein is produced when an additional nitrogen source is added. Also, variations were observed when nitrogen sources were added in a 1:1 ratio. It is interesting that DIA-T strain, when cultured only with PU showed the biggest amount of protein. This could suggest a more aggressive attack towards the polymer. Urease activity was present in several microbial treatments. No important differences were found in the treatments either an important amount of biomass. TGA analysis demonstrated treatment with the fungal strain DIA-T promoted the polyurethane biodegradation. FTIR spectra of polyurethane residues revealed (at 800-1200 cm<sup>-1</sup>) two peaks in PU without microbial treatment which are characteristic of nitrogen-carbon bonds. Loss of one of them or in its intensity after microbial treatment indicated the breakdown of these bonds. This work showed that is possible to degrade polyurethane using filamentous fungi and that an additional nutrient source enhances this degradation.

PS2: 55

**Reactive Black 5 dye decolourisation by free and immobilized cells of *Trametes versicolor* in chemostat under high alkaline and salt conditions****Cristiane Angélica Ottoni, Luis Lima, Cledir Santos, Nelson Lima**

IBB/ Centre of Biological Engineering

The most important group of synthetic colourants, that it is used extensively in the textile industries, is azo dyes. During the dyeing process, a large amounts of alkaline effluents with high concentration of dyes and salts are released without previous treatment for aquatic environment. Nowadays, environmental regulations in most countries require textile effluents to be decolourised before discharging. This led to the study of innovative and environmental friendly technologies. The use of free or immobilized cells of white-rot fungi (WRF) and/or their extracellular enzymes are currently a promising solution as a treatment or as part of a multi-steps treatment of textile wastewater. The purpose of the present work was to compare the dye decolourisation of recalcitrant di-azo Reactive Black 5 (RB5) using the free and immobilized cells of WRF *Trametes versicolor* in chemostat under controlled conditions. *T. versicolor* MUM 04.100 from Micoteca da Universidade do Minho Culture Collection was used. WRF was immobilized in two different inert supports: polyurethane foam (PUF) and scotch brite (SB). The decolourisation and the enzymatic activities of lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lcc) and glyoxal oxidase (GLOX) were assessed during 28 days by continuous and constantly increased addition of a RB5 solution ( $100 \text{ mg l}^{-1}$  at pH 9.5 and  $15 \text{ g l}^{-1}$  of NaCl) to the chemostat. The decolourisation by WRF achieved a range of 90-100% using free and immobilized cells. When the WRF was immobilized using inert supports (PUF and SB) the maximum values of Lcc activity were similar (up to 5.5 U) which represents 1.25 times higher when compared with free cells. Lcc was the most efficient ligninolytic enzyme involved on dye decolourisation. Mechanisms of metabolic regulation of azo dyes degradation for this strain are now under study.

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PS2: 56

**Removal of copper (II), chloride and methylene blue using eggshell residues as low-cost biosorbents****Ana Margarida Castro<sup>1</sup>, Ana Isabel Ferraz<sup>1,2</sup>, Joana Carvalho<sup>3</sup>, Fernando Castro<sup>4</sup>**

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Biosorption, involving the removal of contaminants such as metal ions and dyes by materials of biological origin, can be considered an attractive alternative to traditional technologies. Concerning the process Life Cycle Analysis and economical feasibility, these applications can be particularly interesting when biowastes are used as effective biosorbents. This work aims to study the use of eggshell residues as low-cost biosorbents for copper (II), chloride and methylene blue biosorption. In that regard, eggshell powder (EP) and calcined eggshell powder (CEP) were prepared. Biosorbents physicochemical properties were characterized by Fourier-transform infrared (FTIR) and X-ray fluorescence (XRF) analyses, as well as specific surface area, porosity and density determination. Batch assays were performed in order to assess biosorption optimal conditions concerning medium pH, contact time and biosorbent concentration. FTIR spectra and X-ray fluorescence analysis show that calcium oxide is the main chemical component of EP. Calcination at 1000 °C for 2 h induces changes in eggshell powder composition, with calcium hydroxide and carbonate being identified as CEP main constituents. Further modifications resulting from biosorbent calcination pretreatment concerns the increasing of its specific surface area and porosity. From biosorption assays it is possible to conclude that copper (II) and chloride uptake by EP and CEP is optimal in acid solutions, respectively at pH 6 and 3, while methylene blue is more efficiently removed in alkaline conditions, at pH 8. For the biosorption systems in study, equilibrium was reached rapidly, nearly after 2 h of incubation, a good indicator for their use in large-scale applications. Regarding the optimal biosorbent dosage determined and the overall uptake results, it can be assumed that CEP has greater potential to be used as biosorbent than EP.

PS2: 57

**Salt marsh plants determine the bacterial community composition from Hg-contaminated sediments****Anabela Pereira<sup>1</sup>, Edna Almeida<sup>2</sup>, Isabel Henriques<sup>1</sup>, António Correia<sup>1</sup>**<sup>1</sup>Department of Biology & CESAM, University of Aveiro, Aveiro, Portugal; <sup>2</sup>Department of Biology, University of Aveiro, Aveiro, Portugal

The Ria de Aveiro received continuous mercury discharges from a chlor-alkali industry from 1950s until the mid 1990s. With the restrictions to the anthropogenic emissions, the mercury discharges diminished significantly in the last years, however a considerable amount of this mercury is known to be stored in the sediments of Laranjo Bay, a confined area of the estuarine system. The ecological and toxicological risk of mercury contamination depends on the species that are present in the environment, being the methylated species much more toxic to living organisms. It has been demonstrated that salt marshes are important areas for the methylation production in coastal ecosystems. Recent studies at Laranjo Bay showed that the concentration of mercury in plant colonized sediments were much higher than in the adjacent unvegetated sediments. This study was developed under a project on the biological methylation of mercury in contaminated sediments. The specific objective was to verify in which way salt marsh plants influence the structure and dynamics of bacterial community in contaminated sediments. For that, a green house experience was carried out using a salt marsh plant widely distributed in European estuaries (*Halimione portulacoides*), collected from the reference site (uncontaminated) and transplanted to sediments from the Laranjo Bay (contaminated site). At each sampling time (0, 3 and 6 months) sediment samples were collected from individual pots (5 replicates) with and without plant for Hg and MeHg analysis and for bacterial communities study. Structure and dynamics of bacterial communities were characterized using a 16S rDNA PCR-DGGE approach. Cluster analysis of DGGE profiles revealed the presence of three different clusters. The first cluster congregates samples collected from pots with and without plant in the first sampling moment (T0). Additionally two clusters matching samples from vegetated and unvegetated pots were clearly identified. Within each of those clusters, there were two subclusters corresponding to the last two sampling dates (T1 and T2). Clusters from vegetated and unvegetated sediments shared less than 10% similarity. Thus, indicating that the composition of bacterial communities from contaminated samples was clearly determined by the presence of the plant. Two main effects may be modulating the observed dynamics of the bacterial communities: mercury processes and plant life cycle. Further investigations on both effects are needed.

PS2: 58

### ***Sphingomonadaceae* diversity and antibiotic resistance in water samples**

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*Sphingomonadaceae* are ubiquitous bacteria in environment, including aquatic habitats. Nevertheless, their contribution for the acquisition and spread of antibiotic resistance in nature is poorly understood. We studied the diversity and the antibiotic resistance phenotypes of members of this family in isolates recovered from a drinking water treatment plant (n=6), tap water (n=55), cup filler of dental chairs (n=21) and a water demineralization filter (n=4). Bacterial isolates were identified based on the analysis of the 16S rRNA gene sequence and intra-species variation was assessed on basis of the *atpD* gene sequence analysis. Antibiotic resistance profiles were determined using the ATB PSE 5 strips (Biomérieux). The isolates were identified as members of the genera *Sphingomonas* (n=27), *Sphingobium* (n=28), *Novosphingobium* (n=12), *Sphingopyxis* (n=7) and *Blastomonas* (n=12). Susceptibility patterns were analysed and compared to the different sites of isolation and taxonomic groups. Colistin resistance was observed to be intrinsic (92%). The highest antibiotic resistance percentages were observed for the members of the genera *Sphingomonas* and *Sphingobium*, to the beta-lactams, ciprofloxacin and cotrimoxazol. Comparing the sites of isolation, the highest percentages of resistance were observed for tap and cup filler samples, where isolates of the genera *Sphingomonas* and *Sphingobium* predominated. These two genera presented different patterns of association of antibiotic resistance suggesting different paths of resistance acquisition. Antibiotic resistance patterns were often species- rather than site- or strain-related. This is the first study demonstrating that members of the *Sphingomonadaceae* family may play a role as potential reservoirs of antibiotic resistance in aquatic environments, namely in drinking water.

PS2: 59

## Surface hydrophobicity of solid culture and water biofilm of filamentous fungi

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Fungi are known to produce hydrophobins which are small proteins localised on the outer surface of cells and are involved in several functions in fungal growth and development. Due to characteristics such as dimorphic growth, variety of size and shape of hyphae and spores, and a complex cell wall of filamentous fungi, microsphere adhesion assay (MAA) is said to be the best method to assess cell hydrophobicity. However, contact angle measurement (CAM) is the most common technique applied. This work aimed to perform MAA and CAM to study hydrophobicity of solid cultures and water biofilms of *Penicillium expansum* and *Penicillium brevicompactum*. Contact angles of 7 and 21 days aged solid cultures grown on Malt Extract Agar (MEA) and Water Agar Glucose media (WGA), and 7 and 21 days aged biofilms grown in Malt Extract Broth (MEB) were measured. MAA was applied in 7 and 21 days aged biofilms grown in MEB. As result, both species in solid cultures and 21 days aged biofilms were classified as hydrophobic with contact angles  $> 90^\circ$ . In contrast, for both species, biofilms with 7 days were classified as hydrophilic with contact angles  $\leq 90^\circ$ . When MAA was applied, water biofilms showed different levels of hydrophobicity and was found that biofilms have specific hydrophobic hyphae which may be involved in fungal ecological functions. In conclusion, CAM showed to be more useful to assess hydrophobicity on solid cultures, and MAA was more proficient to assess directly the cells surface hydrophobicity and was useful for characterise different zones of hydrophobicity within the biofilm.



PS2: 60

**Surfactant-resistant and biosurfactant producing *Pseudomonas* from the estuarine surface microlayer**

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The objective of this work was the screening and characterization of the bacteria from the estuarine surface microlayer (SML), for surfactant-resistant and/or biosurfactant-producing strains. Resistance to surfactants was evaluated by colony counts in solid media amended with either sodium dodecyl sulfate (SDS) or cetyl trimethylammonium bromide (CTAB) at critical micellar concentration, in comparison to non-amended controls. Selective cultures for surfactant-resistant bacteria were prepared in mineral medium containing the same surfactants. Surfactant-resistant isolates were tested by PCR for the *Pseudomonas* genus marker *gacA* gene and for the naphthalene dioxygenase encoding gene *ndo*. Isolates were screened for biosurfactant production by the atomized oil assay. A high proportion of culturable bacterioneuston were tolerant to the tested surfactants. *gacA*-targeted PCR revealed that 44% of the isolates were *Pseudomonads*. Biosurfactant production in solid medium was detected in 9.4% of tested isolates, all affiliated with genus *Pseudomonas*. The SML is a potential source of surfactant-resistant and biosurfactant producing bacteria in which *Pseudomonads* emerge as a relevant group. Surfactant-resistant and/or biosurfactant-producing bacteria can be an important tool for bioremediation efforts of hydrophobic pollutants, circumventing the toxicity of synthetic surfactants that often delays microbial bioremediation of these contaminants.

PS2: 61

**Toxicity evaluation of Ionic Liquids – the Microtox toxicological test****Sónia Patrícia Marques Ventura, Fábio Miguel Mendes Ferreira, Joana Lourenço Pereira, Fernando Gonçalves, João Manuel Araújo Pereira e Coutinho**

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Ionic Liquids (ILs) are an important group of compounds, composed of a large organic cation with different alkyl side chains, and an organic or inorganic anion. They are liquid salts characterized by low vapor pressures, high chemical and thermal stabilities and by a higher solvent power. Those properties, associated to their negligible vapor pressure, make them great alternative compounds for organic volatile solvents. Despite those “ecofriendly” properties of ILs, they present water solubility large enough to allow their dispersion into the aquatic ecosystems resulting in water contamination. Thus, the possibility of discharging ILs into the environment through wastewater streams is a real concern and the study of the potential effects of these compounds on aquatic ecosystems is still scarce. This study was accomplished to verify the toxicity of several ILs using a standard toxicological test widely used known as Microtox®, which is performed towards the marine bioluminescent bacteria, formerly known as *Vibrio fischeri*. Several ILs’ structural features were tested, which were based in different cation cores (guanidinium, phosphonium and imidazolium), alkyl chain lengths and types and several hydrophilic anions. In general, the luminescent bacterium, was negatively affected by all the IL structures (cations, anions and alkyl chain lengths). The guanidium- and phosphonium-based ILs seem to follow the established trend that the increase in the alkyl chain length promotes the increase in the toxicity of the IL, normally designated by “side chain effect”, but seems to suffer also the “cut-off” effect (guanidinium-based ILs). Moreover, the introduction of ether or ester groups in the IL side chain leads to the decrease of the toxicity, independently of the cation core (phosphonium or guanidinium). According to those results it was concluded that the phosphonium-based ILs are more toxic than the analogue imidazolium-based ILs (same anion and alkyl chain length). Finally, the hydrophilic anions have a residual toxicological effect when their effect was compared with the influence of the longer alkyl chains or even the cation cores here studied.

PS2: 62

### **Tracking antibiotic resistances in a hospital effluent and in the receiving municipal wastewater treatment plant**

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The use of antibiotics is regarded as a major factor for the selection of antibiotic resistant (AR) bacteria. Numerous evidences suggest that identical AR determinants can be detected in the environment and in human commensal and pathogenic bacteria. The main sources and routes of propagation are often unknown. Given the intensive use of antibiotics, hospital effluents are suspected to contribute to the dissemination of AR bacteria into the environment. Studies on the fate of AR bacteria from hospital effluents in the environment are scarce. This work aimed at tracking AR bacteria in a hospital effluent and in the municipal wastewater treatment plant into which it is discharged. For this purpose, the prevalence of amoxicillin and ciprofloxacin resistance in environmental and clinically relevant bacteria (total heterotrophs, enterobacteria and aeromonads) was assessed in the hospital effluent and in the raw and treated wastewater of the municipal wastewater treatment plant. Cell counts and amoxicillin resistance rates did not differ significantly between the hospital discharge and the municipal sewage (raw wastewater). In contrast, the hospital effluent presented significantly higher prevalence of ciprofloxacin resistance (about three times higher) than the raw municipal wastewater. Apparently, the hospital effluent discharge had a significant impact on the levels of quinolone resistance in the plant inflow. Wastewater treatment led to cell densities reductions of about 100-1000 times, for both total and AR bacteria. Although the final treated effluent had significantly lower resistance rates than the hospital effluent, in general, wastewater treatment did not contribute to significant variations on the rates of amoxicillin and ciprofloxacin resistance. This study confirmed the hospital effluent as an important source of AR into the municipal wastewater. The decrease of ciprofloxacin resistance rates between the hospital effluent and the entrance in the wastewater treatment plant can be attributed to dilution effect. Nevertheless, it is demonstrated that AR bacteria from the hospital effluent enter the municipal wastewater treatment and are not removed efficiently.

PS2: 63

**Uranium immobilization by aerobic bacteria through formation of intracellular crystalline structures****Tânia Sousa<sup>1</sup>, Ana-Paula Chung<sup>1</sup>, Alcides Pereira<sup>1,2</sup>, Ana Paula Piedade<sup>3</sup>, Paula V. Morais<sup>1,4</sup>**

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Severe environmental problems arise from disused uranium mines, which continue to discharge uranium (U) via acid mine drainage waters. Regardless the source, the problem is the leaching and seepage of uranium and radionuclides offsite, and ultimately into soils, subsoils and groundwaters. Recently, bioremediation of U contaminated environments have been attempted using microorganisms which remove U by biosorption, bioaccumulation, bioreduction or biomineralization. Bacteria may use compounds like phosphate and ammonia to enhance the efficient immobilization of uranium over time, a process difficult to occur in the presence of O<sub>2</sub> and thus restricted to anaerobic environments. This work aimed to identify and characterize uranium-resistant bacteria able to immobilize uranium under aerobic conditions, and to assess their uranium reduction capacity by resorting to phosphate and/or ammonia compounds. Bacteria isolated from Urgeiriça Mine were grown at different uranium concentrations and their resistance and reduction ability (U(VI) to U(IV)) were determined. The capacity of strains to use phosphate and/or ammonia was assessed through the determination of quantitative variations of these compounds, and confirmed by SEM and XRD analysis. Two bacterial strains, *Rhodanobacter* sp. A261 and *Burkholderia* sp. A5907, were identified as able to resist in uranium concentrations up to 2 mM. They were able to reduce approximately 30% of U(VI) to U(IV) under aerobic conditions. In the presence of uranium, the strains increased their capacity to uptake phosphate (0.15 μmol Pi). They were also able to produce about 0.5 μg/mL of ammonia, whose levels decreased over time (to 0.2 μg/mL NH<sub>3</sub>), suggesting that these strains resort to these compounds to efficiently immobilize uranium. XRD and SEM analysis identified the presence of black crystalline structures within the cells of the 2 strains, mainly composed of uranium and phosphate, with ammonia residues. This work shows that uranium was effectively immobilized within *Rhodanobacter* sp. A261 and *Burkholderia* sp. A5907 cells through the formation of crystalline complexes mainly composed of uranium-phosphate-ammonia.

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PS2: 64

## Use of an aerobic selector to overcome filamentous bulking in activated sludge

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Secondary biological treatment of wastewater is a complex process and one of the most important steps to ensure the quality of the final effluent in activated sludge plants. In activated sludge systems, the performance of the process largely depends on the balance between filamentous and floc-forming bacteria. When the normal balance of the community is disturbed, filamentous bacteria tend to proliferate, causing various problems. Bulking and foaming phenomena are the major problems referred usually resulting in poor sedimentation of sludge and low quality final effluents. The application of selector reactor technology has become one of the promoted methods for the control of filamentous proliferation, enhancing sludge settle ability in activated sludge systems. The main objective of this research was the use of an aerobic selector to improve the performance of an urban wastewater treatment plant (WWTP), located in North of Portugal with two different parallel lines of treatment during four months. This WWTP receive domestic wastewater with irregular industrial discharges. The evaluation was accomplished through the observation of the diversity and abundance of filamentous microorganisms and the correlations between biological and physical-chemical and operational parameters. This WWTP was followed with recurrent episodes of filamentous bulking caused by *Sphaerotilus natans* and eventual occurrences of Nocardioforms and Type 1863. An aerobic selector was introduced in both lines in the beginning of the studied period, suppressed in one of the lines during 6 weeks, and then put into operation again until the end of the study. A total of 14 filamentous bacteria morphotypes were identified. The results show that the aerobic biological selector in continuous operation prevented the overgrowth of the filamentous Type 1863, of Nocardioforms and, in particular, of *Sphaerotilus natans*. Simultaneously, it allowed to lowering the oxygen levels in the aeration tanks without negative consequences in the overall performance of the WWTP, namely bulking occurrence. In this way, a significant energy save was allowed, even considering the aeration of the selector. The results are more relevant if one considers the fact that the main cause of the bulking problems in this WWTP was the overgrowth of *Sphaerotilus natans*, a filamentous bacterium known to be stimulated by low dissolved oxygen (DO) levels.

PS2: 65

**Walnut shell valorization in the removal of bisphenol A from water****Sofia Pinto Amaro, Isabel Queiroz Macedo**

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Bisphenol-A (BPA) is one of the highest-volume chemicals produced worldwide in the plastic industry. It is an endocrine disruptor classified as an emerging pollutant. In this work we studied the adsorption of BPA from aqueous solutions onto walnut shell-based activated carbon. For the production of activated carbon, walnut shell was soaked with  $\text{ZnCl}_2$  (1:2 m/m) during 9h at 105°C. It was then activated and carbonized in a muffle under  $\text{N}_2$  atmosphere: room temperature to 500°C at 5°C/min, 1h at 500°C and natural cooling to 140°C. Kinetic and equilibrium studies were investigated at initial BPA concentrations ranging from 5 to 60 ppm. Three adsorption kinetic models - first, second and pseudo-second order - were used to fit the experimental data. The first order model gave the best description of the adsorption process ( $R^2 = 0,9888 - 0,9990$ ) with  $k_1$  values ranging from 0,0521  $\text{h}^{-1}$  to 0,0394  $\text{h}^{-1}$  for the initial concentrations studied. The equilibrium experimental data fits better to a Langmuir isotherm ( $R^2 = 0,9986$ ) than to a Freundlich isotherm ( $R^2 = 0,9483$ ), which suggests that the adsorption occurs in a homogeneous surface, with formation of a monolayer and without interaction between adsorbed particles. The equilibrium constant of Langmuir,  $K_L$ , is 1,5 L/mg and the maximum adsorption capacity,  $q_{\text{max}}$ , is 277,8 mg/g (1,22 mmol/g). The fit of experimental data to diffusion models reveals that the adsorption process is preferentially controlled through intra-particle diffusion during the first 9h. When comparing the results with those reported in the literature, it appears that the value of  $q_{\text{max}}$  obtained in this work, 1,22 mmol/g, is similar to the  $q_{\text{max}}$  when a mesoporous carbon was used, 1,30 mmol/g. On the other hand, when a hydrophobic zeolite is used for BPA adsorption, the process is not as efficient,  $q_{\text{max}}$  is 0,49 mmol/g. The results indicate that walnutshell-based activated carbon may represent a useful tool in biotechnological processes of environmental remediation. Since it is an abundant and affordable byproduct, its application in the removal of BPA from urban and industrial effluents, or even from drinking water, seems to be an advantage.

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PS2: 66

### Characterization of molecular factors from plants pathogen *Phytophthora cinnamomi*

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The culture of the chestnut tree is extremely important in the northern region of Portugal, occupying a significant proportion of useful agricultural area. The annual average chestnut production in Portugal can reach 20 000 tons. New plantation areas have increased in the last few decades. However the ink disease caused by the oomycete *Phytophthora cinnamomi* has damage and killed many trees and up to now no concrete solutions have been offered to control the illness. As a consequence, the disease propagation in the orchards of chestnut trees has been causing severe productivity and yield breaks. In addition to the economical losses, the importance of sociological and landscape aspects for the region cannot be neglected. Oomycetes species can manipulate biochemical and physiological processes in their host plants through a diverse array of virulence or avirulence molecules, known as effectors. In susceptible plants, these effectors promote infection by suppressing defense responses, enhancing susceptibility, or inducing disease symptoms. Alternatively, in resistant plants, effectors are recognized by the products of plant resistance genes, resulting in host cell death and effective defence responses known as the hypersensitive response (HR). We've identified and characterized some proteins involved in mechanisms of infection by *Phytophthora cinnamomi*: endo-1,3-beta-glucanase (complete cds), exo-glucanase (partial cds); glucanase inhibitor protein (GIP) (complete cds); necrosis-inducing Phytophthora protein 1 (NPP1) (complete cds), transglutaminase, under the projects Identification, characterization and role of molecular factors associated with the mechanisms of infection of *Fagaceae* species by *Phytophthora cinnamomi*, PTDC/AGR-AAM/67628/2006, funding by FCT; Combating by molecular methods to ink-disease of chestnut and other regional cultures, COMBATINTA/SP2.P11/02 - Interreg IIIA, funding by FEDER, among others. Several technologies, such reverse transcriptase PCR, in vivo expression technology, and Bioinformatics tools have been used to study the expression of selected genes from fungi during infection. In this work we intend to integrate the necessary bioinformatics tools that were used in this investigation. These tools include the use of Databases and associated homology programs as Fasta and Clustal, and several programs for sequence analysis and design of experiments such PCR.

PS2: 67

### Characterization of the filamentous bacteria *Eikelboom* Type 0581 in Portuguese activated-sludge systems

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Universidade do Minho, Portugal

One of the major operational problems of activated sludge systems is the excessive growth of filamentous bacteria. This excessive growth of filamentous microorganisms causes a thick viscous scum or foam on the surface of the reactors (known as *foaming*) and an inadequate solid separation in the clarifiers (or *bulking*). Several different filament morphotypes have been described in systems treating domestic and industrial wastes but, in the absence of pure cultures, many of these have never been sufficiently characterized to resolve their taxonomy or to provide them with valid names. Hence, they are still referred to as numerical types persisting from the early studies of Eikelboom in 1975. The phylogeny of some important filamentous bacteria responsible for *bulking* and *foaming* problems, such as the Type 0092 and the Type 0914, was recently established. Still, there are plenty filamentous bacteria whose phylogeny and ecology remains unknown. In this study, the filamentous morphotype 0581 was studied, aiming at increasing the knowledge of the ecology of these microorganisms. This filamentous type is poorly described and most of its ecological needs unknown. Nevertheless, this is a morphotype frequent and abundant in Portuguese wastewater treatment systems. It morphologically resembles *Microthrix parvicella* or *Microthrix calida*, despite the different response to the Gram and Neisser stains, and a study claims to have found a bound between them based on the positive response of this morphotype to FISH probes developed for *Microthrix parvicella* and *Microthrix calida*. A total of 19 samples were analysed from 7 different Portuguese WWTP. The morphologic characterization was achieved by classical microscopic sludge analysis. Molecular biological methods were used to obtain information about their taxonomic affiliation. Statistical analyses were carried on to determine the operational and the environmental conditions that favour this microorganism.



PS2: 68

**Irradiation effect on some antioxidant system enzymes of hydrocarbon degrading microorganisms****Assemgul Bekturova<sup>1</sup>, Tatayna Karpenjuk<sup>2</sup>, Alla Goncharova<sup>2</sup>, Marat Khanturin<sup>1</sup>**<sup>1</sup>L.N. Gumilev Eurasian National University, Kazakhstan; <sup>2</sup>Al-Farabi Kazakh National University, Kazakhstan

One of the most promising ways of intensification of biodegradation of petroleum and petroleum products are to enhance the metabolic activity microorganisms under the influence of various chemical and physical factors. We have studied the possibility of stimulating the metabolic activity microorganisms under irradiation. The objects of the study were bacteria of *Pseudomonas aeruginosa* and yeast of *Candida sp.*, isolated from active silt. As a source of  $\beta$ -radiation electron accelerator ELU-6 was used. The doses of irradiation were 2 and 5 Gray. The action of ionizing radiation in water radiolysis products are formed that possess oxidizing properties. In order to eliminate reactive oxygen species in cell functioning antioxidant protection in the superoxide dismutase, catalase and peroxidase, etc. Irradiation in 2 Gr dose of bacteria *Pseudomonas* during their growth on the oil and diesel fuel medium stimulates the catalase activity in 1,4 times, and has no affect on polyphenoloxidase and peroxidase activity. The high homology of aminoacid sequences of catalase isoformes (98-100% - for various strains of *Pseudomonas aeruginosa* and 60-82% - for various species of bacteria *Pseudomonas*) in different species of the genus *Pseudomonas*, indicates that the patterns identified in the experiment for the catalase of *Pseudomonas aeruginosa*, may be valid for enzymes from other organisms of this genus. With the growth of yeast *Candida* irradiation does not change activities of these enzymes. Along with this, exposure of stimulated growth processes, increased production of protein, as well as for destructive activity of these microorganisms. This indicates that the antioxidant system microorganisms cope well with the increased levels of free radicals formed during irradiation of microorganisms in small doses. Obtained data indicate the prospects of application of irradiation for the stimulation of metabolic activity of hydrocarbon oxidizing microorganisms.

PS2: 70

**Influence of the sewage composition in the development of the microfauna in a bench-scale activated-sludge system.****João Carlos Silva<sup>1</sup>, Vânia Ferreira<sup>1</sup>, Isabel Mina<sup>2</sup>, Ana Nicolau<sup>1</sup>**<sup>1</sup>Centre of Biological Engineering, University of Minho, Braga, Portugal; <sup>2</sup>Department of Biology, University of Minho, Braga, Portugal

In an activated-sludge system, the efficiency of the wastewater treatment depends on the activity of the microorganisms inhabiting this artificial ecosystem. On the other hand, the microbial communities are largely determined by the physical-chemical and the operation conditions prevailing in the plant. The composition of the influent water is a non-negligible factor to take in account when operating a wastewater treatment plant (WWTP). Some industrial wastes promote the growth of particular bacteria, while easy degradable substrates and low organic contents are said to favour the filamentous microorganisms, altering the floc-forming to filamentous balance, thus endangering the performance of the system. However, only few data like these exist about the eukaryotic populations. The present work aimed at studying the development of the prokaryotic and eukaryotic populations and the plant performance of two bench-scale WWTP, comprising a bioreactor and a decanter, in a series of assays using different types of artificial sewage. These included glucose, peptone, a complex sewage of acetate, milk powder, urea and sucrose and finally a mixture of acetate and trace elements, the two latter with two different flow rates. Monitoring of the WWTP overall performance was performed through the determination of Chemical Oxygen Demand (COD), Total and Volatile Suspended Solids (TSS and VSS) and Biochemical Oxygen Demand (BOD<sub>5</sub>). The microbial communities, comprising Protozoa, metazoa and Filamentous Bacteria were monitored by microscopic analysis. Significant differences were observed in the performance of the WWTP concerning solids and organic matter removal efficiencies. Microscopic analysis also revealed the presence of different of protozoa and metazoan populations in the aerated tanks. Among the most common protozoa were *Arcella sp.*, *Aspidisca sp.* and *Vorticella sp.* *Drepanomonas sp.* was identified only in the WWTP fed with glucose and *Spathidium sp.* when peptone was used. Concerning filamentous bacteria, *Beggiattoa spp.* was the dominant *taxon* in the system fed with glucose, but when the systems were fed with peptone or with the complex mixture, *Sphaerotilus natans* dominated. Acetate plus trace elements favoured the co-dominance of *Sphaerotilus natans*, 021N Type and *Nostocoida limicola l.* The results add important information to the knowledge of how the composition of the sewage determines the microbiological communities in activated-sludge systems.

PS2: 71

**Microbial communities inhabiting a constructed wetland applied to tourism wastewater treatment****Cristina S.C. Calheiros, Albina Cristina Ribeiro Franco, Vânia S. Bessa, António O. S. S. Rangel, Paula M.L. Castro**

CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal

Constructed wetlands (CWs) are man-made wastewater treatment systems that intent to mimic the conditions, mainly in terms of physicochemical and biological processes, of natural wetlands. They represent a low-technology treatment, enabling many times wastewater recycling and reuse. CWs structural components include, in general, a support media, macrophytes, water, microbes and fauna population. Two important groups of these microbial organisms are bacteria and fungi, including mycorrhiza; primarily because of their role in the assimilation, transformation, and recycling of chemical constituents present in various wastewaters. The aim of this study was to assess microbial community shifts over a full year operation on a CW treating wastewater coming from a Portuguese rural tourism unit (Paço de Calheiros, Ponte de Lima). This CW is vegetated by *Canna flaccida*, *Canna indica*, *Zantedeschia aethiopica*, *Watsonia borbonica* and *Agapanthus africanus* in an expanded clay substrate (Leca<sup>®</sup>M). Plant roots were sampled from the inlet and outlet of the CW and microbial community profiles determined using PCR-DGGE. Species diversity were analysed and used to estimate microbial diversity and composition variation over a period of time. Tourism facilities are often characterized by great variations in wastewater quantity and quality over the year, affecting negatively the performance of conventional treatment systems. The use of CWs under this scenario still needs research and knowledge on microbial dynamics can be of great importance to the understanding of such systems.

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# Health Microbiology and Biotechnology

*Poster session thematic symposium 3*

Balcão | Moutinho | Aires | Branquinho | Alves | de  
Abreu | Teixeira | Almeida | Borges | Rocha | Soares |  
Smania | Serra | Duarte | Rodrigues | Peixe | Valença |  
Silveira | Conceição | Duarte | Oliveira | Melo | Portela  
| Gama | Rahimi | Duarte | Caetano | Rodrigues |  
Domingues | Leão | Duarte | Carreira | Jozala |  
Carvalho | Lima | Vasconcelos | Garcia | Costa |  
Seixas | Carneiro | Rodrigues | Sousa | Mourão |  
Moreira | Silva | Botelho | Mussatto | Oliveira | Ribeiro  
| Gonçalves | Bexiga | Dias | Sousa | Garcia | Pinto |  
Dias | Freitas | Rato | Pires | Smania Jr | Correia |  
Guinote | Maia | Pereira | Oliveira

PS3: 1

### **Biopolymeric matrices for structural and functional stabilization of bacteriophages**

**Victor M. Balcão<sup>1,2</sup>, Ana R. Moreira<sup>1</sup>, Carla G. Moutinho<sup>1,2</sup>, José A. Teixeira<sup>2</sup>**

<sup>1</sup>Bioengineering and Biopharmaceutical Chemistry Research Group, Fernando Pessoa University, Porto, Portugal; <sup>2</sup>IBB - Institute for Biotechnology & Bioengineering, Centre Biological Engineering, University of Minho, Braga, Portugal

In the recent past years, bacteriophage research has experienced a renaissance due to their potential application in the pharmaceutical field, especially with the increase of bacterial resistance to antibiotics and the possibility to take part in new methods of early detection and diagnosis of bacterial infections. In that context, the structural and functional stabilization of bacteriophages using biopolymeric microporous hydrogels represents a promising research focus with a broad potential biomedical/biopharmaceutical application. The scope of this work was to develop biopolymeric non-toxic phage-hydrogels of agar and sodium alginate, obtained at neutral pH and mild polymerization conditions, in order to offer adequate characteristics to the maintenance of phage's lytic activity. Disc-like phage-hydrogels were prepared, with a phage and polymer concentration of  $1.3 \times 10^8$  PFU/ml and 1.5% (w/v), respectively. Regarding the alginate hydrogels,  $\text{CaCO}_3$  (22.5 mM) and GDL (48 mM) were also included in the formulation. Agar hydrogels were prepared naturally by jellification, as a function of temperature lowering, and alginate hydrogels were prepared by internal gelation. The matrices were inoculated with a suspension of susceptible (host) bacteria and incubated at 37 °C for 24h. Observation of bacterial lawn's lysis demonstrated that bacteriophages kept their lytic activity, being the method of physical entrapment able to promote their stabilization. Cryo-SEM analysis revealed that both types of phage-hydrogels present interconnective microporous network, which guaranties a facilitated access of the phages to the bacteria, ensuring an efficient lysis of the host bacteria present in the surface of the hydrogels. The developed hydrogels also present appropriate physical and chemical properties for a wider variety of applications in the field of pharmaceutical sciences, such as controlled release of (macro)molecules, cell immobilization and 3D support for tissue regeneration.

PS3: 2

### **Structural and functional stabilization of glycomacropeptide via encapsulation within multiple emulsions**

**Carla G. Moutinho<sup>1,2</sup>, José A. Teixeira<sup>2</sup>, Victor M. Balcão<sup>1,2</sup>**

<sup>1</sup>Bioengineering and Biopharmaceutical Chemistry Research Group, Fernando Pessoa University, Porto, Portugal; <sup>2</sup>IBB - Institute for Biotechnology & Bioengineering, Centre Biological Engineering, University of Minho, Braga, Portugal

Bovine glycomacropeptide (GMP), derived from whey proteins, has been demonstrated to possess an interesting bioactivity that has attracted a lot of attention over the last few years. In particular, its ability to bind *Vibrio cholerae* and *Escherichia coli* enterotoxins, inhibit bacterial and viral adhesion, suppress gastric secretions, promote bifidobacterial growth and modulate immune system responses. Of these, protection against toxins, bacteria and viruses, and modulation of the immune system, are the most promising applications for this bioactive dairy macropeptide. The development of strategies that may allow its structural and functional stabilization via nanoencapsulation within multiple emulsions may increase its food and biopharmaceutical applicabilities. In this research effort, bovine GMP was (thermodynamically) stabilized via entrapment within water-in-oil-in-water (W/O/W) multiple emulsions aiming at mimicking the multifunctional design of biology, with several lipid matrices, and stabilizing layer compositions. Due to their compartmentalized internal structure, multiple emulsions are ideal for encapsulation since they can carry both polar and non-polar (bio)molecules. The composition of the stabilizing layer of the nanosystem was changed by using different poloxamers and proportions of lecithin. Physicochemical characterization of the optimized GMP-encasing nanovesicle formulations encompassed determination of Zeta potential and particle hydrodynamic size over storage time, surface morphology via CRYO-SEM, and microcalorimetric analysis via DSC.

PS3: 3

**Antibacterial activity and antiradical activity of *Nasturtium officinale* hydrophilic extract and their natural compound 2-phenylethyl isothiocyanate, against pathogenic aerobic bacteria****Alfredo Aires<sup>1</sup>, Carla S. P. Dias<sup>1,2</sup>, Eduardo A.S. Rosa<sup>1</sup>, Maria José Saavedra<sup>2</sup>**<sup>1</sup>CITAB-Centre for the Research and Technology of Agro-Environmental and Biological Sciences, Agronomy Department Universidade de Trás-os-Montes and Alto Douro, Vila Real, Portugal;<sup>2</sup>CECAV-Veterinary and Animal Science Research Center, Veterinary Science Department, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal

Several reports have indicated that *Nastrutium officinale* R. Br. (watercress), of the family *Brassicaceae*, has been long used as traditional remedy and medicinal plant in East countries. The aim of this study was to investigate the antibacterial activity of *N. officinale* extracts and evaluate the effect of 2-Phenylethyl isothiocyanate (PEITC), a predominant iosthiocyanate derived from the sulphur compound gluconasturtiin, particularly important in *N. officinale*, against Gram-negative and Gram-positive bacteria, isolated from Human and animal gastrointestinal segments. Using a disc diffusion method and different doses of hydrophilic *N. officinale* extracts and PEITC prepared in dimethyl sulfoxide (DMSO) were tested. Positive (commercial standards antibiotic) and negative control were used. All experiments were conducted in triplicate. The antibacterial activity was also assessed by the application of relative inhibition zone (% RIZD) in relation to the antimicrobial effect of commercial standards antibiotics. Following, total phenolics with Folin-Ciocalteau method, total flavonoids with aluminum-chloride method and the antiradical activity of *N. officinale* extracts was evaluated using the methods of the free radical scavenging activity (the 2,2-diphenyl-1-picrylhydrazyl-DPPH) and the Ferric reducing antioxidant power (FRAP). The results showed that both methanolic extract of *N. officinale* and PEITC was more effective in Gram-positive bacteria. The results indicate that *N. officinale* extracts could be useful as antimicrobial agent and might be used as alternative to, or in combination with current antibiotic-based controls used for treating bacteria. Also, the natural compound PEITC possesses interesting antimicrobial activity and enormous antiradical potential activity probably mediated through direct trapping free radicals, reducing power, and also through metal chelating. Based on its antiradical potential, *N. officinale* could be used in the prevention of free radical diseases.

PS3: 4

### Characterization of antibiotic susceptibility profile and of $\beta$ -lactamase genes content in *Bacillus pumilus* from different sources

Raquel Branquinho<sup>1</sup>, Maria Manuela Amorim<sup>2</sup>, Ângela Novais<sup>1</sup>, Maria Estevez Pintado<sup>2</sup>, Luísa Peixe<sup>1</sup>

<sup>1</sup>REQUIMTE. Serviço de Microbiologia. Faculdade de Farmácia. Universidade do Porto, Portugal;

<sup>2</sup>CBQF – Escola Superior de Biotecnologia – Universidade Católica Portuguesa. Porto, Portugal

Background: *Bacillus pumilus* is associated with a wide range of biotechnological activities, being used as probiotic in humans, animal feed supplement and growth-plant promoter. Concerns about the possibility of dissemination of resistance genes by microorganisms used as human nutrition supplements or in food production for human consumption have lead to the need of demonstration of absence of antimicrobial resistance as a universal qualification of microorganisms' safety (EFSA, 2010). Nevertheless, data on antimicrobial susceptibility using standardized methodology and  $\beta$ -lactamase genes content in isolates of *B. pumilus* is lacking. Four putative  $\beta$ -lactamase genes were found in *B. pumilus* SAFR-032 genome, although their contribution to  $\beta$ -lactams phenotype is unknown. Objective: To determine  $\beta$ -lactam antibiotic susceptibility profile of *B. pumilus* isolates from different origins, and establish its association with putative  $\beta$ -lactamase genes. Methods: Thirty *B. pumilus* isolates, recovered from a variety of sources, including food (n=6), medicines (n=12) and cosmetics (n=4) contaminants; plants (n=4), gastropods' normal flora (n=3), and *B. pumilus* ATCC14884, were assayed. Identification was conducted by biochemical tests, 16S rDNA-PCR and sequencing. Clonal relationship was evaluated by *Apal*-PFGE. Presence of putative narrow-spectrum- $\beta$ -lactamases (B1, B2) and metallo- $\beta$ -lactamases (M1, M2), described in *B. pumilus* SARF-032, was verified by IEF, PCR and sequencing. Susceptibility patterns to 8  $\beta$ -lactams were evaluated by broth microdilution (CLSI/NCCLS/M45-A). Results: All isolates revealed  $\beta$ -lactamase production at a later sporulation phase. They harbored 3 (50%), 4 (23.3%), 1 (16.7%) and 2 (10%)  $\beta$ -lactamase genes, but were susceptible to  $\beta$ -lactams, although isoelectric focusing revealed different bands. Moreover, no correlation was found among MIC values and  $\beta$ -lactamase content. None of  $\beta$ -lactamase gene was consistently found, and the most frequent one (M1) was identified in 80% of the isolates. Twenty-two PFGE-types were defined and associated with high variability of  $\beta$ -lactamases (number/type), being particular pulsotypes associated with isolates' origin. Conclusion: Variable  $\beta$ -lactamase gene content was observed in *B. pumilus* suggesting their mobilization throughout horizontal transfer. Nevertheless, current recommended methodology for testing  $\beta$ -lactams susceptibility tests does not reflect the presence of these putatively transmissible enzymes.

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PS3: 5

### Screening of the antimicrobial activity of wild mushrooms phenolic extracts against clinical isolates

**María Alves<sup>1,2,3</sup>, Isabel Ferreira<sup>3</sup>, Anabela Martins<sup>3</sup>, Manuela Pintado<sup>1</sup>**

<sup>1</sup>Universidade Católica Portuguesa Porto, Portugal; <sup>2</sup>Hospital Trás-os-Montes e Alto Douro;

<sup>3</sup>Instituto Politécnico de Bragança, Portugal

Antimicrobial drugs have long been used for prophylactic and therapeutic purposes; however the drug-resistant bacterial strains have been creating serious treatment problems. This situation has forced the search of new antimicrobial substances effective against pathogenic microorganisms resistant to conventional treatments. Natural resources have been tested and among them mushrooms could be an alternative. This work aimed to screen the antimicrobial activity of phenolic extracts of 13 edible mushroom species, collected in Bragança, against several clinical isolates obtained in Hospital Center of Trás-os-Montes and Alto Douro, Portugal. Microdilution method was used to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). MIC results showed that *Russula delica* and *Fistula hepática* extracts inhibited the growth of Gram negative (*Escherichia coli*, *Pasteurella multocida*, *Morganella morganni*, isolated from urine) and Gram positive (*Staphylococcus aureus*, MRSA- isolated from wound exudates, *Enterococcus faecalis*, *Listeria monocytogenes*- isolated from blood culture, *Streptococcus agalactiae* and *Streptococcus pyogenes*- isolated from vaginal swab) bacteria. A bactericide effect of both extracts was observed in Gram positive bacteria (*L. monocytogenes*, *S. agalactiae* and *S. pyogenes*); regarding Gram negative bacteria, a total inhibition effect was observed for *P. multocida* at the highest concentration tested (20 mg/ml). *Lepista nuda* extract exhibited a bactericide effect upon *P. multocida* at 5 mg/ml, and inhibited *Proteus mirabilis* (isolated from urine) at 20 mg/ml. *Ramaria botrytus* extract did not present activity against Gram-negative bacteria; nevertheless, it showed activity against *E. faecalis*, being bactericide for *S. agalactiae* and *S. pyogenes*. Moreover, this was the extract with the highest activity against *L. monocytogenes* with MBC of 10 mg/ml. *Leucopaxillus giganteus* extract inhibited the growth of *E. coli* and *P. mirabilis*, being bactericide for *P. multocida*, *L. monocytogenes*, *S. pyogenes* and *S. agalactiae*. Among all the studied mushrooms, *Agaricus arvensis* seemed to present the lowest antimicrobial activity against all the tested bacteria. The extracts are chemically characterized and the individual/combined phenolic compounds will be submitted to antimicrobial assays in order to identify compounds responsible for the mushrooms bioactivity.

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PS3: 8

**Occurrence of environmental *Pseudomonas aeruginosa* and other associated species in Hospital de Faro, Portugal****Pedro M de Abreu<sup>1,2</sup>, Susana Santos<sup>1</sup>, Gabriel Paiva<sup>1</sup>, Ana Almeida<sup>2</sup>, Paula V.Morais<sup>1</sup>**<sup>1</sup>IMAR-CMA, 3004-517 Coimbra, Portugal; Department of Life Sciences, FCTUC, University of Coimbra, Coimbra, Portugal; <sup>2</sup>Unidade de Investigação em Ciências da Saúde, CIIERT, Instituto Piaget, Portugal

Nosocomial infections have been recognized for over a century as a critical problem affecting the quality of health care. *Pseudomonas aeruginosa* has been one of the most common pathogens isolated from hospitalized patients and is responsible for many nosocomial diseases. The aim of this study was to assess the prevalence and the genetic variability of *P. aeruginosa* strains present in four hospital units of the Hospital de Faro – Portugal, and identify strains associated to *P. aeruginosa* positive samples. *P. aeruginosa* from environmental and clinical samples were investigated. During 7 months, 290 environmental samples were obtained from mainly humid and moist areas (sink, shower and tap) as well as medical equipment, bed and floor. Additionally 30 clinical samples, from the same clinical units were analyzed. Samples presenting fluorescence on recovering medium (PIA) were then assessed for *P. aeruginosa* by isolation of fluorescent colonies, fluorescence confirmation, DNA extraction, 16S rRNA gene sequencing. Finally, random amplification of polymorphic DNA (RAPD) analysis was performed, in order to type *P. aeruginosa* strains and attempt a relationship between clinical-environmental and environmental-environmental strains. Thirty five percent of the environmental samples were positive for *P. aeruginosa*. In all, 36 environmental strains of *P. aeruginosa* were identified, and the clinical unit presenting the highest level of contamination was the ICU. In samples positive for *P. aeruginosa*, other colony types present have been isolated and identified, such as (Biohazard level 2) *Acinetobacter junii* (3), *Citrobacter freundii* (6), *Microbacterium oxydans* (1), *Stenotrophomonas maltophilia* (8) and *Serratia nematodiphila* (1). RAPD analysis of 30 clinical and 36 environmental samples, show similarities between 7 and 3 clinical-environmental and 4 environmental-environmental, strains respectively, collected in different months, indicating possible occurrence of cross-colonization, suggesting an epidemic population structure for *P. aeruginosa* in this hospital. This confirms the need of continuous molecular-microbiological monitoring of hospital microflora in order to early detect potentially dangerous epidemic hospital strains, which are able to cause nosocomial infections.

PS3: 10

### Multilocus sequence typing of Portuguese strains of *Aspergillus fumigatus*

**Joana Maria Santiago Teixeira**<sup>1,2</sup>, **Rita Caramalho**<sup>1,2</sup>, **Ricardo Araújo**<sup>1</sup>, **Leonor Gusmão**<sup>1</sup>, **Antonio Amorim**<sup>1,2</sup>

<sup>1</sup>IPATIMUP, Portugal; <sup>2</sup>Faculty of Sciences, University of Porto, Portugal

*Aspergillus fumigatus* is a saprophytic mould involved in recycling the environmental carbon and nitrogen. The fungus has an extensive distribution and it may colonize all environments. *A. fumigatus* conidia are frequently referred in medical information as responsible for most cases of invasive aspergillosis (IA) - about 90% of mould infections. In order to evaluate the genetic diversity of *A. fumigatus* isolates, we employed the multilocus sequence typing (MLST) which is a successful technique for genotyping isolates based on single nucleotide polymorphisms. MLST was employed to a group of *A. fumigatus* isolates obtained from clinical and environmental samples in Porto, Portugal. Primers suggested by Bain et al. (2007) were employed for amplification and sequencing. The sequences obtained in this study were compared with the polymorphic strains published on MLST website. The number of polymorphisms ranged from 8 to 14 polymorphisms in each gene, considering online and personal collections. And based on these polymorphisms, it was possible to differentiate about 3 to 12 different sequences per gene. Considering the total group of sequences analysed, the most polymorphic gene was LIP (2.8%) and the least polymorphic gene was SODB (1.2%). Through this study was possible to find a set (1 to 5) of exclusive polymorphisms on the gene fragments from Portuguese isolates. A small group of polymorphisms was selected giving similar genotyping information compared to MLST. Some polymorphisms were only found in Porto area what may suggest the existence of some microbial endemism. It is imperative that strains from several sources/countries enrich the MLST online database to get correct assumptions on the geographic distribution of fungal strains.

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PS3: 11

### The PneumoEL project: epidemiology of pneumococcal colonization among the elderly

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*Streptococcus pneumoniae* (or pneumococcus) is a human commensal that frequently colonizes the nasopharynx and is a major cause of otitis media, pneumonia, bacteremia and meningitis worldwide. Risk groups for pneumococcal disease include children under 5 years old, immunocompromised patients, and the elderly. This epidemiological pioneer study conducted in Portugal aimed to evaluate pneumococcal carriage in adults aged over 60 years. Between April and November of 2010, the nasopharynx (NP) and oropharynx (OP) of adults over 60 years, living in Oeiras (n=650), an urban area, and in Montemor-o-Novo (n=650), a rural area, were swabbed. Pneumococci were identified by susceptibility to optochin, solubility in sodium deoxycholate, and PCR detection of genes *cpsA* (present in most pneumococci capsular *loci*) and *lytA* (ubiquitous gene in pneumococci that encodes for the major autolysin) genes. Pneumococcal strains were tested by agar disk diffusion for susceptibility to chloramphenicol, erythromycin, clindamycin, tetracycline and cotrimoxazole according to the CLSI guidelines. MICs to ciprofloxacin and penicillin were determined by E-test. Pneumococcal strains were also serotyped by multiplex PCR and/or by the Quellung reaction. Of the 1,300 adults swabbed, the mean age was 74 years, (range 60-99 years); 43.8% were male. Most of the participants (91.1%) lived at home, among which 3.1% lived with children younger than 6 years old. In the rural area, 17.5% of the participants lived in nursing homes. Chronic respiratory diseases were reported from c.a. one-fifth (21.8%) of the participants. More than half of the participants (58.5%) had taken the influenza vaccine in the season, while only a small percentage (3.5%) had taken the 23-valent pneumococcal polysaccharide vaccine. Thirty adults (2.3%) carried *bona fide* capsulated pneumococci. One adult had two different pneumococcal strains in the nasopharynx. The 31 strains expressed capsular types 11A (4 strains), 22F, 23B, and 37 (3 each), 3, 6A, 19A, and 35F (2 each), 5, 9L, 15A, 15B, 16A, 18A, 21, 23A, 31 and 36 (1 each). Four strains were resistant to macrolides, three were penicillin non-susceptible ( $0.1 \text{ mg/L} \leq \text{MIC} \leq 1 \text{ mg/L}$ ) and two had a ciprofloxacin MIC  $\geq 2 \text{ mg/L}$ . To our best knowledge, this is the first study on pneumococcal colonization in adults aged over 60 years in Portugal. The prevalence of pneumococcal carriage in the elderly is low and large serotype diversity seems to exist.

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PS3: 14

### The effects of allylithiocyanate and 2-phenylethylisothiocyanate on biofilm control of pathogenic bacteria

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Biofilms are multicellular communities and represent the prevalent mode of microbial life in nature, industrial processes, and health. It is estimated that biofilms contribute to more than 80% of all infections in humans. A particular characteristic is their extreme resistance to antimicrobial treatments. Moreover, the emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required. Glucosinolates and their hydrolysis products particularly isothiocyanates have long been accepted for their distinctive benefits to human nutrition and plant defense. Recent findings indicate that some glucosinolate hydrolysis products (GHP) have effects against pathogenic bacteria. The purpose of the present study was to evaluate the activity of two isothiocyanates [allylithiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC)] against biofilms of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. Each product was tested at a concentration of 100, 500 and 1000 µg/ml. The anti-biofouling activities of AITC and PEITC were tested against the bacteria using a microtiter-plate assay for biofilm mass quantification (crystal violet staining) and viability activity assessment (alamar blue staining). The isothiocyanates tested showed a higher potential to reduce the mass of biofilms formed by the Gram-negative bacteria, comparatively to those Gram-positive. The highest reduction in biomass was found for strain *E. coli* and the smallest was for *L. monocytogenes* with both isothiocyanates used. No isothiocyanates showed a total reduction in biofilm formed by the strains in study. In terms of viability, AITC and PEITC promoted reductions higher than 80% for all the biofilms tested except for *P. aeruginosa* where the biofilm reduction is approximately 70%. AITC and PEITC demonstrated potential to control biofilms of important pathogenic bacteria. Further studies will be developed in order to assess the effects of their combination in biofilm control and their mechanisms of antibacterial action.

PS3: 15

### Urinary tract infection - etiology and antimicrobial susceptibility, Centro Hospitalar do Médio Ave

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Urinary Tract Infections (UTIs) are very frequent worldwide, particularly among women. Because of the high impact of UTI, it is essential to know the most common etiologic agents and their susceptibility to different antibiotics profiles, so that empiric treatment is more effective. We conducted a cross-sectional study designed to characterize the most frequent UTI etiologic agents and their antimicrobials susceptibility profiles, in Centro Hospitalar do Médio Ave, Unidade de Vila Nova de Famalicão (CHMA-UVNF). Studied sample included all positive urocultures admitted to the Clinical Pathology Service of CHMA-UVNF between 1 January 2008 and 31 December 2010. We registered 2419 positive urocultures, of which 70,48% were from female patients and 29,52% from male patients. *Escherichia coli* was the most frequently isolated microorganism (58,69%), followed by *Klebsiella pneumoniae* (6,52%), *Pseudomonas aeruginosa* (6,52%), *Proteus mirabilis* (5,59%) and *Enterococcus faecalis* (4,99%). The observed frequency of *E. coli* in males (39,92%) was lower than the frequency registered among women (66,82%), due to this microorganism vaginal mucosa tropism. In the age groups above 60 years old, the percentages of *E. coli* infection gradually diminished, while other microorganisms infections increased with age in those age groups. *P. aeruginosa* is more frequently isolated in males (13,22%) than in females (3,63%). Regarding antibiotics susceptibility, imipenem showed the highest susceptibility percentage, while ampicillin presented the lowest, except for *E. faecali*. The low susceptibility of the uropathogenic to ciprofloxacin registered may be due to its massive and uncontrolled use in clinical practice. In order to control the growing trend of antibiotic resistance in uropathogenic agents, periodic analysis of susceptibility testing to these microorganisms, allows clinicians to better management and effective antimicrobial therapy in UTI.

PS3: 16

**Antibacterial mode of action of two phenolics acid on pathogens****Anabela Portela Borges<sup>1,2</sup>, Maria José Saavedra<sup>1,3</sup>, Manuel Simões<sup>2</sup>**<sup>1</sup>CECAV-University of Trás-os-Montes e Alto Douro, Vila Real, Portugal, Portugal; <sup>2</sup>LEPAE-Department of Chemical Engineering, Faculty of Engineering, University of Porto, Portugal;<sup>3</sup>Department of Veterinary Sciences, School of Agriculture and Veterinary Science, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal

Antimicrobial resistance is a major public health concern, particularly in hospitals and other health care settings. Infectious diseases that could be readily treated by antimicrobials are almost untreatable. The evolution of multidrug-resistance of important pathogenic microorganisms can be directly attributed to the use and overuse of antibiotics and transmission of resistance within and between individuals. To counter the emergence of resistant microorganisms, substantial resources have been invested in the research of new antimicrobials, mainly of microbial and plant origin. Plants synthesize a diverse array of secondary metabolites (phytochemicals) known to be involved in plant defence, and in the last few years it is recognized that some of these molecules, such as phenolics, have health beneficial effects by preventing the risk of some diseases. Phenolics are widely distributed in plants and have been shown to possess antimicrobial properties. In this study, the mechanism of action of gallic acid (GA) and ferulic acid (FA) was assessed on *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. Each product was tested at concentrations of 100, 500 and 1000 µg/ml. The targets of antimicrobial action were studied using different bacterial physiological indices: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), membrane permeabilization, intracellular potassium release, physico-chemical surface properties and surface charge. The MIC was >1000 µg/ml for all strain with GA. The MIC of FA was 1000 µg/ml for *S. aureus* and *L. monocytogenes*, 500 µg/ml for *E. coli* and 100 µg/ml for *P. aeruginosa*. The MBC was >1000 µg/ml for Gram-positive bacteria and for *E. coli*, and 500 for *P. aeruginosa* with both compounds. GA and FA led to a significant change in cell surface hydrophobicity and induced propidium iodide uptake. Such results suggest cytoplasmic membrane damage, corroborated by the release of intracellular potassium. The results obtained from the zeta potential measurement demonstrate that exposure to phenolics acid changes the surface charge of cells to less negative values particularly for Gram-negative bacteria. We can conclude that phytochemicals tested, mainly FA is effective compounds against the tested bacteria. It was found changes in membrane properties through loss of integrity with consequent leakage of essential intracellular constituents after exposure to the phenolic acids.

S3: 17

**Characterization of *Staphylococcus* strains obtained from individuals with atopic dermatitis****José C. Soares<sup>1</sup>, Freni K. Tavoria<sup>1</sup>, Cristina Lopes<sup>2</sup>, Rosa Silva<sup>3</sup>, Vera Oliveira<sup>3</sup>, José Morgado<sup>3</sup>, Luís Delgado<sup>2</sup>, Maria Manuela Estevez Pintado<sup>1</sup>**<sup>1</sup>CBQF - Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal;<sup>2</sup>Serviço & Laboratório de Imunologia Faculdade de Medicina da Universidade do Porto; <sup>3</sup>Centro Tecnológico e das Indústrias Têxtil e do Vestuário de Portugal, Vila Nova de Famalicão, Portugal

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disorder in the general population worldwide, where the majority of patients are colonized with *Staphylococcus aureus* capable of producing some virulence factors that further exacerbates the disease condition. However, coagulase-negative staphylococci (CNS) are also medically important pathogens characterized by an increasing prevalence of toxin related genes. In the light of this development, a better understanding of the diversity and virulence factors physiology among these bacteria is an essential prerequisite. Thus, the aim of this research project is to study the staphylococcal biodiversity and some virulence factors associated from AD patients and normal healthy individuals without any kind of disturbance in the skin. Species identification was performed using different molecular methods, viz. Multiplex-PCR and partial sequencing of the *sodA* gene sequencing amplification. The target species chosen for the Multiplex-PCR were: *S. aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus* and *S. hominis*. The prevalent strains in AD patients were *S. aureus* with 67 (34.9%) isolates, followed by *S. epidermidis* 59 (30.7%), *S. hominis* 54 (28.1%) and *S. capitis* with 12 (6.3%) isolates. In healthy subjects the highest incidence revealed *S. aureus* with eight (40.0%) isolates, *S. haemolyticus* with five (25.0%), *S. capitis* with four (20.0%) and *S. epidermidis* 3 (15.0%) isolates. The ability of the isolates to produce and express superantigen (SAg) genes and on the presence of other virulence factors was also assessed. In the virulence factors detection it was demonstrated that most isolates were coagulase negative and presents non-hemolytic activity. Existence of enterotoxin genes were also demonstrated in some strains. These results provided evidence that the presence of *Staphylococcus* species in DA context is very common and mostly represented by *S. aureus*, however with low levels of virulence factors associated.



PS3: 18

**Influence of substituent in the basic ring on antibacterial activity of chalcones****Hugo Pereira Avila<sup>1</sup>, Franco Delle Monache<sup>2</sup>, Artur Smania Junior<sup>1</sup>, Elza de Fatima Albino Smania<sup>1</sup>**<sup>1</sup>Universidade Federal de Santa Catarina, Brazil; <sup>2</sup>Universita di Roma, Italy

Chalcones are open chain flavonoids in which two aromatic rings are joined together by a three-carbon  $\alpha,\beta$ -unsaturated carbonyl system, *i.e.* 1,3-diphenyl-2-propen-1-one derivatives. These compounds have shown a wide variety of anticancer, anti-inflammatory, anti-invasive, and antimicrobial activities. This study aims to establish SAR of chalcone-base compounds to antibacterial activity. The antibacterial activity of thirty-one chalcones was evaluated, by determining the minimum inhibitory concentration (MIC) using the microdilution broth method. The compounds were tested against bacterial strains, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. Some of the tested chalcones showed significant activity against Gram-positive bacteria, with MIC values ranging from 3.9 to > 2.000  $\mu\text{g/ml}$ , and from 1.000 to > 2.000  $\mu\text{g/ml}$  for Gram-negative bacteria. Analysis of the structure of compounds showing positive activity indicates that antibacterial activity can be related to structural features such as the presence of a C-4 hydroxyl group, a C-4' oxygenated substituent or a C-3' isoprenoid side chain, while the C-2' hydroxyl group might have importance for the stability of the molecules. The inhibitory effect of chalcones on human pathogenic microorganisms can be correlated with the substitution patterns of the aromatic rings. In conclusion, these studies showed the potentiality of the chalcones as antibacterial agents, pointing out the importance of the positions of phenolic hydroxyl groups and/or the isoprenyl side chain in the substitution pattern for the antibacterial activity.

PS3: 20

### Is hormesis effect an evolutionary enhancer of antimicrobial resistance in *Stenotrophomonas maltophilia*?

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Opportunistic pathogens from natural environmental sources are submitted to strong bottleneck effect by antimicrobial use in medical environments, leading to infections of problematic management. Conversely, in its natural environment opportunist pathogens are exposed to minute concentrations of antibiotics, which can increase its fitness. These antagonist effects suggest that these microbial populations can be under hormesis. However, no information is available regarding the effect of antimicrobial growth stimulating concentrations (hormetic concentrations) on the selection and development of antimicrobial resistance. Therefore, to assess the impact of hormesis effect in antimicrobial susceptibility, in the presence and absence of antimicrobial bottleneck, we used as model a Tn5-mutant library of 2160 isogenic mutants from *Stenotrophomonas maltophilia* D457 strain. After establishment of hormetic and bottleneck antimicrobial concentrations for norfloxacin, chloramphenicol and carbenicillin, evolution experiments were performed until 157 generations during 1296 hours. These experiments were controlled for the emergence of spontaneous mutations. Hormesis effect was observed at concentrations 16, 160 and 64 fold lower than minimal inhibitory concentrations (MICs) for norfloxacin, chloramphenicol and carbenicillin, respectively. The populations evolved in the presence of hormetic concentrations show lower antimicrobial susceptibility relatively to those in absence of antimicrobial agent. Furthermore, growth was recovered in populations exposed after bottleneck to hormetic concentrations of chloramphenicol, in opposition to the absence of such exposure. Moreover, lower antimicrobial susceptibility to chloramphenicol was observed in populations exposed to hormetic concentrations of norfloxacin prior to bottleneck. Thus, our results show exposure to hormetic sub-inhibitory concentrations to enhance the evolution of cross-linked antimicrobial resistance and survival, conferring a potential adaptive advantage. In nature, this can favor the behavior of susceptible bacteria and its selection in high antimicrobial use environments. Being *S. maltophilia* an important reservoir for resistance determinants in medical environments, these results highlight the importance of hormesis effect in the enhancement of spread of antimicrobial resistance.

PS3: 21

### Molecular survey of viral pathogens in free-ranging carnivores in northern Portugal

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Viral diseases can play an important role in the population dynamics of wild species and further threaten the ones endangered. Likewise human activities have direct and indirect effects in wildlife health by increasing the contact between humans/domestic animals and wildlife, or by subjecting populations to stress, increasing their susceptibility to eventual outbreaks. Information regarding the serological status of free-ranging wild carnivores in Portugal is already documented, whereas detection of Antigen, viral protein of nucleic acid, more thorough to assess viral prevalence and genetic diversity is scarce. Considering the potential spillover of viral pathogens from domestic animals to wildlife we performed a molecular survey of canine distemper virus (CDV), feline and canine parvovirus (PV), canine adenovirus (CAV) and feline and canine coronavirus (CoV) in order to evaluate the viral incidence in the sampled population. The screening was performed on different tissues (spleen, liver, lymph node, intestine, lung and bone) of wolf (*Canis lupus*) and red fox (*Vulpes vulpes*), eurasian otter (*Lutra lutra*), stone marten (*Martes foina*), genet (*Genetta genetta*) and european badger (*Meles meles*), available through the ICNB tissue bank. Samples were collected in northern Portugal between 2005 and 2011. After co-extraction, total DNA/RNA samples were tested by conventional RT-PCR or PCR for detection of viral RNA or DNA. CDV RNA (intestine; lung) was detected in 2/42 wolves (4,8%) and 2/2 genet (100%). PV DNA (spleen, lymph node, intestine) was detected in 2/21 wolves (9,5%) and 3/12 red foxes (25%). CoV RNA (spleen, intestine) was detected in 13/22 wolves (59,1%), 1/1 eurasian otter (100%), 4/12 red foxes (33,3%) and 2/2 genets (100%). CAV DNA (liver, intestine, lung) was not detected. Our results indicate the presence of important viral pathogens in the wolf (CDV, PV and CoV), the red fox (PV, CoV), the eurasian otter (CoV) and the genet (CDV, CoV) species, emphasizing the need to clarify the genetic diversity of these viruses and to identify the source of infection. The continuing evaluation and monitoring of the health status of free ranging wild carnivores will provide valuable insights to the molecular epidemiology of these viruses and may supply important information on the potential impacts of viral pathogens on the conservation of Iberian carnivores.

PS3: 22

### Occurrence of ESBLs among *Enterobacteriaceae* isolates from swine and piggeries environment in Portugal

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Recent studies have alerted for the wide presence of extended-spectrum beta-lactamases (ESBLs) in bacteria from food-producing animals. In Portugal, previous surveys (1998/2004) showed a low incidence of ESBLs (only SHV-12) in swine from Portugal. We aim to investigate the ESBL-types currently spread in *Enterobacteriaceae* from diverse Portuguese piggeries. We analyzed 43 samples (fresh/dry faeces, nasal/hide, drinking/waste water, feed, air, powder, surfaces, xerume) from 5 geographically distant Portuguese piggeries (2006-07). Samples were plated on MacConkey agar with/without antibiotics and production of ESBLs was searched by DDST. Bacterial identification, antibiotic susceptibility testing and conjugation experiments were performed by standard methods.  $bla_{ESBL}$  characterization was accomplished by PCR ( $bla_{TEM/SHV/CTX-M}$ ) and sequencing. The genetic context of  $bla_{CTX-M}$  genes (*ISEcp1*, *IS903*, *ISCR1*) was also evaluated (PCR and sequencing). We identified 173 *Enterobacteriaceae* corresponding to different morphotypes/susceptibility patterns. ESBL-producers (n=22) were found in 21% (9/43) of the samples analyzed (North region), being all identified as *Escherichia coli*.  $bla_{TEM-52}$  was the most common ESBL identified (13/22, 59%), being recovered from animal (n=7), feed (n=3) or environmental (n=3) samples of Piggery F, although  $bla_{CTX-M-1}/bla_{CTX-M-32}$ , commonly found in food-producing animals from Southern Europe, were found in animal/non-animal samples of Piggeries F or E, respectively. *ISEcp1* was detected upstream  $bla_{CTX-M}$  in two isolates producing CTX-M-32 (2/6, 33%), although among other CTX-M-producers we could not identify its presence under the conditions tested. *IS903* was detected in CTX-M-32-producing isolates (4/6, 67%) and *ISCR1* was not detected. Most *bla* genes were transferred by conjugation (21/22, 95%). Co-resistance to other antimicrobial classes was observed in all ESBL-producers. A piggery reservoir of  $bla_{TEM-52}$ , widely spread in other niches in the EU (humans, poultry and broilers) is described. The identification of ESBL-types commonly detected in Southern European pigs might be associated with trade of common animal lineages for fattening.

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PS3: 23

### Phenotypic detection of acquired AmpC beta-lactamases using the phenylboronic acid test

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Acquired AmpC  $\beta$ -lactamases (qAmpC) are usually implicated in false cephalosporin susceptibility reports and therapeutic failures, and hence detection methods need to be routinely performed in clinical laboratories. Some inhibitors can be used in their detection, but a standard method has not yet been established. To evaluate the accuracy of a recently described test for phenotypic detection of qAmpC producers based on the inhibitory activity of phenylboronic acid (PBA) in a well characterized collection of qAmpC-producing *Enterobacteriaceae*. Eighty-nine clinical isolates [29 *E. coli* (EC), 50 *K. pneumoniae* (KP), 9 *K. oxytoca* (KO), 1 *P. mirabilis* resistant to cefoxitin and intermediate/resistant to amoxicillin-clavulanic acid and at least one oxyimino-cephalosporin (CLSI disk diffusion) were selected for qAmpC production confirmatory tests. Isolates were recovered during a 7 year period (2002-08) and included DHA-1 (n=7), DHA-1+ESBL (n=40) and CMY-2 (n=3) producers, as well as qAmpC(-)/ESBL(-) (n=6) and qAmpC(-)/ESBL(+) (n=33) isolates. The PBA test was performed in Mueller-Hinton agar using disks containing only cefotetan (CTT) (30 $\mu$ g) and CTT plus PBA (30 $\mu$ g/400 $\mu$ g). A DHA-1-producing clinical isolate and EC ATCC 25922 were used as positive and negative controls, respectively. A positive test for the presence of qAmpC was considered if an increase of  $\geq$  5 mm in the inhibition halo occurred after the addition of PBA to CTT. Identification of known *bla*<sub>qAmpC</sub> genes was carried out by PCR and sequencing. The PBA test was positive for 59 isolates (44 KP, 10 EC, 5 KO). DHA-1-producing KP (n=41) and KO (n=4), and CMY-2-producing EC were correctly detected, but the test failed for DHA-1-EC (n=2). PBA test was positive in 11 isolates (7 EC, 3 KP and 1 KO) which yield a negative result in PCR assays for *bla*<sub>qAmpC</sub>, those from EC being attributable to natural AmpC hyperproduction. Sensibility and specificity of the method for this bacterial collection were 96% and 71.8%, respectively. Positive and negative predictive values (NPV) were 81.4% and 93.3%, respectively. PBA test combined with our screening method of qAmpC phenotypes displayed a high sensitivity with a good NPV, but only moderate specificity for detection of qAmpC producers. Other cephalosporin/boronic acid combinations might be incorporated to the confirmatory tests, especially to rule out EC natural AmpC hyperproduction.

PS3: 24

### Screening of Portuguese propolis antimicrobial activity in a wide range of microorganisms

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Propolis is a bee product composed by several compounds such as waxes, resins, essential oils and other organic compounds. Over the last decades, several studies have shown a wide range of biological and pharmacological properties for propolis from different provenance. Due to this large spectrum of bioactivities, such as antimicrobial, antioxidant, anti-inflammatory, and antitumor, among others, this product can become an alternative, economic and safe source of natural bioactive compounds. Propolis' antimicrobial action has been widely reported, either *in vivo* or *in vitro*, using propolis extracts or its isolated compounds. However, Portuguese propolis has earned little attention and its antimicrobial potential remains to be studied and characterized. This study reports the *in vitro* screening for the antimicrobial potential of a Portuguese propolis ethanol extract against a panel of Gram-positive and Gram-negative bacteria, as well as a group of yeasts, using the broth microdilution assay. Propolis was collected in the Summer of 2010 and extracted three times with ethanol at room temperature. The obtained solutions were filtered and pooled giving a propolis ethanol extract (PEE). Microorganisms were incubated with PEE and cell viability was assessed with serial concentrations, after 24 or 48 hours. Results showed that Portuguese propolis exhibits an efficient antimicrobial action either against yeasts or bacteria. In general, Gram-positive bacteria are more sensitive than Gram-negative bacteria, as has been widely documented for propolis samples from different origins. The present work also allowed the selection of the most sensitive bacteria and yeasts to be used as indicator strains in further studies.

PS3: 25

### Spread of large plasmids carrying antibiotic and copper resistance genes among enterococci from different ecological niches

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Association between the use of biocides and metals and selection of antibiotic resistant (AB<sup>R</sup>) bacteria has been suggested. Copper (Cu) is widely used in veterinary with different purposes (e.g. animal growth promoter). Our goal was to analyze the occurrence of Cu<sup>R</sup> genes (*tcrB*, *cueO*) among enterococci from different sources and to identify their genetic context and the link with AB<sup>R</sup> genes. *Enterococcus* isolates were collected from clinical samples (C, 103); healthy humans (HV, 125), poultry (P, 155), piggeries environment/swine (PE, 232) and sewage/river (SR, 52) (1997-2007). AB<sup>R</sup>/CuSO<sub>4</sub><sup>R</sup> phenotype/genotype was studied by disk diffusion/agar dilution (CLSI) and PCR. Clonality among isolates was established by PFGE/MLST. Plasmid analysis included location of Cu<sup>R</sup> gene (S1-PFGE hybridization) and identification of RepA from pLG1 (multi-R megaplasmid with *tcrB* operon). Transferability of Cu<sup>R</sup> genes was done by standard methods. Enterococci carrying *tcrB* and/or *cueO* genes (19%, 127/667) were detected from several sources and species (PE-27%, 25%; P-8%, 5%; HV-16%, 16%; H-4%, 5%; SR-19%, 13%; 83 *E. faecium-Efm*, 19 *E. faecalis-Efl*, 25 other enterococcal species). Susceptibility against CuSO<sub>4</sub> was lower among 35 representative isolates containing *tcrB* and/or *cueO* than among those lacking such genes (MIC<sub>CuSO<sub>4</sub></sub> 12->36mM vs 8-16mM; *Efm*). Isolates carrying *tcrB/cueO* were clonally diverse for both *Efm* [44 PFGE clones; CC17(ST18/ST393/ST431), CC5(ST5/ST185/ST150), 6 other ST] and *Efl* [10 PFGE clones; CC21-ST224, CC2-ST49, ST260, ST53, ST288 and ST53]. Cu<sup>R</sup> genes were located in plasmids of 90-120kb among *Efl* (3-*tcrB*; 1-*tcrB+cueO*) and 130-320kb in *Efm* (29-*tcrB*; 2-*cueO*; 24-*tcrB+cueO*). They often carried *ermB* (15 isolates), *tetM* (10), *tetL* (11), *aac6-aph2* (1). The *repA*-pLG1 was identified in 69% of *Efm* plasmids carrying Cu<sup>R</sup> genes. Cu<sup>R</sup> genes were transferred in all 25 *Efm* tested. Resistance to erythromycin, tetracycline, streptomycin, vancomycin, HLR-gentamycin, or ampicillin were detected in 95%, 83%, 45%, 19%, 14%, 5% of transconjugants. Co-transfer of Cu<sup>R</sup> and AB<sup>R</sup> genes occurred in the same or in different plasmids in representative transconjugants (12 and 2, respectively). Our data suggest that Cu<sup>R</sup> genes are often co-located with AB<sup>R</sup> in large plasmids carrying RepA-pLG1 which are widely spread among enterococci from different niches. The intensive use of Cu might favor the selection of AB<sup>R</sup> enterococci both by clonal expansion and spread of large mobile genetic elements.

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PS3: 26

### ***Staphylococcus epidermidis* nasal carriage: population structure and genetic diversity in single hosts**

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*Staphylococcus epidermidis*, a commensal of human skin and mucosa, is a major pathogen in nosocomial infections. Invasive isolates frequently belong to a major genetic lineage (CC2-SCC*mec*IV). However, limited information is available concerning the population structure of *S. epidermidis* in nasal colonization. Sixty-seven Danish volunteers with different risk factors for staphylococcal colonization (young hospitalized children, patients attending a Dermatology outpatient clinic or with previous MRSA carriage) were screened for *S. epidermidis* nasal carriage. Swabs were plated onto staphylococcal selective medium and colonies with different morphology were identified at species level and characterized by PFGE, MLST and SCC*mec* typing. The arginine catabolic mobile element (ACME) was detected by PCR and Southern blotting. Antibiograms were performed by disk diffusion. Fifty-four *S. epidermidis* isolates (30 methicillin resistant [MR] and 24 methicillin susceptible [MS]) were recovered from 36 individuals. Molecular typing distributed the MR isolates into three major lineages: PFGE type A/ST5/SCC*mec*IVa or IV related (*mec* complex: IS1272-ISSep1-like- $\Delta$ *mecR1-mecA*) (n=20); G/ST59/IVa or a non-subtypeable form of SCC*mec* IV (n=5); B/ST22/NT (n=2), and three singletons. All isolates belonged to CC2. MS isolates were distributed into 16 PFGE types of which 10 belonged to CC2 (n=18) and six were singletons. ACME was detected in 28 MR and 10 MS isolates. Thirteen individuals were found to be multiple carriers of genetically distinct MRSE (n=7), MSSE (n=3) or both (n=3), with one case of MRSE and MSSE sharing a PFGE profile with a single band difference. The concomitant presence of *S. epidermidis* and *S. aureus* was found in 10 carriers (including 2/13 *S. epidermidis* multiple carriers). Although MRSE coexisted with MSSA, in no case MRSE and MRSA was found in the same host. The results suggest that invasive *S. epidermidis* (CC2-SCC*mec*IV) is commonly carried in the nose. ACME-SCC*mec*IV, described first in several HA- and CA- MRSA lineages, was common in MRSE carriage, supporting the proposal of *S. epidermidis* as a reservoir of these elements. Moreover, the coexistence of MRSE and MSSA in a host enhances the potential for horizontal gene transfer, namely of *mecA* and ACME, promoting the emergence of novel MRSA, which is a major concern in Denmark, a country with a low but increasing prevalence of CA-MRSA.

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PS3: 27

**Virulence characterization of staphylococci of animal origin****Rui Seixas, Carla Margarida Duarte, José Pedro Santos, Carla Carneiro, Ricardo Bexiga, Cristina Lobo Vilela, Manuela Oliveira**

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Staphylococci have emerged as relevant animal pathogens, being responsible for conditions such as dermatitis, abscesses, and mastitis, among others. They can express a wide array of virulence factors, including surface proteins, exoenzymes and extracellular toxins that facilitate immune evasion, bacterial growth, tissue colonization and destruction. We characterized the virulence traits of methicillin-resistant staphylococci (n=26), assessed by oxacillin resistance. Isolates belonged to a collection of clinical isolates obtained from 2004 to 2010 (cats n=3, dogs n=3), and to a collection of bovine mastitis isolates obtained in 2008 (n=20), both from the Faculty of Veterinary Medicine, Technical University of Lisbon. Staphylococci species tested included *Staphylococcus aureus* (n=5), *S. epidermidis* (n=15) and *Staphylococcus* spp. (n=6), isolated by conventional microbiological procedures. Isolates identification was performed using biochemical galleries and confirmed by Multiplex PCR. Virulence factors production was screened by phenotypic methods. Coagulase production was tested using rabbit plasma; DNase production was evaluated in DNase Test Agar; gelatinase activity was tested in gelatinase test agar; hemolysin production was tested in Columbia agar supplemented with 5% sheep blood; lipase activity was evaluated in Spirit Blue Agar; and biofilm production was determined in Congo Red Agar supplemented with Comassie Blue. It was observed that four isolates were coagulase-positive (15.4%); 19 isolates produced hemolysin (73.1%), 7 produced DNase (26.9%), 25 were gelatinase-positive (96.2%), 18 were lipase-positive (69.2%) and 10 isolates were able to produce biofilm (38.5%). Only one isolate did not showed any of the virulence traits tested. The high frequencies of virulence traits present in these isolates suggest that staphylococci from veterinary origin have an increased ability to colonize the animal host and cause disease. Outbreaks in human medical centers promoted by virulent and antimicrobial resistant staphylococci are becoming frequently reported worldwide, rising concerns about human and animal health safety and on potential transmission of virulent bacteria in the wider concept of "One Health".

PS3: 28

### Antibacterial activity on opportunistic *Pseudomonas aeruginosa* pathogen by a novel *Salmonella* phage endolysin

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The Gram-negative pathogen *Pseudomonas aeruginosa* can cause severe infections of burn wound or cystic fibrosis on patients. Bacteriophage endolysin based strategy can offer a new alternative antimicrobial therapy. Endolysins are lytic enzymes that break down the peptidoglycan of bacterial cell wall at the late phage lytic cycle, however they are inactive on their own against Gram-negative bacteria when applied exogenously as recombinant proteins due to the peptidoglycan (endolysin substrate) protective outer membrane. We propose an innovative strategy to target Gram-negative *Ps. aeruginosa* based on the combination of endolysin enzymes and an outer membrane permeabilizing agent - ethylenediamine tetraacetic acid (EDTA). To validate this approach, we have isolated a novel *Salmonella* phage endolysin (68gpLys). Cloning this gene into *E. coli* expression system and subsequent large scale protein expression led to a high soluble yield of 14,3 mg/L of expression culture. In order to characterize it, muralytic assays on chloroform/Tris-HCl pretreated *Ps. aeruginosa* strain PAO1k (to remove the outer membrane) were made to check activity levels on substrate (398.05 Units/mM). The pH range was also determined with pH 7 being the optimum for the endolysin activity. For antimicrobial test, *in vitro* assays showed that incubation of  $10^6$  *Ps. aeruginosa* cells/mL with 0.5 mM EDTA and 5000 nM of 68gpLys, led to a strain inactivation of  $3.42 \pm 0.02$  logarithmic reduction units in a time-frame of 30 min. Here we prove that the synergistic effect of endolysin 68gpLys with EDTA can significantly reduce *Ps. aeruginosa* contamination. These results suggest, the great potential of this strategy for prevention and/or control of other Gram-negative pathogens. Current work has been also development to engineer new endolysins with incorporated cell penetrating peptides (CPP), employing site- and random-mutagenesis molecular techniques, to further enhance outer membrane permeabilization.

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PS3: 29

### Isolation and optimization of the production of *Staphylococcus epidermidis* bacteriophage from environmental samples

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*Staphylococcus epidermidis* is now among the most important nosocomial pathogenic agents owing its virulence to the adhesion and biofilm-forming abilities on medical surfaces, such as catheters. Biofilm control by antibiotics is often ineffective and new strategies of biofilm control are being sought. One promising strategy is the use of bacteria-specific virus, known as bacteriophages, to control infections by pathogenic bacteria. Bacteriophages, also known as phages, have been suggested to be one of the most abundant biological agents on the planet. Phages are currently suggested as possible alternatives to antibiotics for the treatment of bacterial diseases in humans to minimize the pathogen loads in medical devices. The goal of this study was to isolate new phages with potential bactericidal activity against *S. epidermidis* clinical isolates. Bacteriophages were isolated from an effluent from Waste Water Treatment Plants or from Hospital effluents, using a set of 40 bacterial strains as background. Five phages were isolated but when determining the phage titer the achieved concentration was around  $10^5$  pfu/ml and this titer was reduced 1 fold in two week's time. In order to increase the concentration of bacteriophages, since the obtained concentration was not sufficient to use in biofilm assays, several optimization steps were performed, using previous described isolation protocols, namely: using different concentrations of  $\text{CaCl}_2$ , using different concentrations of top agar, using different buffers, and using different phage filtration systems. For the optimization protocols we selected the bacteriophage with the higher titers and found that an optimized protocol was achieved by using Tris Buffer, Top Agar at 0.4%, and purification with  $\text{CsCl}_2$  gradient ( $q = 1.3, 1.5, \text{ and } 1.7$ ) with ultracentrifugation at  $100,000g$  for 1 h at  $4^\circ\text{C}$ . This phage titer was determined to be around  $10^8$ - $10^9$  pfu/ml. The optimized produced phage was then characterized by determining the lytic spectrum. The phage was able to lyse 13 strains, and of these strains 10 had the biofilms genes present. Finally, the 10 selected strains were tested for biofilm formation, using the microtiter assay, and it was confirmed that they formed biofilms in TSB supplemented with 1% glucose. For future work; we need to determine if we have lytic or temperate phages with DNA sequence analyses and to test the phage against to the Biofilm formation of relevant bacterial strains.

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PS3: 30

**Assessment of the effect of different antibiotics on bacterial cell integrity by rheology****Raquel Pereira Portela<sup>1,2</sup>, Ana Madalena Ludovice<sup>1,3</sup>, Pedro L. Almeida<sup>4,5</sup>, Maria Teresa Cidade<sup>5</sup>, Catarina R. Leal<sup>4,5</sup>, Rita Gonçalves Sobral<sup>1,2</sup>**

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The classification of antibiotics relies on the type of interaction between the drug and its target, which frequently determines its bactericidal or bacteriostatic nature. Treatment with most bactericidal compounds results in cell lysis and the most clinically relevant are  $\beta$ -lactams, which inhibit peptidoglycan biosynthesis. We are currently developing a new and high-speed method to determine if a specific compound has bacterial lytic activity by directly measuring the change in the shear viscosity of a bacterial culture upon antibiotic treatment, in order to assess cell integrity. We used as a model organism *Staphylococcus aureus*, an important pathogen with high capacity to develop antibiotic resistance. The mechanical properties of *S. aureus* cultures were analysed by simple shear flow measurements resorting to a rotational stress controlled rheometer using a cone and plate geometry. Exponential-phase grown cells were treated with a  $\beta$ -lactam, oxacillin, and allowed to further grow. Samples of treated and untreated cultures were collected along time. Their viscosity, as a function of the shear rate, was immediately measured at 20 °C. The upper rotating cone allows the application of a shear deformation at controlled shear rate and also the measurement of the stress induced within the sample. By measuring the viscosity, at fixed shear rates, for different growth time, the evolution of the bacterial growth was evaluated. In all samples a shear thickening behaviour was observed. The viscosity of the culture was significantly amplified by the action of oxacillin which damages the cell wall, resulting in lysis and release of the cell contents. Treatment with chloramphenicol, a bacteriostatic antibiotic that inhibits protein synthesis, is also being tested. In an initial approach, the assay is expected to discriminate between bacteriolytic and bacteriostatic drugs. We also aim to test the method's sensitivity to discriminate between susceptible and resistant strains to bacteriolytic antibiotics. Furthermore, the viscosity of the isolated cellular components is being tested to evaluate the contribution of each macromolecule to the overall viscoelastic properties of the bacterial lysate. The mechanical role of the cell wall constituents, such as peptidoglycan or teichoic acids is of particular interest. The described technique appears as a rapid and simple way to assess bacterial cell wall integrity and characterize the lytic impact of antimicrobial compounds.

PS3: 32

### **Direct genotyping of enteroviruses in children with aseptic meningitis in Iran.**

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Viral meningitis is an inflammation of the leptomeninges as a manifestation of central nervous system (CNS) infection. The clinical course in viral meningitis is usually self-limited, with complete recovery in 7-10 days. More than 85% of viral meningitis cases are caused by nonpolio enteroviruses which belong to the family of Picornaviridae. 120 cerebral spinal fluid (CSF) from children between 0-13 years old with primary diagnosis of viral meningitis were transported on ice to department of virology, Pasteur Institute of Iran and stored in -70 freezer. Identification of enteroviruses was performed using RT-PCR with Pan-EV primer pairs and Real-Time PCR Taq Man probe. Serotype identification of enteroviruses was done by sequence analysis of the amplified VP1 region by semi-nested RT-PCR method. Enteroviruses were detected in 13 (10.8%) of 120 CSF specimens. Enterovirus genotyping was achieved in 10 specimens including echovirus type 14 (15.4%), human polio enteroviruses type 1 (15.4%), echovirus type 30 (7.7%), echovirus type 5 (7.7%) and EV 71 (30.8%). Enteroviruses have an important role in childhood aseptic meningitis cases, and the predominant serotypes vary according to years. The phylogenetic and molecular epidemiologic analysis of strains circulating during the EV season are possible by the sequence analysis of the VP1 region derived from the RT-snPCR. The overwhelming majority of meningitis cases are caused by serotypes of coxsackievirus and echovirus. However, the enterovirus 71 is a well-known pathogen. Although the molecular assays for detection of enteroviruses in CSF specimen is significantly more sensitive than the conventional assays, detection and typing of enteroviruses directly in clinical specimens especially in CSF should be considered more problematic. This was the first molecular epidemiological study of enterovirus meningitis in Iran.

PS3: 33

**Dolphin morbillivirus in Portugal: first report in stranded cetaceans****Carolina Bento<sup>1</sup>, Ana Isabel Duarte<sup>1</sup>, Marisa Ferreira<sup>2</sup>, Fernando Afonso<sup>1</sup>, José Vingada<sup>2,3</sup>, Luis Tavares<sup>1</sup>**<sup>1</sup>Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, Portugal; <sup>2</sup>Dep. Biologia e Sociedade Portuguesa de Vida Selvagem, Universidade do Minho, Braga, Portugal; <sup>3</sup>Centro de Estudos sobre Ambiente e Mar, Universidade de Aveiro, Aveiro, Portugal

Dolphin morbillivirus (DMV) and Porpoise morbillivirus (PMV) are members of the subfamily Paramyxovirinae, family Paramyxoviridae. These viruses are important pathogens of cetaceans, causing serious respiratory and central nervous system infections, leading to stranding and death. Since 1987 DMV and PMV are responsible for several epidemics in cetacean populations worldwide [1]. To date no information regarding the presence of these viral pathogens in stranded cetaceans in the coast line of Portugal is available. Due to the pathogenic impact of DMV a molecular survey was conducted for detection of viral RNA from biological materials of stranded cetaceans available through the National Marine Mammal Stranding Network and through the CRAM-Q - Quiaios Marine Animal Rehabilitation Centre, Portugal. Lung and brain samples (n=40) were collected between 2004-2011 from 25 *Delphinus delphis* (Dd), 4 *Globicephala melas* (Gm) and 8 *Stenella soeruleoalba* (Sc), 2 *Phocoena phocoena* (Pp) and 1 *Mesoplodon mirus* (Mm). After total RNA extraction the samples were screened by conventional one step RT-PCR. The primers targeting a 550bp fragment included in the M gene of the DMV/PMV genome were chosen by the Primer designing tool available through <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>. The expected amplicon was detected in the lung samples of two Sc and one Gm, collected in 2007, 2011 and 2008 respectively. After sequencing its specificity was confirmed against the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) sharing 99% homology with the two available sequences of DMV M gene. Our results confirmed the presence of DMV RNA in samples collected from stranded cetaceans which may indicate subclinical infection of these animals. Interestingly one Gm (2008) was found positive and this species may act as virus reservoirs for other cetacean species [2]. The biological significance of this finding emphasizes the need for a more thorough investigation regarding the virus temporal pattern in Atlantic waters and its potential correlation to the occurrence of past and future epizootics of the disease [3].

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PS3: 34

**Encapsulation of *Mycobacterium bovis* BCG in biodegradable alginate-chitosan microparticles for mucosal immunization****Liliana Aranha Caetano<sup>1,2</sup>, António José Almeida<sup>1</sup>, Lúdia Maria Diogo Gonçalves<sup>1</sup>**<sup>1</sup>iMed.UL, Portugal; <sup>2</sup>ESTeSL, Portugal

Tuberculosis remains the leading cause of death from infectious diseases throughout the world, killing an estimated 1.9 million people every year. The only available preventive vaccine, using *Mycobacterium bovis* bacille Calmette–Guérin, is considered to be ineffective against adult pulmonary tuberculosis, the most prevalent form of the disease. In addition, the current BCG vaccine faces some technological problems, since it is intradermally administered and supplied in multi-dose vials, with risk of contamination and loss of efficacy within subsequent administrations. Given the lack of success of the *M. bovis* BCG vaccine, efforts are ongoing to develop more effective vaccines using a range of strategies, including the development of microparticle based delivery systems. It is clear that the uptake of antigen by APCs is favored in particulate form rather than soluble; particles may therefore improve the immune response through stimulating cellular uptake, as well as affecting its adjuvant and immunological properties and its eventual clearance. Therefore, we propose to use a new strategy with particulate delivery systems to design an improved anti-TB vaccine, capable of elicit a strong pulmonary mucosal response and to induce protective cell-mediated immunity, in a more stable and long-lasting formulation. Our goal is to change antigen presentation by APC's by modifying surface physicochemical properties of BCG through coating/encapsulation of whole mycobacteria in suitable polymeric particulate carriers with immunological adjuvant properties.

Alginate-chitosan particles containing whole live monodisperse BCG were formulated avoiding solvents, high shear stress and high temperature. Best encapsulation systems were assessed according to loading capacity, encapsulation efficiency, particle size and surface charge. Cell viability and formulations stability after freeze-drying and reconstitution were evaluated. Current work includes infection of macrophage cell lines, in order to assess how efficiently bacteria-beads will be phagocytised by macrophages in culture and to evaluate the intracellular traffic of these beads to the phagolysosome compartment.

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PS3: 35

**Enterobacteriaceae from Portuguese piggeries as potential reservoirs of acquired quinolone resistance genes****Elisabete Machado<sup>1,2</sup>, Ana Monteiro<sup>1</sup>, Rafael Cantón<sup>3</sup>, Teresa M. Coque<sup>3</sup>, Luísa Peixe<sup>2</sup>**<sup>1</sup>Universidade Fernando Pessoa, Porto; <sup>2</sup>REQUIMTE. Faculdade de Farmácia, Universidade do Porto; <sup>3</sup>Hospital Ramón y Cajal, Madrid, Spain

Plasmid-mediated quinolone resistance (PMQR) genes are increasingly identified worldwide in *Enterobacteriaceae* of human and animal origin. They confer reduced susceptibility to quinolones that could facilitate the selection of mutants with high-level resistance. To evaluate the occurrence and diversity of genes coding for acquired resistance to quinolones among *Enterobacteriaceae* from Portuguese piggeries. Forty-three samples (fresh/dry faeces, nasal/mucoid, drinking/waste water, feed, air, powder, surfaces, xerume) from 5 Portuguese piggeries located in different regions were analysed (2006-07). Samples were plated on MacConkey agar with/without antibiotics. Isolates were identified by API ID32GN and susceptibility testing was performed by CLSI disk diffusion method. *qnrA*, *qnrB*, *qnrS*, *qepA* and *aac-(6)-Ib-cr* genes were searched by PCR and further sequenced. Co-presence of *bla*ESBL genes was also evaluated (PCR, sequencing). We identified 173 *Enterobacteriaceae*, corresponding to different morphotypes/susceptibility patterns. A low rate of resistance to nalidixic acid (17%) and ciprofloxacin (2%) was observed. Occurrence of PMQR genes was found in 3% (5/173) of the isolates (4 piggeries, 3 regions). *qnrB* was the most frequent, being detected in four *Citrobacter freundii* isolates recovered from water, powder and swine feed of three piggeries (North, Centre and South of Portugal). *qnrS1* was found in one CTX-M-32-producing *E. coli* strain from feed. *qnrA*, *qepA* and *aac-(6)-Ib-cr* were absent. Our results constitute the first description of *qnr* genes among *Enterobacteriaceae* from Portuguese swine or piggery environments, highlighting the piggeries as potential reservoirs of PMQR determinants. The role of *C. freundii* as relevant reservoir of *qnr* genes is also confirmed.



PS3: 36

### Epidemiological shift and expansion of widespread *Escherichia coli* clones from Portuguese hospitals

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In a previous survey (2003-04), TEM-24 and TEM-116 *Enterobacter aerogenes* were the most frequent ESBL-producing isolates in a hospital in the Centre region of Portugal. Preliminary data on ESBL-producing isolates recovered during 2006 and 2007 in the same hospital suggest an increase of CTX-M-producing *E. coli*. We aim to characterize the population structure and epidemiological features of recent *E. coli* isolates from this institution and a recently opened hospital located nearby, in order to understand mechanisms of selection and epidemiological shifts of ESBLs occurring at Portuguese hospitals. Fifty two recent ESBL-producing *E. coli* isolates from two Portuguese hospitals located in the Centre Interior region were included (2006-2008). Species identification and antibiotic susceptibility testing were performed by standard methods. ESBL characterization included DDST and identification of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub> and *bla*<sub>CTX-M</sub> genes by PCR and sequencing. Clonal relatedness was investigated by XbaI-PFGE and MLST. *E. coli* phylogenetic groups were identified by a multiplex PCR. Most isolates were recovered from urines (81%) and UTIs (63%), produced mostly CTX-M enzymes (91%; CTX-M-1, -14, -15, -32), although TEM (4%; TEM-52) and SHV (6%; SHV-12) types were also detected, and belonged to particular clonal complexes. A B2-ST131 *E. coli* clone was the most frequently identified in both institutions (n=42, 81%; 5 PFGE-types), exhibiting resistance to kanamycin (93%), tobramycin (90%), gentamicin (83%), tetracycline (90%) and ciprofloxacin (88%). It harboured *bla*<sub>SHV-12</sub> (n=1) or *bla*<sub>CTX-M-15</sub> (n=41) frequently associated with *bla*<sub>OXA-1</sub> and/or *bla*<sub>TEM-1</sub>, and occasionally *bla*<sub>TEM-10</sub> or *bla*<sub>TEM-116</sub>. *E. coli* isolates belonging to phylogroup D were ST117 (n=2, encoding CTX-M-1 plus TEM-116 or CTX-M-14) and ST648 (n=1, TEM-52). A-*E. coli* belonged to ST10 complex (n=2, CTX-M-15, CTX-M-32), ST2230 (n=1, CTX-M-1) and ST2228 (CTX-M-15). B1-*E. coli* isolates were diverse and associated with different ESBL-types: 1 ST1431 (SHV-12), 1 ST58 (TEM-52) and 1 ST348 (CTX-M-14). A, B1 and D clones were frequently resistant to streptomycin, sulphonamides and tetracycline. We detected an epidemiological shift characterized by current widespread of B2-ST131 (CTX-M-15/SHV-12) in different Portuguese hospitals, and the emergence of particular A (CTX-M enzymes) and B1 (CTX-M-14, SHV-12, TEM-52) clones. The detection of additional TEM-10/-116 variants might indicate diversification or recent plasmid acquisition.

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PS3: 37

**Evaluation of antigenotoxic effects of phytochemicals: DNA protection from oxidative stress and improved DNA repair ability****Alberta Domingues<sup>1,2</sup>, Alberto Dias<sup>1</sup>, Rui Oliveira<sup>2</sup>**

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In recent years, plant extracts and their phytochemicals have received increasing attention, especially in the field of pharmaceutical sciences and medicine, due to the potential prevention activity of a number of chronic and degenerative diseases including cancer and cardiovascular diseases. Most of the health-promoting effects have been attributed to antioxidant action including the ability to scavenge reactive oxygen species and to chelate metal ions. The yeast *Saccharomyces cerevisiae* is perhaps the best studied eukaryotic organism and exhibits striking similarities in molecular mechanisms such as transcription, translation, replication, and DNA repair with higher eukaryotes. For these reasons, it is a good model for studying the DNA damage and search for antigenotoxic effects of plant compounds. We aim to study the protective effect of the flavonoids quercetin, isoquercitrin, quercitrin, rutin and hyperoside, in DNA and the potential inducing activity of DNA damage repair. Experiments involved pre-treatment, co-treatment and post-treatment of *S. cerevisiae* cells with phytochemicals (regarding the order of incubation with phytochemicals in relation to oxidative stress imposed by H<sub>2</sub>O<sub>2</sub> or KMnO<sub>4</sub>) and DNA damage was subsequently assessed by the yeast comet assay, using tail length as the parameter for DNA damage. The results obtained from pre-treatment experiments with quercetin, isoquercitrin, quercitrin, rutin and hyperoside show that tail lengths are shorter, suggesting that these compounds protect cells against oxidative DNA damage caused by 10 mM H<sub>2</sub>O<sub>2</sub> and 500 µM KMnO<sub>4</sub>. Furthermore, we have observed quercetin-mediated DNA protection against oxidative stress, which has improved with the time of incubation. In co-incubation experiments with 10 mM H<sub>2</sub>O<sub>2</sub>, quercetin promoted decreased DNA damage in cells, suggesting reactive oxygen species scavenging activity. In assays of pre and post-incubation with different concentrations of quercetin, we have observed a decrease of DNA damage in a dose-dependent manner. This suggests that quercetin stimulates the DNA repair machinery besides direct protection of DNA from oxidation. The observed improvement in DNA repair depends on the DNA repair machinery since post-treatments of mutants affected in Base Excision Repair (BER) with quercetin showed no reduction of comet tails, indicating that quercetin presumably could activate DNA repair enzymes from BER pathways, which repair oxidative damage.

PS3: 38

**Genetic heterogeneity of *Mycobacterium avium* subspecies *hominissuis* isolated in Portugal****Célia Leão<sup>1</sup>, João Inácio<sup>1</sup>, Ilda Sanches<sup>2</sup>, Ana Botelho<sup>1</sup>**

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*Mycobacterium avium* subsp *hominissuis* (MAH), a member of the *Mycobacterium avium* complex (MAC), is an opportunistic zoonotic pathogen. MAH causes disease mainly in humans and pigs, evidenced by localised granuloma-like lesions in the lymph nodes of the digestive tract. MAH has a relevant economic impact in livestock industry and became an increasing concern in public health in the last decades. Between 2004 and 2006, a large number of lymph nodes collected from pigs at some Portuguese regional abattoirs had lesions compatible with tuberculosis lymphadenitis. MAH was identified in the majority of the mycobacteria-positive samples. A selection of these isolates were characterized by an IS 1245-RFLP typing analysis and revealed to be highly variable [1]. Recently, a variable number tandem repeat (VNTR) analysis using *Mycobacterium avium* tandem repeats (MATR-VNTR) revealed to have an excellent discriminatory power for MAC strains [2]. In this work, we evaluated the MATR-VNTR typing scheme to retrospectively characterize the genetic heterogeneity of MAH isolates responsible for the 2004-2006 tuberculosis lymphadenitis outbreaks. The Portuguese MAH strains revealed a high diversity of MATR-VNTR profiles, with the loci MATR-3, MATR-8 and MATR-11 showing the highest allelic diversity. There was no apparent relationship between the geographic origin of the samples and the MATR allelic profiles of MAH strains, corroborating the results of the previous typing study using IS 1245-RFLP [1]. MATR-VNTR typing revealed to be simple, fast and affordable, having a high level of reproducibility and discriminatory power for MAH.

[1] Domingos et al. 2008. *Vet. Rec.* 163: PO12161-1-9. [2] Inagaki et al. 2009. *J. Clin. Microbiol.* 47: 2156-2164. Célia Leão is a recipient of a PhD grant from the Portuguese "Fundação para a Ciência e a Tecnologia" (SFRH/BD/62469/2009)

PS3: 39

**HepG2 cells under starvation-induced autophagy are susceptible to cell death caused by oleanolic acid but not by ursolic acid****C. L. Duarte<sup>1</sup>, C. Pereira-Wilson<sup>2</sup>, C. F. Lima<sup>1</sup>**<sup>1</sup>CITAB - Department of Biology, School of Sciences, University of Minho, Braga, Portugal; <sup>2</sup>CBMA

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Cancer incidence is increasing worldwide mainly due to changes in diet, life style and increased lifespan. In particular, liver cancer is the fifth most common cancer in the world and the third most common cause of cancer mortality. Plant phytochemicals are a good and promising source of anticancer compounds. In a previous work, we reported the potential of ursolic acid (UA) to induce cell death and to inhibit proliferation in colorectal cancer cells [1]. This natural triterpenoid UA was also shown to activate JNK and to modulate molecular markers of autophagy (Xavier *et al.*, unpublished data). In the present study, we tested the ability of two isomer triterpenoids, UA and oleanolic acid (OA), to induce cell death and modulate autophagy in human hepatocellular carcinoma cell line (HepG2 cells). For that, the effect of these phytochemicals on cell death was evaluated by MTT assay and propidium iodide staining, in complete and starvation medium. Autophagy markers were evaluated by western blot and fluorescent microscopy. Contrary to our previous data with other cell lines, HepG2 cells were less susceptible to UA and, unexpectedly, OA was a more potent inducer of cell death than UA. Interestingly, starvation-induced autophagy sensitized HepG2 cells to cell death caused by OA, but not by UA. The IC<sub>50</sub> of OA decreased from 50 µM in complete medium to 3.5 µM in starvation medium. Although UA and OA increase the levels of autophagy markers LC3-II and p62, as well as the number of acidic vacuoles (as assessed by MDC staining), the cell death induced by OA was not prevented by inhibitors of autophagy and of lysosome proteases. Overall, the results seem to indicate that autophagy is not involved in cell death induced by OA. Interestingly, methyl-β-cyclodextrin (a polymer able to decrease membrane cholesterol content) prevented OA-induced cell death. In conclusion, these results seem to indicate that cellular membrane biophysics are affected by OA, in particular during starvation and with involvement of cholesterol, which leads to sudden cell death. In the future OA can be viewed as specific drug for cancer treatment in particular cell physiological conditions.

[1] Xavier *et al.*, *Cancer Letters*, 2009, 281: 162-70. *Acknowledgements*: This work is supported by FCT research grant NaturAge – PTDC/QUI-BIQ/101392/2008, which is co-funded by the program COMPETE from QREN with co-participation from the European Community fund FEDER.

PS3: 40

**Human leptospirosis in Azores: from past to present****Teresa Marqarida Carreira**<sup>1,2</sup>, **Mónica Susana Nunes**<sup>1,2</sup>, **Maria Luísa Vieira**<sup>1,2</sup><sup>1</sup>Unidade de Microbiologia Médica – Grupo de Leptospirose e Borreliose de Lyme, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Lisboa, Portugal;<sup>2</sup>Centro de Recursos Microbiológicos, Faculdade de Ciências e Tecnologia, UNL, Lisboa, Portugal

Leptospirosis is probably the most widespread and frequent zoonotic disease in the world. Since 1993, Azores islands (Portugal) have been considered endemic for this worldwide zoonosis. High precipitation and moderate temperature, together with high densities of rodents (the primary reservoirs of leptospires), provide an extensive dissemination of these spirochetes allowing their survival in nature. The high prevalence and mortality rates by Leptospirosis, the growing number of detected human cases and the lack of local laboratory facilities for diagnosis, contributed to the implementation and development of a research project entitled “*Epidemiology and Control of Leptospirosis in Azores*” under USA Scientific-Cooperative Agreement No. 58-4001-3-F185. The project, undertaken between 2004 and 2008, was founded on four research areas: *i*) human epidemiology; *ii*) reservoir’s ecology; *iii*) laboratory diagnosis, including the technology transfer from Leptospirosis Reference Laboratory (LRL) at IHMT, to local hospitals on São Miguel and Terceira islands; and *iv*) evaluation of the knowledge attitudes and practices of the population facing exposure to *Leptospira* infection. The purpose of this study was to evaluate the current trend of the Leptospirosis in Azores, based on human positive cases registered on LRL databases concerning the timing, before, during and after project completion. A total of 1182 sera, received at LRL (IHMT), between 1993 and 2010, from Azorean patients with Leptospirosis clinical suspicion, were analyzed during the referred period, by the reference Microscopic Agglutination Test (MAT) in order to detect anti-*Leptospira interrogans* sensu lato (s.l.) antibodies. Serological positive results obtained by MAT showed the following distribution: from 2008 to 2010, 60<sup>+</sup>/226 (27%); from 2004 to 2007, 148<sup>+</sup>/370 (40%), and from 1993 to 2003, 248<sup>+</sup>/586 (42%). These findings reveal an important impact of the project’s involvement area since the number of cases of Leptospirosis significantly decreased during and after the project’s completion. It is assumed that this important reduction is a consequence of the mapping of the major *Leptospira* transmission risk-areas to humans, and the creation of laboratory facilities, namely the diagnosis implementation by a serological screening and PCR technique, avoiding the extreme clinical pictures. Globally, these results promote a continuous and active surveillance in Azores.

PS3: 41

**Incorporation of antimicrobial peptide into bacterial cellulose produced through food waste****Angela Faustino Jozala<sup>1</sup>, Carolina Alves dos Santos<sup>2</sup>, Renata Aparecida Nedel Pértille<sup>3</sup>, Francisco Miguel Gama<sup>4</sup>, Adalberto Pessor Jr.<sup>1</sup>**<sup>1</sup>School of Pharmaceutical Science, University of São Paulo, Brazil; <sup>2</sup>Chemical Engineering Department, University of São Paulo; <sup>3</sup>Department of Chemical Engineering, UFSC/SC;<sup>4</sup>Department of Biologic Engineering, UMINHO/Portugal

The incorporation of antimicrobial, such as nisin, or other drugs in bacterial cellulose has a wide applicability in pharmaceutical, medical, chemical, cosmetic, food and other areas. Nisin is a natural antimicrobial peptide used as food preservative; being effective at controlling a broad range of gram-positive bacteria, including the multidrug-resistant pathogen. Bacterial cellulose (BC) is an extracellular polysaccharide produced by strains of *G. xylinus*. BC production using waste as culture media is a novelty, stimulating scale-up for industrial production and extended applications in medical devices. For this reason, the objective of this work was evaluated nisin activity after incorporation into BC standard and produced using waste. For BC production, the following culture media were employed (i) Hestrin and Schramm (standard); (ii) Waste fruits, collected from the disposal of free markets; (iii) Mixture of the waste fruit and milk whey, under 30 °C for 96 hours in static conditions. BC standard and from waste were submerge in 1mL of nisin (Sigma<sup>®</sup>, 1g contain 2.5% of nisin), at different concentrations 250, 125, 62.5, 31.25, 15.63 and 7.81 µg/mL, at phosphate buffer saline (PBS) pH 4.5, sterilized by filtration, 0.22 µm. They were incubated at 30°C under 100rpm for 4 hours, followed by protein analysis and nisin activity, which was determined by agar diffusion with *L. sakei* as bioindicator. Results demonstrated that all types of BC have the ability to incorporate nisin after 4 hours. Nisin activity was higher in BC, although only 43% of proteins from the initial solution were transferred into the membrane. The same behavior on nisin incorporation was observed in BC standard and from waste, indicating that waste can be applied on BC production. This work can be considered a profitable alternative, generating high-value products with extended applications and contributing to decreasing disposals in the world.

PS3: 42

**Induction of antioxidant defenses by diterpenic phenolics in human fibroblasts for nutritional applications toward anti-aging interventions****Ana Cristina Carvalho<sup>1</sup>, Andreia C Gomes<sup>2</sup>, Cristina Pereira-Wilson<sup>2</sup>, Cristovao F Lima<sup>1</sup>**<sup>1</sup>CITAB - Department of Biology, University of Minho, Braga, Portugal; <sup>2</sup>CBMA - Department of Biology, University of Minho, Braga, Portugal

Human lifespan is increasing in developed and developing countries, rising considerably the percentage of elderly people. Aging is characterized by the progressive accumulation of molecular damage, which leads to altered cellular functioning, reduced stress tolerance and susceptibility to diseases, such as cancer, neurodegenerative and cardiovascular diseases. Consumption of fruits, vegetables and herbs has been related with health promotion due to their content in bioactive phytochemicals. In a previous work, we have shown the ability of curcumin to induce antioxidant stress responses in human fibroblasts, protecting the cells to a further oxidant challenge, which suggests curcumin's potential in anti-aging interventions [1]. In the present work, we propose to evaluate the ability of the phenolic diterpenes carnosic acid (CA) and carnosol (CS) to induce intracellular antioxidant defenses in normal human skin fibroblasts and relate that with anti-aging effects. The ability of these compounds to induce cellular antioxidant defenses was investigated by western blot and biochemical determinations. We observed that human fibroblasts are more susceptible to CS than CA. Carnosic acid was not toxic up to 40  $\mu\text{M}$  (the highest concentration tested), CS induced toxicity in human fibroblasts only at 40  $\mu\text{M}$ . Both CA and CS at non-toxic concentrations induced significantly glutathione levels, an important intracellular antioxidant, in a concentration dependent manner. Both compounds were also able to induce cytoprotective enzymes such as HO-1, NQO1 and Hsp70, assessed by western blot. We also tested the capacity of these compounds to afford a cytoprotective action by pre-incubating cells with CA and CS before a further oxidant challenge with *tert*-butyl hydroperoxide (*tert*-BOOH). In conclusion, the induction of antioxidant defenses in human skin fibroblasts by CA and CS may be applied for nutritional applications toward cytoprotective and anti-aging interventions.

[1] Lima *et al.*, *Mol. Nutr. Food Res.* 2011, 55: 430-42. *Acknowledgements*: ACC is supported by B11-PTDC/QUI-BIQ/101392/2008 grant. This work is supported by FCT research grant NaturAge – PTDC/QUI-BIQ/101392/2008, which is co-funded by the program COMPETE from QREN with co-participation from the European Community fund FEDER.

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PS3: 43

### Induction of antioxidant defenses in human HepG2 cells by a methanolic extract of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens*

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*Hypericum perforatum* (HP) commonly known as St. John's wort is an important medicinal plant traditionally used in several ailments including mild to moderate depression. In a recent study, elicitation of HP cells (culture of cells in suspension) with *Agrobacterium tumefaciens* (AT) increased significantly the production of compounds with antioxidant and antimicrobial properties [1]. The present investigation has been conducted to test the efficacy of methanolic extracts of elicited and control HP cells in protecting human hepatocytes (HepG2 cell line) upon oxidative stress. Briefly, HepG2 cells were treated with *tert*-butyl hydroperoxide (*tert*-BOOH) to induce oxidative stress, and the ability of the HP extracts to protect against cell death were measured by MTT and LDH leakage assays. The ability of these extracts to induce cellular antioxidant defenses were investigated by western blot and biochemical determinations. When HepG2 cells were co-incubated with 800  $\mu$ M *tert*-BOOH and HP extracts (80  $\mu$ g/ml), only the extract from the elicited HP cells was able to significantly prevent *tert*-BOOH-induced cell death. The extract from control HP cells did not show any protective effect, on the contrary, stimulated *tert*-BOOH toxicity. When HepG2 cells were pre-incubated for 5 h with HP extracts, followed by a period of 16 h of recovery with fresh medium, prior to incubation with *tert*-BOOH, only the HP extract from elicited cells significantly protected against cell death. This suggested the ability of HP extract from elicited cells to induce intracellular antioxidant defenses in HepG2 cells. That was confirmed by the induction of about 40% in the content of glutathione in HepG2 cells, whereas control extract increased only 10%. As well, only the extract from elicited HP cells were able to induce the levels of cytoprotective enzymes such as HO-1 and NQO1. In conclusion, we observed that elicitation of HP cells with AT produced bioactive compounds present in the methanolic extract able to protect HepG2 cells against oxidative stress, and also to induce intracellular antioxidant defenses of this human cell line.

[1] Franklin *et al.*, *Phytochemistry*, 2009, 70: 60-8. ACC is supported by B11-PTDC/QUI-BIQ/101392/2008 grant. This work is supported by FCT research grants NaturAge – PTDC/QUI-BIQ/101392/2008 and (HyperFood)-PTDC/AGR-ALI/105169/2008, co-funded by the program COMPETE from QREN with co-participation from the European Community fund FEDER.



PS3: 44

**Effect of algae and plant lectins against bacterial biofilm formation****Mayron Alves Vasconcelos<sup>1,3</sup>, Francisco Vassilíepe Sousa Arruda<sup>2,3</sup>, Victor Alves Carneiro<sup>2</sup>, Kyria Santiago Nascimento<sup>1</sup>, Benildo Sousa Cavada<sup>1</sup>, Edson Holanda Teixeira<sup>2</sup>, Mariana Henriques<sup>3</sup>, Maria Olívia Pereira<sup>3</sup>**

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Biofilms are composed by microbial cells that are irreversibly associated with a surface and are enclosed in a matrix of polymeric material. The search for potential phytochemicals as anti-biofilm agents has become an active area of research. Lectins are sugar binding proteins of non immune origin that agglutinate cells and precipitate glycoconjugate molecules. Due to their capacity to bind and recognize specific carbohydrates, lectins can be a potent tool against biofilms. Thus, this work aims to evaluate, *in vitro*, the activity of a set of plant and red algae lectins against clinical relevant bacteria, *Staphylococcus aureus* and *Klebsiella oxytoca*, by the assessment of their capacity to interfere on biofilm formation. Lectins were added to bacteria ( $2 \times 10^6$  CFU/mL) on the moment of biofilm formation in concentrations ranging from 25 to 250  $\mu\text{g/mL}$ . Subsequently, the resultant biofilms (48 h) were analyzed in terms of biomass by crystal violet staining and in terms of cell viability by assessing the number of colony forming units. Additionally, the effect of lectins on planktonic growth was also assessed following the optical density of the bacterial cultures along time. Although both groups of lectins were able to reduce the growth of *S. aureus* and *K. oxytoca*, the plant lectins from *Vatairea macrocarpa* (VML) and *Cratylia floribunda* (CFL) showed the better activities. It should be highlighted that VML at 250  $\mu\text{g/mL}$  reduced around 90% the planktonic growth of *S. aureus*. Curiously, this lectin had also a good effect in avoiding the establishment of biofilms of the same bacteria. Regarding *K. oxytoca*, CFL and red algae lectins (from *Hypnea musciformis* and *Bryothamnion triquetrum*), reduced its biofilm mass. However, the effect of the lectins on the biofilm viable cells was not so notorious. Interestingly the reduction of biomass observed for *S. aureus*, in the presence of the highest concentration of VML, was accompanied by a decrease on the number of biofilm viable cells. In this study, it was showed that some lectins are able to interfere with biofilm growth of *S. aureus* and *K. oxytoca*. However, the most promising lectins showed differences in their activities, which can be explained by their different binding specificities for sugars.

PS3: 45

### Penicillin Ionic Liquids: “salting” antibiotic properties

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New forms of Active pharmaceutical ingredients (APIs) have been recently developed by the Pharmaceutical Industry in order to improve chemical-physical properties like solubility, stability and hygroscopy. The improvement of those properties can be reflected in better dissolution, biocompatibility and delivery dynamics of APIs. Designing liquid phase APIs will not only improve those properties but also prevents the formation of polymorphisms during the storage and manufacturing process. Ionic liquids (ILs), which are usually defined as salts that have melting temperatures below 100°C, are one of the most promising solutions to obtain liquid phase APIs. The interest in ILs has grown exponentially in the last decade, mostly motivated by their tailor-made properties. We report here different ionic compounds based on the combination of two  $\beta$ -lactam antibiotics (Ampicillin and Penicillin G) with Oleylamine. Our aim was to evaluate if a liquid form of Penicillin G and Ampicillin could be useful to enhance their antibiotic properties. Moreover motivated by the fact that ionic modifications could enhance and scope APIs action, we have also evaluated the possibility of using these types of ionic antibiotics as anticancer drugs. Our results show that both ionic antibiotics (AMP-OLEY and PEN-OLEY) are highly viscous liquids at room temperature and form a micellar system in water. Based on this latter result we have tried to evaluate if this micellar system could eventually sustained the release of ampicillin in an aqueous media. For this purpose we have set up a sustained delivery detection system based on the rate of death of *Escherichia coli* K12. Our results show that the ionic association between oleylamine and ampicillin could be used to sustain the release of this antibiotic in aqueous media. Finally we also evaluate the possibility of AMP-OLEY and PEN-OLEY exhibit anti-tumor activity. For that purpose we have used two different cell lines, namely breast cancer cells (MFC-7) and Lung cancer cells (NLC-H460). Our results suggest that the viability of both cells lines decrease in the presence of both ionic antibiotics.

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PS3: 46

### **Functional analysis of the uncharacterized *Candida glabrata* drug:H<sup>+</sup> antiporter CgQdr2 (ORFCAGL0G08624g): role in antifungal drug resistance**

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In recent years, an increase in the emergence of resistance to antifungal drugs was observed, posing as a severe clinical problem. Multidrug resistance (MDR) often results from the action of drug efflux pumps from the ABC (ATP-Binding Cassette) and MFS (Major Facilitator Superfamily) superfamilies. However, the role of the putative drug:H<sup>+</sup> antiporters from the MFS [1] in pathogenic yeasts has largely escaped characterization. In this work, the function of the uncharacterized ORF *CAGL0G08624g*, encoding a putative drug:H<sup>+</sup> antiporter from the pathogenic yeast *Candida glabrata*, was examined. Using a GFP fusion, this protein was found to be targeted to the plasma membrane in *S. cerevisiae* and *C. glabrata*. The effect of its functional expression in *S. cerevisiae* and *C. glabrata* especially in the resistance to antifungal drugs was inspected. This ORF was seen to confer multidrug resistance, being largely involved in the resistance to azoles, such as clotrimazole, tioconazole and miconazole. Also, and similarly to its close homolog from *S. cerevisiae*, the *QDR2* gene [2], ORF *CAGL0G08624g* was seen to confer resistance to the antimalarial and antiarrhythmic drug quinidine. Its transcript levels were seen to be up-regulated in *C. glabrata* cells in the presence of both clotrimazole and quinidine, in the dependency of the CgPdr1 transcription factor, the major regulator of MDR in this pathogenic yeast. The role of this transporter in drug efflux will be discussed.

[1] Sá-Correia et al., 2009, *Trends Microbiol*, 17, 22-31. [2] Vargas et al., 2007, *Eukaryotic Cell*, 6, 134-142.

PS3: 47

### Antimicrobial resistant characterization of Methicillin-Resistant Staphylococci from animal origin

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The widespread use of antimicrobial compounds has led to the emergence of resistant staphylococci. Two years after the introduction of methicillin into clinical practice, the first methicillin-resistant *Staphylococcus aureus* (MRSA) appeared in a British hospital. A few years later, the first MRSA in animals was reported, obtained from mastitic cow milk. Nowadays, MRSA is a common nosocomial pathogen responsible for severe infectious both in humans and animals. Recently, Methicillin-Resistant *Staphylococcus epidermidis* has emerged as an important agent of nosocomial infections. The present work performed the antimicrobial resistance characterization of methicillin-resistant staphylococci (n=26) by the disk diffusion method (DD) according to the Clinical and Laboratory Standards Institute (CLSI). Isolates were obtained from clinical samples (cats, n=3; dogs, n=3; bovine mastitis, n=20). Species tested included *Staphylococcus aureus* (n=5), *S. epidermidis* (n=15) and *Staphylococcus* spp. (n=6), isolated by conventional microbiological procedures. Isolates identification was performed using biochemical galleries and confirmed by Multiplex PCR. Methicillin resistance was screened by DD using oxacillin and cephoxitin. Presence of *mecA* was detected by PCR. Antimicrobials tested included compounds commonly used in veterinary and human medicine belonging to several classes, such as aminoglycosides (n=6), carbapenems (n=1), first generation cephalosporins (n=2), second generation cephalosporins (n=1), third generation cephalosporins (n=2), glycopeptides (n=1), lincosamides (n=2), macrolides (n=1), penicillins (n=3) and penicillin combinations (n=1), fluoroquinolones (n=4), oxazolidinones (n=1), phenicols (n=1), sulphonamides (n=1), tetracyclines (n=1), and others (n=1). All discs were purchased from Oxoid. Antimicrobial resistance ranged from 0% (Chloramphenicol, Imipenem, Vancomycin) to 100% (Cloxacillin). None of the isolates was susceptible to all antimicrobials tested, while most showed multi-resistance profiles. Although staphylococci resistance to newer drugs is still low, one MRSA tested was also linezolid resistant. The high antimicrobial resistant levels observed suggest that methicillin-resistant staphylococci from animal origin may represent a serious problem regarding public health, rising concerns about human and animal health safety. Multi-resistant staphylococci isolates represent a complex challenge to human and veterinary medicine.

PS3: 48

### Effectiveness of prevention and control measures of multidrug-resistant microorganisms infections

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Infections caused by multidrug-resistant microorganisms are a major problem in the healthcare provider services. Our study aimed to characterize the a multidrug-resistant microorganisms infections (MRMI) and evaluate the impact of Healthcare-Associated Infections (HCAI) reduction measures taken by the Infection Control Committee (ICC) of Centro Hospitalar Médio Ave - Unidade de Famalicão, EPE (CHMA-UF, EPE) on MRMI frequency. We conducted a cross-sectional study. The sample included all positive bacteriological tests for MRMI diagnosis: Methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp., from Medicine, Surgery and Orthopedics Services, analyzed between January 2006 and December 2010. There were registered 1088 MRMI among the total of 25264 Medicine, Surgery and Orthopaedics Services hospitalizations. During the study time, the number of MRMI decreased while the number of hospitalizations increased slightly. Despite 47% of hospitalizations occurred in Surgery, 78% of MRMI occurred in Medicine Service. In the two years prior to the implementation of measures to reduce the number of HCAI, there were a total of 694 MRMI, being MRSA and *Pseudomonas* spp. the most frequent (48% and 28%, respectively). Afterwards, there were 231 infections, of which 59% caused by MRSA and 27% by *E. coli* spp.. Regarding the MRMI, we verified a significant prevalence reduction among hospitalized patients, and currently Medicine continues to have higher prevalence of infection (5 in every 100 hospital patients). The adopted infections prevention and control measures had a strong impact, reducing of about 1/3 of the multidrug-resistant infections. Medicine was the Service that registered as greater decrease in MRMI. The microorganisms with the highest decrease of infection cases were MRSA and *Pseudomonas* spp. MRMI considered epidemiologically important in this study are among the most studied in other publications on MRMI and HCAI. The inverse proportion between the number of hospitalizations and the number of infections shows to the effectiveness of implemented infection prevention and control measures. Upon the results of this study, we concluded that the implemented plan for prevention and reduction of HCAI in CHMA-UF reduced considerably MRMI frequency.

PS3: 49

### **Efficacy studies of a *S. epidermidis* bacteriophage against stationary and biofilm cells**

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*Staphylococcus epidermidis* has recently become known as a common cause of nosocomial infections, predominantly in patients with indwelling medical devices. Although, *S. epidermidis* infections only rarely develop into life-threatening diseases, they are very frequent and difficult to treat due to the ability of this bacterium to adhere to the surfaces of indwelling medical devices and form biofilms. A biofilm is a three-dimensional microbial structure consisting of a multicellular community composed of cells embedded in a matrix composed, at least partially, of material synthesized by the sessile cells in the community. When *S. epidermidis* cells are in a biofilm they are more resistant to antibiotics and to the immune system. The importance of biofilms in the pathogenesis of the *S. epidermidis* infections is becoming more understandable, consequently several studies are needed, in order to develop effective methods for biofilm control. The use of (bacterio)phages to eradicate biofilms has been considered a potentially valuable approach. Phages are virus that infect bacteria and are the most abundant organisms on Earth. They are generally very efficient antibacterial agents and possess many advantages over antibiotics. Our aim is to search for virulent phages with broad host range for *S. epidermidis* biofilm therapy. Using wastewater treatment plants raw effluents we were able to isolate 5 phages. Their activity against 40 clinical *S. epidermidis* isolates with different genetic profiles was screened and was found to be different ranging from 46% to 95% of positive results. Further morphologic and genetic characterization of these isolated phages is now being performed. Preliminary results show that, one of the phages (phiIBB-Se1), using a MOI of 1 is able to cause a 6 Log CFU/ml reduction of the cell titre in <2h for some of the clinical strains at exponential phase and in <4h for stationary phase cells. This phage has also the capacity of reducing by up to 2 Log CFU/ml 24h biofilm cells. These are promising results, since phage phiIBB-Se1 presents a broad host range and ability to control *S. epidermidis* under different metabolic states. Ongoing studies are being performed with 4 other phages, with the purpose of developing a phage cocktail to be used against *S. epidermidis* biofilm infections.

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PS3: 50

### Emergence of multiple carbapenem resistant clinical isolates from different *Enterobacteriaceae* species and widespread clones

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The emergence of multiple ertapenem resistant *Enterobacteriaceae* isolates (*Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter asburiae*, *Enterobacter aerogenes* and *Enterobacter cloacae*) presumptively identified as carbapenemase producers in a Portuguese hospital was studied (March–July 2010). Characterization of antibiotic susceptibility patterns,  $\beta$ -lactamases, population structure (PFGE, MLST), plasmid content and major enterobacterial porins was performed in ertapenem resistant (ERT-R, n=15) and also ertapenem susceptible (ERT-S, n=13) strains identified in the same period and hospital wards. Although acquired carbapenemases were not detected, deficiencies in major porins were identified. Most isolates were CTX-M-15-producing ERT-R (n=8, 2 PFGE-types, 1 outbreak variant) and ERT-S (n=10, 1 PFGE-type) ST15 *K. pneumoniae* or B2-ST131-*E. coli* (n=1 ERT-R, n=3 ERT-S; 4 PFGE types). *bla*<sub>CTX-M-15</sub> was identified in variable plasmid types (IncFII in *E. coli* and IncR in *K. pneumoniae*) and genetic contexts. Sporadic ST14 and ST45 *K. pneumoniae* and D-ST354 *E. coli* ERT-R clones were also identified. *K. pneumoniae* ERT-R strains contained non-sense mutations (*ompK35*, *ompK36*; MIC<sub>ERT</sub>=12–32  $\mu$ g/ml), IS1-mediated porin disruptions (*ompK36*, MIC<sub>ERT</sub>=12  $\mu$ g/ml) or alterations in the L3 Loop (*ompK36*, MIC<sub>ERT</sub>=4–16  $\mu$ g/ml). Porin changes were also observed in ACT-4-producing *E. asburiae* (n=1, MIC<sub>ERT</sub>>32  $\mu$ g/ml), TEM-24-encoding *E. cloacae* (n=1, MIC<sub>ERT</sub> = 4  $\mu$ g/ml), *E. coli* and *E. aerogenes* (MIC<sub>ERT</sub> = 2–>32  $\mu$ g/ml). We describe the emergence of multiple ERT-R *Enterobacteriaceae* species and clones in a short period of time with serious impact in clinical outcomes. The emergence and the nosocomial spread of ERT-R widespread *E. coli* (ST131) and *K. pneumoniae* (ST15) clones deserve closer attention due to the risk of further expansion of carbapenem-resistant bacteria.

PS3: 51

### High-throughput methodologies for tracing *Acinetobacter baumannii*

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*Acinetobacter baumannii* (Ab) are, nowadays, a relevant public health issue due to the incidence and epidemicity of multidrug-resistant (MDR) strains of this species. The development of rapid, effective and low-cost typing methodologies is a priority for promptly contain the spread of infectious agents and namely of MDR *Acinetobacter* isolates. Spectroscopic typing methods are, when compared with traditional genotypic methods, less time consuming, free of reagents, quickly and easy to execute. In this work we assessed Fourier Transform Infrared Spectroscopy (FTIR) for differentiation of carbapenem-resistant Ab clinical isolates. Representative isolates (n=22) of disseminated Ab lineages producing acquired carbapenem-hydrolysing carbapenemases (ST92 producing OXA-23, ST98 producing OXA-40 and ST103 producing OXA-58) were selected from a collection of hospital isolates (1995-2008). The acquired spectra were analysed with the support of chemometric methods. Performing a Partial Least Square Discriminant Analysis (PLSDA) 3 clusters were obtained, one with ST-92-OXA-23 isolates and the other two, less distinguishable from each other, comprising ST98-OXA-40 and ST103-OXA-58 isolates. The discrimination obtained with a spectroscopic methodology combined with chemometry was able to differentiate the carbapenem-resistant Ab lineages spread in Portugal. FTIR ability to discriminate the MDR Ab lineages associated with infections in Portugal, strengths the potential of this quick and environmentally friendly method for epidemiological bacterial purposes.



PS3: 52

### Molecular and phenotypic characterization of the emerging *Salmonella enterica* serotype 4,[5],12:i:-

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*Salmonella enterica* serotype 4,[5],12:i:-, a monophasic variant of serotype *S. typhimurium* (4,[5],12:i:1,2), has become in recent years one of the most common serotypes associated with human infection transmitted throughout food chain and with worldwide distribution. We intended to implement and validate a genotypic methodology for identification of this serotype, to characterize phenotypic and genotypic resistance to antibiotics and to assess the clonal relationship among the isolates obtained in Portugal. The study included 165 isolates serologically identified as *S. 4,[5],12:i:-* (2002-2010): human (n=146), food (n=10), environment (n=5) and piggeries (n=4). The serotype was confirmed by PCR (gene *fljB* and intergenic region *fljB-fljA*). Antibiotic susceptibility to 11 antibiotics was tested by disk diffusion method (CLSI). Characterization of antibiotic resistance genes, search of  $\beta$ -lactamases and class 1 integrons was performed by PCR, PCR-RFLP and/or sequencing. Clonality was established by *Pulsed Field Gel Electrophoresis* (PFGE) and *Multilocus Sequence Typing* in representative isolates. Only 132 isolates were confirmed as *S. 4,[5],12:i:-* and selected for further studies. All but 2 isolates (99%) were resistant at least to one antibiotic and 93% were multidrug resistant (MDR, 2-8 antibiotics). The major resistance was expressed to sulfamethoxazole (Su/92%), tetracycline (T/91%), streptomycin (S/88%), ampicillin (A/67%), chloramphenicol (C/45%), trimethoprim (Tr/35%), and gentamicin (G/27%). The study of clonal relationships by PFGE, identified nine clones associated with MDR profiles and different genotypes. Three major groups were associated with three different genotypes: i) ASSuT profile (n=48; *blaTEM*, *strA-strB*, *sul2*, *tetB*) associated with 4 clones and ST34 (SLV of ST19); ii) MDR profile (n=45), most of them ACGSSuTTr phenotype (n=27; *blaTEM*, *cmlA1*, *aac(3)-IV*, *aadA*, *sul1-sul2-sul3*, *tetA*, *dfrA12*), belonging to clone O/X, widespread ST19 and phage type DT104/U302 iii) CSSuTTr profile (n=15; *cmlA1*, *aadA*, *sul3*, *tetB*, *dfrA12*) associated with a clone and ST19. This was the first study characterizing isolates *S. 4,[5],12:i:-* serotype in Portugal. Of concern is the detection of three MDR genotypes prevalent in humans, foods of animal origin and pig farms. The resistance to multiple antibiotics and strains belonging to globally disseminated clonal lineages could explain the recent emergence/persistence and success of this serotype.

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PS3: 53

### **Pulmonary co-infection by *Burkholderia dolosa* and *Burkholderia cenocepacia* and clonal variation during long-term colonization of a cystic fibrosis patient**

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The *Burkholderia cepacia* complex (Bcc) is a group of genetically distinct but phenotypically similar bacteria that comprises 17 recognized species. These bacteria are the most threatening pathogens in Cystic Fibrosis (CF), contributing to a more rapid decline in lung function that, in some cases, culminates in a fatal necrotizing pneumonia known as the "cepacia syndrome". Epidemiological surveys carried out in several countries indicate that all the Bcc species can be recovered from respiratory secretions of CF patients but *B. cenocepacia* and *B. multivorans* are predominant. A remarkable exception to these studies is the result of the epidemiological analysis carried out by our laboratory in the major Portuguese CF Center at Santa Maria Hospital (HSM), in Lisbon, where an exceptionally high representation of *Burkholderia cepacia* occurs [1-2]. The systematic and longitudinal study of this CF Portuguese sub-population during the last 16 years also indicate that around 70% of the patients infected with Bcc species are persistently colonized. In the present study we carried out the molecular identification and the phenotypic characterization of 18 sequential isolates recovered at HSM, from sputa of a CF patient chronically colonized for 5.5 years, until the patient's death following severe pulmonary deterioration. *B. dolosa* was found to be involved in chronic colonization during the whole period of infection. A transient co-infection with *B. cenocepacia* (*recA* lineage III-B) during three months was also registered. The prevalence of *B. dolosa* in CF patients worldwide is low and cases of co-infection involving *B. dolosa* and *B. cenocepacia* were never reported. As recently described for *B. cenocepacia* [3], phenotypic clonal variation during long-term infection was demonstrated based on the systematic comparison of phenotypes of *B. dolosa* serial isolates. Clonal expansion found to occur during long-term colonization involves the alteration of a number of phenotypes relevant in bacterial pathogenesis. In particular, the antimicrobial susceptibility and swarming motility of the first isolate retrieved from the patient, and thought to have started the infection, exhibiting consistently higher values compared with those for the isolates obtained during the course of infection, antimicrobial therapy and disease progression.

[1]. Cunha et al. 2003. *J Clin Microbiol*, 41: 4113. [2]. Cunha et al. 2007. *J Clin Microbiol*, 45: 1628. [3]. Coutinho et al. 2011. *Infect Immun*, 79: 2950.

PS3: 54

### The role of *Candida* species in colonization, invasion and damage of an *in vitro* reconstituted human oral epithelium

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Oral candidosis is a common problem in immunocompromised patients, and whilst *Candida albicans* is regarded as the principal cause of infection, other *Candida* species are increasingly being recognized as human pathogens. Moreover, relatively little is known about the role of *Candida* species in oral infections. Thus, this work aimed to examine *Candida* species infection of oral epithelium, and to assess their ability to colonize, invade and damage an oral epithelium. The ability of *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* to colonize and invade a reconstituted human oral epithelium (RHOE) was examined by confocal laser scanning electron microscopy (CLSM). Simultaneously, the levels of lactate dehydrogenase (LDH) release by the epithelium cells were determined to assess the extension of tissue damage. A comparison of *Candida* species was made in terms of secreted aspartyl proteinase (SAP) gene expression. CLSM images showed that all *Candida* species were able to colonize RHOE however this was in a species dependent manner. Low invasion of RHOE occurred with *C. parapsilosis* cells after 12h, whereas extensive tissue damage was evident after 24h when assessed by histological examination and LDH determination. Conversely, *C. tropicalis* and *C. albicans* cells exhibited higher tissue invasion after 12h, with extensive tissue damage occurring at 24h. Molecular analysis of SAP gene expression, for *C. tropicalis* and *C. parapsilosis*, suggested that Saps are not involved in invasion. In addition, pepstatin A (Sap Inhibitor) was unable to inhibit the invasion of RHOE by both species. Furthermore, after 24h of infection it was evident that a reduction of tissue damage occurred in case of *C. parapsilosis*, but not in case of *C. tropicalis*. These findings suggest that Saps could play an important role in tissue damage induced by *C. parapsilosis*. *C. glabrata* single infection studies revealed no invasion of the RHOE. Moreover, mixed infections showed that *C. albicans* enhanced the invasiveness of *C. glabrata*, and led to increase of LDH released by the RHOE, which paralleled the observed histological damage. Overall, this work demonstrated that Saps are not involved in the invasion of RHOE by *C. tropicalis* and *C. parapsilosis* but seems to be highly responsible for tissue damage for *C. parapsilosis*. In addition we were able to demonstrate that *C. glabrata* alone is not able to invade RHOE however in presence of *C. albicans* causes significant tissue damage.

PS3: 55

### Towards the understanding of *Coxiella burnetii* impact in public health: PCR diagnostic of Q-fever in animals

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Q fever, caused by *Coxiella burnetii*, is a zoonosis with a worldwide distribution and an emerging impact in Public Health: recently (2007-2009), a large rural area in the southeast of the Netherlands was heavily affected by the disease. Domestic ruminants are regarded as one of the key-elements in disease epidemiology. The animals usually present asymptomatic chronic infections but shed bacteria in feces, milk and vaginal mucous, mainly during birth or abortion that constitutes one clinical alert of suspected infection. Notification of Q fever in humans is mandatory in Portugal since 1999, but the source and route of infection of *C. burnetii* and its importance in Public Health remains unevaluated. Some evidences already exist for the occurrence of infection both in zoo (1) and domestic animals (2). PCR assays for the detection of *C. burnetii* DNA in veterinary matrices of suspected Q-fever affected animals are, therefore, pertinent to get information about the incidence of the disease and the risk of transmission to humans. Screening for *C. burnetii* DNA was performed in this work, by nested PCR (3), in lung and/or liver of small ruminants, with undefined cause of death, and placenta, aborted fetus and still birth of bovine and small ruminants, in cases of abortion. These samples were received at National Veterinary Research Laboratory for routine laboratory diagnosis. *C. burnetii* co-infection with *Chlamydophila abortus* was observed in two bovine fetus, seeming to be the cause of abortion, since no other bacteriological, virological or parasitological suspected pathogen was detected. This co-infection was also observed in a goat fetus/placenta, and in lung/liver from two other small ruminants and also in a black-face impala from the Lisbon Zoo. However, there were cases where the cause of abortion seemed to be due to *C. burnetii* alone, since *Chlamydophila*, *Salmonella* or *Brucella* were not detected. This was the case of three goat still births from the same herd, and two fetus from goat and sheep, respectively. The lack of awareness for Q-fever in Portugal is remarkable, leading to an underestimation of the real number of cases both in animals and humans. This results in economic losses in animal production and increased public health risks. This work represents one of the first steps to invert this situation, looking at the two counterparts, human and animal health, as one single health.

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PS3: 56

**Antibacterial activity of crude methanolic extract and fractions obtained from *Larrea tridentata* leaves****Silvia Martins<sup>1</sup>, Solange I. Mussatto<sup>1</sup>, Cristóbal N. Aguilar<sup>2</sup>, Elba Amorim<sup>3</sup>, Tadeu Peixoto<sup>3</sup>, Antonio Saraiva<sup>4</sup>, José A. Teixeira<sup>1</sup>**

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*Larrea tridentata* (Zygophyllaceae), commonly known as creosote bush, is a plant that grows in semiarid areas of Southwestern United States and Northern Mexico and was traditionally used for medicinal purposes. This plant is a notable source of natural compounds with approximately 50% of the leaves (dry weight) being extractable matter. This study was designed to evaluate the antibacterial activity of the crude methanolic extract (CME) and fractions [hexane (H), dichloromethane (DCM), ethyl acetate (EA) and ethanol (Et)] obtained from *Larrea tridentata* leaves. A preliminary study of the antibacterial activity was performed using the agar diffusion method against six strains of Gram-positive and Gram-negative bacteria. The micro-dilution method was applied for the determination of the minimal inhibitory concentration (MIC) of selected bacteria strains. HPLC analyses of tested samples were also carried out. The antibacterial activity of the tested samples was noticeable more effective inhibiting the growth of Gram-positive bacteria comparing with Gram-negative bacteria, by the CME, DCM and EA fractions. EA fraction showed the highest antibacterial activity against methicillin-resistant *Staphylococcus aureus* isolated from secretion; with a MIC value (31.3 µg/mL) lower than the reference antibiotic tetracycline (64 µg/mL). Low MIC values (62.5 µg/mL) were also obtained for crude methanolic extract and DCM fraction compared to tetracycline. The highest concentrations of quercetin, kaempferol and nordihydroguaiaretic acid were observed in CME (8.67, 21.52 and 35.75 mg/g, respectively); nevertheless, EA fraction also showed considerable levels of these compounds compared with the remaining fractions. Other compounds were observed in the HPLC chromatograms and further studies are needed in order to identify them. The antibacterial activity of the samples studied might be explained by the synergistic or additive effects of several components rather than arising from a single compound. In conclusion, EA fraction showed the most promising results against the bacterial strain methicillin-resistant *Staphylococcus aureus*, which represents an important step for the search and development of a new antibacterial agent. Nevertheless, further toxicological and pharmacological studies are needed in order to confirm the hypothesis of using phytochemicals from *Larrea tridentata* leaves.

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PS3: 57

### Antimicrobial resistance in Gram-positive cocci from the skin and oral microbiota of East Timor individuals

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Characterization of zoonotic bacteria antimicrobial resistance traits is of major importance for assuring the safeguarding of health in the wider concept of "one health". Nevertheless, the role of individuals from developing countries as potential reservoirs and vehicles for bacterial virulence dissemination is yet to be established. We aimed at characterizing the antimicrobial resistance profile of Gram-positive cocci (GPC) from the skin and oral microbiota of East Timor persons without antibiotherapy reported. Skin and oral swab samples were taken from 74 individuals in Dili, East Timor, in July and August 2006. Bacteria were isolated according to conventional microbiological procedures. GPC were identified according to biochemical characteristics. Antimicrobial resistance was determined by the disk diffusion method (Clinical and Laboratory Standards Institute guidelines), for amikacin (AK 30µg), clindamicin (DA 2µg), erythromycin (E 15µg), gentamicin (GN 10µg), norfloxacin (NOR 10µg), penicillin P (P 10µg), sulphamethoxazol + trimethoprim (STX 25µg), tetracycline (TE 30 µg) and vancomycin (VA 30µg). Macrolide resistance phenotypes (M and MLS<sub>B</sub>) were detected by double disk test with E and DA. GP and catalase-negative cocci (n=6) were typed by Smal-pulsed-field gel electrophoresis (Smal-PFGE). We obtained a total of 507 isolates (oral n=228, and skin n=279), from which Gram-positive (n=327) and Gram-negative (n=180) bacteria. GPC (n=37, 22 oral and 15 skin isolates) were identified as micrococci, enterococci, staphylococci, aerococci, *Kocuria* and streptococci. The majority of the CPC isolated showed a broad susceptibility to antimicrobials. Oral isolates were mostly susceptible to GN (80%) and VA (87%), and the skin isolates to GN (82%), NOR (77%) and TE (82%). Resistance to E was 36% and 40% and resistance to DA was 59% and 87% in the oral and skin GPC, respectively. E resistant isolates were of the M (n=3) or MLS<sub>B</sub> (n=11) phenotypes. Multiresistance, although not common, was observed among the six genera. Two *Enterococcus faecium* shared a same Smal-PFGE pattern, two *Streptococcus dysgalactiae* isolates were unrelated and two *Aerococcus* spp. isolates were nontypeable by Smal-PFGE. Our results highlight the importance of individuals from developing countries as vectors for resistant bacteria dissemination and for the fact that antimicrobial resistance in these countries may be significant, particularly to macrolides, glycopeptides or quinolones.

PS3: 58

**Antioxidant properties and hepatoprotector effect of extracellular glycopeptides complexes from *Trametes versicolor* cultures****Rita Sofia Garcia Ribeiro<sup>1</sup>, José Sampaio<sup>1</sup>, Ana Teresa Caldeira<sup>1,2</sup>, Maria de Fátima Candeias<sup>1,3</sup>, Cátia Salvador<sup>1,2,3</sup>, José Arteiro<sup>1,2</sup>, Maria do Rosário Martins<sup>1,3</sup>**<sup>1</sup>Departamento de Química da Escola de Ciências e Tecnologia, Universidade de Évora, Portugal;<sup>2</sup>Centro de Química de Évora, Universidade de Évora, Portugal; <sup>3</sup>Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Portugal

In recent years, biological and pharmacological properties of compounds obtained from some *White Rot Fungi*, namely *Trametes versicolor*, have received great attention from the scientific community. Although some recent studies focus on the therapeutic effects of the compounds extracted from these mushrooms, little information about its antioxidant and protective properties are available. The main objectives for this work were to produce and evaluate biological properties of the extracellular protein-polysaccharide complexes (EPS) extracted from *T.versicolor* cultures. Previous studies with protein-polysaccharide complexes obtained from *T.versicolor* cultures with different waste residue as carbon source showed that these complexes have important antioxidant properties [1]. In this study, submerged fermentation cultures were carried out in a liquid medium containing sunflower waste as carbon source under orbital shaking at 25°C, during 14 days. After that, protein-polysaccharide complexes were extracted from supernatant (EPS) and lyophilized. The lyophilized EPS extracts showed antioxidant activity either by DPPH<sup>•</sup> radical method ( $0.402 \pm 0.054$ mg ascorbic acid/mg polysaccharide) and  $\beta$ -carotene/linoleic acid system ( $54.845 \pm 0.001$ mg ascorbic acid/mg polysaccharide). Moreover, the EPS extracts showed ability to mimic the superoxide dismutase (SOD) and catalase (CAT) enzyme activities, with SOD activity of  $54.845 \pm 0.10$ U/mg EPS and CAT activity of  $1.44 \pm 0.14$ U/mg EPS. Additionally, the hepatoprotector effect of EPS freeze-dried extracts and dried mycelium was evaluated, by oral administration in *Wistar* rats, with the determination of AST, ALT, ALP and  $\gamma$ -GT serum enzyme activities. Results showed that oral administration of EPS extracts and dried mycelium seems to indicate reduction of liver damage induced by ethanol-administration, presenting a hepatoprotective activity equivalent to that observed for the standard drug, *silymarin*. Based on these studies, the protein-polysaccharide complexes produced by liquid cultures of *T.versicolor* may be an important source of bioactive compounds with great potential medicinal value and important biotechnological applications.

[1] Arteiro et al (2011). *Medicinal Chemistry Research*, DOI: 10.1007/s00044-011-9604-6.

PS3: 59

### Children faecal colonization with *Stenotrophomonas maltophilia* in the North of Portugal

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Our previous work on detection and characterization of extended-spectrum-beta lactamase (ESBL) producing *Enterobacteriaceae* in the faecal flora of children attending day-care centres (DCC), in Northern Portugal, showed that some children were colonized with carbapenem resistant non fermenters. The main purpose of this work, was the characterization of these isolates. Fresh nondiarrheal stools of children from few months to 6 years old, attending two DCC, from the North of Portugal, were collected from April to July 2009. Faecal samples were suspended in BHI. Isolates were selected in MacConkey agar with aztreonam (2mg/l) in one case and in the other two cases, in MacConkey agar with imipenem (1mg/l). Non fermenter colonies were randomly selected and susceptibility to antimicrobial agents was determined by agar diffusion methods according to the CLSI. Identification of the selected carbapenem resistant isolates was achieved by ID 32 GN. Of 105 faecal samples of children attending two DCC in the North of Portugal, we found 3 children showing faecal colonization with carbapenem resistant *Stenotrophomonas maltophilia*, from one centre. Our results showed 3 young children colonized with carbapenem resistant *Stenotrophomonas maltophilia*. This is a ubiquitous organism associated with opportunistic infections showing intrinsic resistance to many antimicrobials, including carbapenems. This finding alerted us for the risk of infection by this carbapenem natively resistant opportunistic pathogen and also to the hypothesis of "in vivo" acquisition of resistance mechanisms by other opportunistic bacteria colonizing the gut of these children, influencing antimicrobial resistance of the enteric flora. These children might constitute a dissemination focus of this resistant threat and an infection risk to at-risk patient populations. This drug-resistant potentially pathogenic bacteria, underrecognized as a reservoir of resistance to therapeutically important antimicrobial agents, might be excreted by some people, despite the lack of exposure to the particular drugs. The sources of this resistant organism in children faecal microflora, are unknown. The 3 Kids are from the same institution, what alerted us to the hypothetical cross contamination of children by DCC routine procedures. This potential pathogen might pose public health and infection control unexpected challenges, namely in this young healthy population.



PS3: 60

### Dynamics of subclinical bovine mastitis due to non-aureus staphylococci on 4 farms

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Bovine mastitis is the inflammation of the bovine mammary gland, most often of infectious origin. It is the most costly disease and the first reason for antimicrobial use in dairy cows. Non-aureus staphylococci (NAS) are the most frequently isolated bacteria from bovine milk in several studies worldwide, but the impact of individual staphylococcal species on udder health is not well characterised. The objectives of this study were to compare the impact of different NAS species on udder health measured in terms of number of inflammatory cells (somatic cell count, SCC) present in the milk and duration of intramammary infection. The strain diversity was also compared between farms to try to gain some insight into most likely forms of infection (contagious or environmental). This longitudinal observational study was performed on 4 farms that were sampled at 4 week intervals for a total of 12 visits each. Quarters infected with NAS were followed through time with milk samples being submitted for bacteriological culture and SCC determination. PCR amplification of the internal transcribed spacer region and sequencing of the *sodA* and *rpoB* genes were used for species allocation. Pulsed-field gel electrophoresis was performed to assess strain identity. The percentage of quarters affected per farm varied between 35 and 6%, with the most frequently isolated NAS species being *S. epidermidis*, followed by *S. simulans*, *S. chromogenes* and *S. haemolyticus*. It was possible to follow 111 intramammary infections due to NAS through time. Duration of infection had a mean of 188 days and was not significantly different between NAS species. Geometric mean quarter SCC overall was 132.000 cells/ml and was also not significantly different between NAS species. Estimation of the Simpson diversity index showed significant differences between NAS species when all farms were considered, with *S. epidermidis* and *S. simulans* showing less diversity than *S. haemolyticus*. Comparing *S. epidermidis* diversity between 2 of the farms showed a significant difference, whereas comparing *S. simulans* diversity between the same 2 farms showed no difference. Despite the possibility of a different epidemiology of infection, the impact in terms of udder health seems to be similar for different NAS species. It remains to be clarified if certain species are more likely to behave in an environmental or contagious way, or if these characteristics of infection are mainly a feature of each farm.

PS3: 61

**First portuguese case report on sporotrichosis caused by *Sporothrix mexicana*****Nicolina Dias<sup>1,2</sup>, Manoel Oliveira<sup>3</sup>, Manuel Portela<sup>2</sup>, Cleidir Santos<sup>1</sup>, Rosely Zancope-Oliveira<sup>3</sup>, Nelson Lima<sup>1</sup>**<sup>1</sup>IBB/Centre of Biological Engineering, University of Minho, Braga, Portugal; <sup>2</sup>CITS - Centro de Investigação em Tecnologias da Saúde, CESPU, Paredes, Portugal; <sup>3</sup>Laboratório de Micologia, Fundação Oswaldo Cruz, RJ, Brazil

Sporotrichosis is a worldwide subcutaneous fungal infection caused by a traumatic inoculation or inhalation of spores of the dimorphic fungus *Sporothrix schenckii*, which natural habitat is soil and plants. Previous molecular studies revealed that this species constitutes not a single species but a complex of several cryptic species, genetically different. Distinct phenotypic characters, pathological behavior and different antifungal responses suggest that the appropriated treatment depends on the strain which caused the infection. We report, in this work, the first isolation of *Sporothrix mexicana* on human from a Portuguese patient. A 34 year-old male sought care at a podiatry clinic in Vila Nova de Famalicão, Portugal, in 2009 for multiple polymorphous eruptions and ulcers in both feet. He referred working in Malaysia in 2003 wearing open footwear, although he did not recall receiving a skin wound. Back to Portugal in 2004 subcutaneous nodules in both feet ulcerated, and spontaneously healed. He claimed for chronic infection in both feet and lower limbs since 2005 when more severe lesions had appeared. Fragments of skin were collected from lesions and submitted for mycological assessment. Filamentous fungus with hyaline septate hyphae, hyaline and dematiaceous conidia compatible with *Sporothrix* sp was microscopically observed. The isolate was accessed and preserved in the Micoteca da Universidade do Minho (MUM, Braga, Portugal) and given the accession code MUM 11.02. Yeast-like form was achieved incubating the fungus in Brain Heart Infusion Agar in culture slants at 35±2° C. Carbohydrates assimilation pattern was compatible with *Sporothrix schenckii* and *Sporothrix mexicana*. Genomic DNA was obtained from the mycelial phase of MUM 11.02 at Fundação Oswaldo Cruz, RJ, Brazil, and the partial sequencing of the nuclear calmodulin (CAL) gene was based on the amplicon generated by PCR by using CL1 and CL2A primers. A BLAST analysis comparing the sequence of the CAL gene with sequences AM398382/AM398393/AM117444/AM116899/AM116908 in the GenBank database confirmed the isolate identity as *S. mexicana*. MUM 11.02 showed 99% similarity with the sequences of *S. mexicana* with high bootstrap support values. *In vitro* susceptibility testing with antifungals fluconazol, itraconazol and terbinafine revealed MIC 128, 32 and 0.5-1.0 µg/mL respectively. Those results are compatible with *S. mexicana*. This work reports that *S. mexicana* is an emerging cause of human sporotrichosis.

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PS3: 62

### Genetic diversity and natural resistance to Maraviroc in HIV-1 strains circulating among intravenous drug users in the Greater Lisbon

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Maraviroc (MVC) is currently the only CCR5 licensed inhibitor, being active against HIV-1 R5 strains resistant to other antiretrovirals. It binds to a hydrophobic pocket in the transmembrane helices of CCR5 and it is believed to alter the conformation of the extracellular loops that HIV interacts with during coreceptor binding, thus preventing their recognition and subsequent binding by gp120. Several viral mutations have been described, especially located in the gp120 V3 loop, which are somehow associated to MVC resistance. To determine the genetic diversity of the V3 loop of HIV-1 strains circulating in intravenous drug users in the Greater Lisbon, and to describe the presence of natural genetic polymorphisms and their association with MVC resistance, 52 serum samples were studied. Specific viral cDNA fragments, corresponding to the C2V3C3 region of gp120, were amplified by RT-PCR from 35 samples, being subsequently sequenced and genotyped by phylogenetic analysis. The genetic polymorphisms and mutations associated with resistance to MVC were analyzed, after translation of the nucleotide sequences. Coreceptor prediction was also performed resorting to the 11/25 rule and PSSM and Geno2pheno programs. Phylogenetic analysis revealed 23 subtype B sequences (2 possibly CRF14\_BG), 8 subtype A, 3 subtype G and 1 sub-subtype F1. Subtype B sequences revealed high diversity, allowing the distinction of several phylogenetic subgroups. A high frequency of natural genetic polymorphisms was observed, with the C2 region being the most conserved. Several mutations associated with MVC resistance were observed, including some patterns found in resistant strains *in vivo*. The mutation pattern 11S+26V was observed in 2 strains (subtype B, R5) and the pattern 20F+25D+26V in one strain (subtype B, R5). Although the resistance profile for MVC is highly complex and still poorly defined, the presence of these mutations in naïve populations may have important clinical implications.

PS3: 63

### First identification of *Leptospira interrogans sensu lato* in patients of Lobito Region (Angola)

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Leptospirosis, an emerging zoonotic disease caused by pathogenic bacteria from the genus *Leptospira*, occurs in environments of industrialized and developing countries. However, in some regions, the real prevalence is unknown and only the outbreak reports show that leptospirosis is a global and important Public Health problem. In tropical regions, the clinical diagnosis can be problematic, where similar febrile illnesses are common and can be confused with other disorders such as Malaria, Viral hepatitis, Dengue, Typhoid fever, etc. Leptospirosis may represent 20-40% of febrile illness of unknown origin. In Angola, the diagnosis is done only by the patient history and medical suspicion, which can lead to misdiagnosis and may contribute to the increase in antibiotic treatment and resistance in the population. Thus, it is important to increment the Leptospirosis research in these tropical regions, in order to include it in the differential diagnosis of fever diseases, improving the population health. The aim of this preliminary study, the first on human Leptospirosis carried out in Angola, was to investigate the occurrence of disease in Lobito (Benguela Province) and identify the circulating species of *L. interrogans sensu lato* (s.l.), using serological and molecular methods. Serum samples (n=141) were collected from patients admitted in the Lobito General Hospital whose principal signs and symptoms were fever and/or headache symptoms. The serological evaluation was performed, at the local lab, by the Macroscopic Agglutination test (MACRO), and by Microscopic Agglutination Test (MAT). *Leptospira* DNA detection was performed by a PCR targeting the *hap1* gene. MAT and PCR were developed at the Leptospirosis Reference Lab (IHMT). The main clinical manifestations were headache (73,8%), fever (65,2%), myalgia (41,1%) and jaundice (33,3%). Serological results showed the following MACRO reactivities: 14 (10%) positives and 18 (13%) doubtful. Of these samples, five (16%) were confirmed by MAT (presence of agglutinins anti-*L. interrogans* s.l.). In parallel, was detected leptospiral DNA in 13/81 (16%) of sera. Our results showed the unequivocal occurrence of pathogenic leptospirae in Lobito Region, being reasonable to assume a similar situation in other regions of the country. This study is the first of further studies in Angola and in other countries where Leptospirosis situation is not known, putting it on the list of emerging fever illness in tropical regions.

PS3: 64

### Enumeration of major bacterial groups in faecal samples of children with type 1 diabetes

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Type 1 diabetes (T1D) is a T cell mediated autoimmune chronic disease whose incidence is increasing worldwide at an annual rate of around 3–5%, particularly in children under 5 years old. In 2005, in Europe, the number of new cases of T1D in people under 15 years was estimated to be 15 000, and this number is predicted to rise to 24 400 in 2020. Genetic and environmental factors were associated to its pathogenesis. Environmental factors such as various components of the diet, toxic chemicals and virus infection have been investigated. The influence of the gut microbiota in the development of T1D has also caught the interest of some researchers. The main objective of this work was the determination of the load of major bacterial groups that have a recognized role on health, namely *Bifidobacterium* spp., *Lactobacillus* spp., *Escherichia coli*, *Eubacterium rectale*, *Eubacterium eligens* and *Clostridium difficile*, in faecal samples of 15 children with T1D by quantitative real-time PCR, in order to identify possible disorders in these important group of bacteria. Every three months over a period of six months stool samples of each child were collected. Information on diet was collected through a questionnaire. In one sampling period the load of *Bifidobacterium* spp. was significantly higher ( $P < 0.05$ ) in comparison with the control children and no significant differences were observed for the remaining periods. No significant differences ( $P > 0.05$ ) were observed for *Lactobacillus* spp and *C. difficile* during the three sampling collection times. The bacterial load of *E. rectale* and *E. eligens* was significantly higher in comparison with the control children. The percentage of colonization was higher for diabetic children in comparison to the control children for the following bacterial groups: *E. coli*, *Bifidobacterium* spp., *Lactobacillus* spp., *Eubacterium rectale* and *E. Eligens*, and these values were more pronounced in the first sampling collection time for *Bifidobacterium* spp., second collection time for *E. coli* and *Lactobacillus* spp. The enumeration of the target bacterial groups evidence a tendency to the overload of the important gram negative *E. coli* but this group of children may be protected by the presence of *Bifidobacterium* spp. *E. rectale* and *E. eligens*.

PS3: 65

### Natural volatile extracts and terpenic hydrocarbons as potential antimicrobial agents for the treatment of *tinea pedis*.

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Cutaneous mycoses persist in the XXI century, as the most common infections in humans. Some of these infections, such as *tinea pedis*, can often originate chronic conditions whose clinical disorder may lead to relative immobility of the affected limb and consequent inability to perform daily tasks. Occasionally, they can cause serious infections in immunocompromised patients that have increased over the last few decades. Otherwise, increasing resistance of microorganisms to conventional therapy, low efficacy of drugs, side effects and high costs, makes urgent the development of new antifungal agents. Plant extracts, due the diversity and complexity on secondary metabolites, are valuable collections of compounds for screening biological activities. Particularly, extracts from aromatic plants, are used for centuries for their antimicrobial properties. In this study we have evaluated the effect of a selected group of volatile plant extracts and pure natural compounds in order to establish their effect on the growth and viability of dermatophytes *in vitro*. Plant volatile extracts were isolated by hydrodistillation and analysed by GC and GC/MS. The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) were determined against *Trichophyton rubrum* (ATCC MYA-4438) and *Trichophyton mentagrophytes* (ATCC MYA-4439). According to the determined MIC and MLC values, dermatophytes were sensitive to some of the tested extracts, namely from *Juniperus* sp. and *Lavandula* sp, and isolated compounds, monoterpenes mainly (MIC and MLC values ranged from 0.1 to 1.2 mg mL<sup>-1</sup>). Results confirmed the huge potential of these natural products as antifungal agents and one of the most promising groups of natural products for the development of broad-spectrum, safer and cheaper antifungal agents.

PS3: 66

**Optimization of a protocol for gene expression using biofilm cells from *S. epidermidis*****Ana Isabel Freitas<sup>1</sup>, Ana Filipa Henriques<sup>1</sup>, Ângela França<sup>1</sup>, Carlos Vasconcelos<sup>2</sup>, Manuel Vilanova<sup>3</sup>, Nuno Cerca<sup>1</sup>**

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Gene expression assays are one of the most common tools used nowadays to evaluate the importance of genes in many different life sciences areas, namely, in clinical microbiology. Since most gene expression kits for qPCR have been optimized for assays with planktonic cells it is important to also optimize protocols for this type of assays, to be used with biofilms. Biofilms are communities of bacteria that grow attached to a surface and embedded in an extracellular matrix, what poses some difficulties to RNA extraction. Proper RNA quality is of the utmost importance during all the downstream processes, namely cDNA synthesis and qPCR quantification. The aim of this work was to optimize a protocol for gene quantification from biofilm samples of *S. epidermidis*, a known biofilm forming nosocomial pathogen. This optimization was made in many different steps, from the RNA extraction (a crucial step) to complementary DNA (cDNA) synthesis and qPCR reactions, using growth conditions well described in the literature, so that the results obtained could be anticipated beforehand. The expression of the *icaA* gene was tested from RNA extracted with a custom made protocol and then quantified using a combination of 4 commercial kits of cDNA synthesis and 4 commercial kits of qPCR quantification. Furthermore, the volumes of reaction were either the volume recommended by the manufacturer (20 µl) or half that volume. From our results, we conclude that there were no significant differences of *icaA* expression when using any of the qPCR kits used in this study. However, using different cDNA synthesis kits, a statistical difference was found in the results obtained using one of the kits, with an *icaA* expression near 4-fold different than that obtained using the other kits. Interestingly, the 10 µl reaction generally resulted in higher *icaA* expression than when using the 20 µl reaction volume, but within the expected range of values, indicating that any of the two volumes could be used for quantification studies. Excluding the cDNA kit with low *icaA* levels expression, the average of *icaA* expression induced by glucose was similar in both cDNA and qPCR optimization steps (9.5 and 9.4 fold, respectively). The obtained protocol provides reliable results, comparable to the ones in literature, with the advantage of saving reagents. Furthermore, our results confirm that cDNA synthesis is a more crucial step than previous thought.

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PS3: 67

**PCR diagnosis of *tinea unguium* with specific detection of *Trichophyton rubrum* on clinical samples****Nelson Linhares<sup>1</sup>, Cláudia Escórcio<sup>2</sup>, Nicolina Dias<sup>2,3</sup>, Nelson Lima<sup>3</sup>**<sup>1</sup>CBMA - Centro de Biologia Molecular e Ambiental, Universidade do Minho, Braga, Portugal;<sup>2</sup>CITS - Centro de Investigação em Tecnologias da Saúde, CESPU, Paredes, Portugal;<sup>3</sup>IBB/Centre of Biological Engineering, Portugal

Dermatophytes are keratinolytic fungi responsible for skin infections. The affinity for keratinised tissues implies that the infection remains restricted to the cornified layers of the skin, nails, and hair. Onychomycosis is the general term used for fungal infection of the nails and dermatophytes, non-dermatophytes moulds or yeasts are causative agents. However, *tinea unguium* is the term more appropriate when mycological diagnosis confirms that the agent is a dermatophyte being *Trichophyton rubrum* the most frequently isolated species all over the world. Conventional methods routinely used in laboratory for mycological analysis included both direct microscopic examination and cultures. Therefore the confirmation of the etiologic agent relies on macroscopic and microscopic morphology. This approach is time-consuming (3-4 weeks); it requires specialized staff and frequently delays the treatment. Thus false-positives and false-negatives are reported in a high percentage of cases. Alternatively molecular-based methods have been proposed by some authors. Amplification of DNA sequences specific for certain classes of fungi by PCR allows the identification of the etiologic agent most likely in about two days. In this study we applied the multiplex PCR-based method developed by Brillowska-Dabrowska *et al.* (2007) directly from 96 nail specimens clinically suspected of onychomycosis and comparison with conventional mycological analysis was performed. PCR diagnosis was positive for dermatophytes for 43 samples and in 91% *T. rubrum* was the etiologic agent. Nineteen samples that could not grow in culture media were found positive for *tinea unguium* and from those 17 (90%) *T. rubrum* was present. The application of multiplex PCR-based diagnosis and the *T. rubrum* detection allow obtaining a rapid, specific and low-cost diagnosis for *tinea unguium* in routine clinical laboratory.

Brillowska-Dabrowska *et al* 2007. *J. Clin. Microbiol.* 45:1200-4.



PS3: 68

### Phenotypic and molecular characteristics of *Streptococcus agalactiae* from bovine mastitis in Portugal

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*Streptococcus agalactiae* (Group B *Streptococcus*, GBS) is a pathogen causative of bovine mastitis, a highly prevalent and costly disease in dairy industry due to antibiotherapy and loss in milk production, and it is also a relevant human pathogen due to severe invasive neonatal infections. Our aim was the evaluation of *S. agalactiae* antimicrobial drug resistance patterns, particularly important for streptococcal mastitis control and the identification of strain molecular features. Comparison with human *S. agalactiae* isolates was also carried out. Antimicrobial resistance was assessed by disk diffusion against amoxicillin-clavulanic acid, cefazolin, cefoperazone, cefquinome, pirlimycin-PRL, rifaximin, streptomycin, chloramphenicol, erythromycin-ERY, gentamicin, tetracycline-TET and vancomycin. Genotypic relationships were identified using pulsed-field gel electrophoresis (PFGE), macrolide and/or tetracycline resistance gene profiling, molecular serotyping, virulence gene profiling and multi locus sequence typing (MLST). The majority of the isolates were susceptible to all drugs except to aminoglycoside, macrolide, lincosamide, tetracycline and to one of the cephalosporins. Close to half of the TET resistant isolates have *tetO* and *tetK* and almost all ERY-PRL resistant isolates have *ermB*. The *S. agalactiae* bovine isolates belong to ST-2, -554, -61, -23 lineages, and five new molecular serotypes and insertion sequences in the *cpsE* gene considered to be specific of human *S. agalactiae* were found. Bovine *S. agalactiae* of serotype V with *scpB* and *lmb* seem to be related with *S. agalactiae* isolates of human origin (same ST-2 and similar PFGE). In conclusion, a contagious source for *S. agalactiae* was assessed. Serotype-specific capsular polysaccharides appear to have evolved in a herd-associated manner. Dissemination of antimicrobial-resistance was clonal and by lateral gene transfer. The bovine isolates of serotype V carry *scpB* and *lmb* virulence genes, which are considered to be required for human streptococcal species to colonize and to infect human suggesting that these bovine strains may be able to infect the human host. The majority of the bovine isolates of serotypes other than V do not carry the *scpB* - *lmb* region probably due to deletion during putative adaptation to the bovine host.

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PS3: 69

***Pseudomonas-Candida* interaction in dual-species biofilms****Diana Priscila Pires, Sónia Silva, Mariana Henriques, Sanna Sillankorva, Joana Azeredo**

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Bacteria and fungi co-inhabit in a wide variety of environments and the interactions between them can result in huge medical and economic impacts. *Pseudomonas aeruginosa*, a Gram-negative bacterium, and *Candida albicans*, a dimorphic fungus, are two important opportunistic pathogens frequently identified as the major causes of nosocomial infections, mainly due to their ability to form virulent biofilms. A pathogenic interaction between *P. aeruginosa* and *C. albicans* was recently identified and, it was also found that *C. albicans*' morphology and virulence are significantly affected in the presence of *P. aeruginosa*. In the present work, the interaction between *P. aeruginosa* and *C. albicans* in dual-species biofilms was studied. Biofilm formation was carried out in 24-well microplates containing 1 ml of Yeast Peptone Dextrose medium and 10 µl of each cellular suspension with an OD<sub>600nm</sub> of 1. Biofilms were formed during 24 and 48 hours with medium renewal every 12 hours. The results revealed that in mixed biofilms *C. albicans* proliferation was inhibited by the presence of both *P. aeruginosa* ATCC 10145 and PAO1 strains. The number of *C. albicans* viable cells was reduced by 2 and 3 logs in 24 and 48 hours old biofilms compared to single *C. albicans* biofilms. Conversely, *P. aeruginosa* was not influenced by the presence of *C. albicans* and so, the amount of viable cells of *P. aeruginosa* was similar in single and dual-species biofilms for both *P. aeruginosa* strains studied. To better understand the cause of *C. albicans* inhibition, biofilms of *C. albicans* with *P. aeruginosa* LPS mutant strains were also studied. These results showed that the LPS chain of *P. aeruginosa* has a great impact on *C. albicans* proliferation – mutants with full LPS chain inhibit the greatest while mutants with truncated A and B chains and also truncated outer core allow the growth of *Candida*. According to this study, the inhibition of *C. albicans* biofilm formation is directly correlated with the composition of the *P. aeruginosa* LPS chain.

PS3: 70

### **Targeting quorum-sensing for antimicrobial therapy: identification of bacterial adhesion inhibitors for bacterial control**

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Indiscriminate use of antibiotics for control and prophylaxis of bacterial pathogens has promoted the development of resistance to antimicrobial drugs commercially available, leading pharmaceutical companies to either abandoned antibiotic development programs or base their research on the conventional approach of improvement of pre-existing biocide drugs which have already proven their effectiveness and safety in use. New unconventional approaches for the development of antimicrobial drugs target inhibition of virulence, rather than bacterial viability. Such drugs are less prompt to induce bacterial resistance, being therefore a more cost-effective investment for pharmaceutical research. Amongst the most promising unconventional targets for the development of new antimicrobial drugs figures bacterial adherence and biofilm formation, and their control system, Quorum-sensing (QS), a mechanism of communication used to coordinate bacterial activities. The present study describes the identification and characterization of compounds active against bacterial bacterial biofilm. Compounds were selected or synthesized based on their similarity to QS inhibitors described, and tested against a panel of clinic relevant Gram-positive and Gram-negative bacterial strains. Biofilm inhibition tests were carried out in polystyrene microtiter plates. Inhibition was accessed by staining the adherent bacteria with crystal violet, followed by solubilization with SDS and quantification on a microplate reader. Three amongst the 40 compounds tested displayed significant activity against biofilm. Based on their structure, compounds identified as positive for biofilm inhibition can be classified as: (i) analogues of AI-2 QS inhibitors previous reported, such as pyrogallol, and (ii) chalcone derivatives. Studies on the mechanisms of biofilm inhibition and cytotoxicity against human cells are under way to evaluate the potential use of these compounds for biofilm control *in vivo*.

PS3: 71

### The divergent metallo- $\beta$ -lactamase Sfh-I: biochemical characterisation, structure and genetic context of the gene

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Carbapenemases, both serine and metallo- $\beta$ -lactamases (MBLs) constitute an increasingly important group of  $\beta$ -lactamases that render bacteria resistant to  $\beta$ -lactam-containing antibiotics, including carbapenems used as last resort antibiotics in treatment of multidrug resistant bacteria. The research of the resistance mechanisms to carbapenems in an environmental strain isolated from untreated drinking waters, *S. fonticola* UTAD54, conducted to the characterisation of a new gene coding for a metallo- $\beta$ -lactamase, Sfh-I. Further characterisation revealed that this strain also contains a class A  $\beta$ -lactamase designated SFC-1. The resistance profile of SFC-1 is typical of the class A carbapenemases. In order to better understand the structure-function relationship among MBLs we pursued the biochemical and structural characterisation of Sfh-I. The enzyme was overexpressed in *Escherichia coli* and purified by liquid chromatography. Like previously characterized B2 MBLs Sfh-I exhibited substrate selectivity toward carbapenems, hydrolysing imipenem and meropenem and reduced activity against penicillins and cephalosporins. However, Sfh-I also displays some distinctive properties when compared to other B2 enzymes, showing some hydrolytic activity against cefepime and being only weakly inhibited by excess zinc in a substrate-dependent fashion. Sfh-I is thus the most divergent member of the B2 MBL subgroup that has been identified to date. Purified Sfh-I binds one equivalent of zinc, as shown by mass spectrometry. The Sfh-I crystal structure is the first of an unliganded B2 MBL. This structure reveals the disposition of water molecules in the active site supporting a catalytic mechanism for subclass B2 MBLs in which His118, activates the nucleophilic water molecule. The genomic context of the gene encoding Sfh-I was investigated by DNA sequencing. The presence of several transposable elements suggests an exogenous origin of the gene. From a genomic library it was possible to obtain recombinant plasmids carrying large DNA inserts that, based on the amplification of the target genes by PCR, contain the genetic determinants of both carbapenemases of UTAD54, SFC-1 and Sfh-I.

Fonseca et al. 2011. *Antimicrob Agents Chemother*, in press. doi:10.1128/AAC.00429-11. Fonseca et al. 2011. *J Biol Chem* 411:951. Saavedra et al. 2003. *Antimicrob Agents Chemother* 47:2330. Work supported by the FCT grants SFRH/BD/19443/2004 and SFRH/BPD/21384/2005

PS3: 72

**Resistome: Characterization of antibiotic resistance pathways at the environment/animal interface****Inês Batista Guinote<sup>1</sup>, Gonçalo da Costa<sup>2</sup>, Catarina Meireles<sup>1</sup>, Sofia Carvalho<sup>1</sup>, Teresa Albuquerque<sup>1</sup>, Ana Botelho<sup>1</sup>, Carlos Cordeiro<sup>2</sup>, Patrick Freire<sup>1</sup>**<sup>1</sup>Laboratório de Bacteriologia, Unidade de Sanidade Animal, INRB, IP/LNIV, Portugal; <sup>2</sup>Departamento de Química e Bioquímica, Centro de Química e Bioquímica, FCUL

The continuous use, often abuse, of antimicrobial agents transversally through society exerts a strong selective pressure on microorganisms, leading to the emergence of resistance to “first line” antibiotics. Resistances can be transmitted between strains by horizontal gene transfer and rely on cellular mechanisms that have emerged in parallel to the evolution of bacterial populations. However, the origin of these mechanisms remains poorly defined. Recent studies have demonstrated that environmental microorganisms have not only a strong capacity for resistance to antibiotics, but antibiotics can even be used as a carbon source by them. Thus, this data suggest a prior existence of cellular mechanisms that lead to resistance in environmental microorganisms, furthermore selected by anthropogenic pressure, leading to the emergence of resistance observed. It is therefore essential to identify and characterize the Resistome, as the set of resistance mechanisms in environmental reservoirs, rural and urban, hence estimating the potential risk to public health. For that, around 450 Gram-negative bacteria were isolated from soil samples, resistant to beta-lactam and (fluoro)quinolone from industrially polluted urban land, as well as rural soils cross-contaminated with animal feces. It was thus possible to determine an overall high prevalence of resistant microorganisms in various locations, from animal production centers where higher values of minimum inhibitory concentration (MIC) were reached, in line with the strong selective pressure by the use of antibiotics as prophylaxis, but also in regions that, despite having high levels of industrial pollutants, are less likely to contain antibiotic residues in high concentration, which was nevertheless possible to isolate bacteria resistant to the tested agents. The molecular mechanisms that underlie resistance were analyzed and the presence of genes encoding the presence of several broad spectrum beta-lactamases screened, as well as genetic mutations inducing resistance to quinolones. The comparison of protein expression profiles in selected isolates has allowed a better understanding of changes in cellular mechanisms that may underlie the differences in antibiotic resistance profiles. The deepening of this analysis, infers on the origin and process of bacterial response to antibiotics, also enabling the detection of new bio-cellular targets that can be used in complementary approaches to combat bacterial growth.

PS3: 73

### Biodegradation of four fluoroquinolone antibiotics by a bacterial consortium followed by a validated HPLC-FD method

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The present concern in pharmaceuticals in the environment is well known and research studies in this area have been regularly reported. Pharmaceuticals reach the environment by several ways but mostly due to their incorrect disposal and the incomplete elimination during the treatment processes in Wastewater Treatment Plants (WWTP). These residues continuously enter aquatic environments and many of them are resistant to degradation, being normally found in aquatic ecosystems at  $\text{ng L}^{-1}$  to  $\mu\text{g L}^{-1}$  range. Antibiotics are a therapeutic class extensively studied due to its persistence and implications on development of multi-resistant bacteria. This work describes the biodegradation of four fluoroquinolones, Ofloxacin (OFL), Norfloxacin (NOR), Ciprofloxacin (CPF) and Moxifloxacin (MOX), by a bacterial consortium constituted by three bacterial species isolated by the work group: *Rhodococcus* sp. Strain FP1, *Labrys portucalensis* strain F11, and *Rhodococcus qingshengii* strain S2, known to degrade different aromatic fluorinated compounds. The experiments were conducted in batch mode using minimal salts medium supplemented with acetate and  $10 \text{ mg l}^{-1}$  of each compound or  $10 \text{ mg l}^{-1}$  of a mix of the four fluoroquinolones. The bacterial consortium was capable of aerobic biodegradation of OFL, NOR, CPF, and MOX during successive feedings of the compounds to the medium, as measured by monitoring the removal of the compounds by a validated HPLC-FD method and fluoride release by potentiometry. Degradation profile of the fluoroquinolones used in this study indicated that intermediate metabolites were accumulated. Two of the initial constituting strains, F11 and S2, were recovered from the medium, F11 predominating in cultures fed with MOX and CPF whereas S2 was mostly found in OFL and NOR cultures. No cultivable isolates were recovered from the mixture assay. After 19 days, OFL presented the higher extent of degradation according to the compound removal followed by HPLC-FD, with a degradation rate of 98.3%.

*This work was financially supported by CESPU - 09-GCQF-CICS-09 and FCT - PTDC/EBB-EBI/111699/2009.*

PS3: 74

### Production and purification of a novel canine norovirus nucleocapsid protein in yeast for use in serological studies

**Clara Pereira<sup>1,3</sup>, Edite Lopes<sup>3</sup>, João R. Mesquita<sup>2,3</sup>, M. São José Nascimento<sup>2,3</sup>, Lucília Saraiva<sup>1,3</sup>**

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Human noroviruses (NoVs) are recognized as the most frequent cause of foodborne outbreaks of acute gastroenteritis and of sporadic enteric illness. Though the most important mode of transmission is person-to-person contact, zoonotic transmission has been recently suggested. In 2007, studies focusing in the search of NoVs in dogs led to the discovery of a novel canine NoV in the North of Portugal. This constituted a relevant achievement in the emerging field of canine NoVs, compelling to explore the ecology and biology of this new virus, particularly a possible zoonotic transmission. Since this is a new virus, with no commercial Virus-Like Particles (VLP)-based Enzyme Immunoassay (EIA) available, the development of an in-house EIA will be crucial. To establish this assay, a substantial amount of VLPs to be used as an antigen is required. The yeast *Saccharomyces cerevisiae* has been extensively used for the expression of viral proteins, including viral core and nucleocapsid proteins. Hence, the aim of this work was to use *S. cerevisiae*, as a cost-effective and high-level expression system, to produce sufficient amounts of the novel NoV capsid protein, VP1 (which self-assembles into VLPs), to be used as antigen in the in-house EIA assay. With this goal, the VP1 gene was cloned into a multicopy yeast expression vector (pRS) and the protein was fused, at the C´ terminus, to a 6His tag. 6His tag allows not only the detection and quantification of recombinant proteins in Western blot (using a commercial anti-His) but also, and most importantly, to perform a one-step purification of VP1 by immobilized metal affinity chromatography (IMAC). The production of VP1 in yeast was optimized for the strain and growth conditions used and the purification performed by IMAC was analyzed by Coomassie blue staining of SDS-PAGE and Western-blot. The obtained product corresponded to an expected molecular weight of 63 kDa and the yield obtained was 1,38 mg/g wet weight of yeast. The immunological properties of the recombinant VP1-6His protein were evaluated by Western blot and by in-house EIA and its efficiency proved by confirming the serology of several canine samples. Together, in this work a fast and efficient system of production and purification of VP1 is presented.

We thank REQUIMTE (grant PEst-C/EQB/LA0006/2011), FCT (PTDC/CVT/113218/2009), POCl, FEDER and U.Porto/Santander Totta for financial support. J.R. Mesquita (SFRH/BD/45407/2008) is recipient of a FCT fellowship.

PS3: 75

### Virulence traits of *Pseudomonas* spp. carried by free-living sea turtles (*Eretmochelys imbricata*) from the Príncipe Island, Gulf of Guinea

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*Pseudomonas* spp., is an important human and animal opportunistic pathogen, assuming *P. aeruginosa* particular relevance as a multi-drug resistant organism (MDRO). Besides frequently resistant to antibiotherapy, *Pseudomonas* virulence can be further enhanced by other enzymes contributing to infection progression. Nevertheless, little is known about the role of the environment and wildlife in the spread of virulence traits. A total of 22 *Pseudomonas* spp. isolates, from oral (n=11) and cloacal (n=11) swabs taken in 2010, were obtained from 10 free-living sea turtles (*Eretmochelys imbricata*) free of gross signs of disease, in the nearshore waters of Príncipe Island (Gulf of Guinea). Bacteria were isolated in CFC Agar. Antimicrobial susceptibility to tetracycline (TE), beta-lactams [carbenicillin (CAR), imipenem (IPM), piperacillin (PRL)], aminoglycosides [amikacin (AK), and tobramycin (TOB)], 3<sup>rd</sup> generation cephalosporins [ceftazidime (CAZ), cephotaxim (CTX)], 2<sup>nd</sup> generation quinolones [ciprofloxacin (CIP), ofloxacin (OFX)] and quinolones for veterinary use [enrofloxacin (ENR)], was evaluated by the disk diffusion method (Clinical Laboratory Standards Institute Guidelines). Virulence factors production was phenotypically assayed: DNase production in DNA Test Agar; gelatinase activity in gelatin test agar; hemolysin production in Columbia agar with 5% sheep blood; and biofilm production in Congo Red Agar supplemented with Coomassie Blue. The majority of isolates was resistant to CAR (86%), but all were susceptible to the other beta-lactams tested (IMP, PRL). Some degree of resistance was also observed to 3<sup>rd</sup> generation cephalosporins (CTX 68%; CAZ 36%). The majority of isolates showed very low levels of antimicrobial resistance to TE (14%), quinolones (CIP 0%, OFX 5%; ENR 14%) and all isolates were susceptible to aminoglycosides. All but 2 isolates produced hemolysin (91%), 7 produced DNase (32%), 7 were gelatinase-positive (32%) and only one expressed biofilm (5%). Data suggest that *Pseudomonas* spp carried out in free-living sea turtles from the Príncipe Island do not represent a threat in terms of dissemination of important virulence traits. These isolates, most likely of environmental origin, are not MDRO and only one expressed biofilm. However, as *Pseudomonas* spp. rapidly change their susceptibility patterns and are opportunistic pathogens for turtles and the knowledge on prevalence of virulence traits may allow for the timely detection of highly mutable strains.



# Next Generation Sequencing, comparative Genomics and Evolution

*Poster session thematic symposium 4*

Cariço | Delgado | Osório | Calado | Soares-Castro |  
David-Palma | Valério | Eusébio | dos Santos | e  
Castro | Almeida | Cunha | Félix | Gonçalves | Tavares  
| Brito | Ponce

PS4: 1

## Fooled by trees: when simple can be misleading

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<sup>1</sup>KDBIO, INESC-ID, Lisboa, Portugal; <sup>2</sup>IST, Universidade Tecnica de Lisboa, Lisboa, Portugal; <sup>3</sup>ISEL, Instituto Politécnico de Lisboa, Lisboa, Portugal; <sup>4</sup>Network Modeling Instituto Gulbenkian de Ciência, Oeiras, Portugal; <sup>5</sup>IM/IMM, Faculdade de Medicina de Lisboa, Lisboa, Portugal

The use of trees for phylogenetic representations started in the middle of the 19<sup>th</sup> century. One of their most popular first uses was by Charles Darwin in "The Origin of Species". The simplicity of the tree representation still allows researchers today to easily convey the diversification and relationships between species. Yet trees suffer from several drawbacks that are not always clear to researchers. Since several different algorithms can be used to infer and draw the tree, one must be aware of each algorithm's set of assumptions. This variability has important repercussions, in particular in the analysis of the relationships of microbes in epidemiological and evolutionary studies. In the analysis of sequence-based microbial typing methods, Minimum Spanning Trees are becoming the standard for representing relationships between strains. However, these suffer from several limitations that can mislead in the interpretation of the resulting tree. The fact that a single tree is reported from a multitude of possible and equally optimal solutions and that no statistical metrics exist to evaluate them, justified a recent heuristic approach to address these issues [1]. We suggest an improvement of this approach by determining the number of possible trees and the relevance of each link based on an expansion of the Kirchhoff's Matrix Tree theorem. This will provide researchers with an exact evaluation of the number of possible trees and a precise confidence value for the presence or absence of a particular link, given all possible trees. This algorithmic advance will also run much faster than the current alternative and be able to handle much larger datasets, such as those arising from next-generation sequencing. Furthermore, in order to select the "best" tree an extension of the goeBURST [2] algorithm for the creation of a MST-like tree is proposed, providing a biologically significant model to support this choice. These novel tools will be made available in the PHYLOViZ software, freely downloadable at [www.phyloviz.net](http://www.phyloviz.net).

[1] Salipante et al (2011). *J Clin Microbiol*. In press. doi:10.1128/JCM.00919-11 [2] Francisco et al (2009). *BMC Bioinformatics*, 10(1), 152. doi:10.1186/1471-2105-10-152

## PS4: 2

**A new user-friendly web infrastructure for the study of the molecular epidemiology of *Staphylococcus aureus***

**Cândida de Castro Delgado<sup>1</sup>, Helen Andrade Arcuri<sup>1</sup>, Jonas Silva Almeida<sup>1,2</sup>, Maria Miragaia<sup>3</sup>, Herminia de Lencastre<sup>3,4</sup>, Helena Futscher Deus<sup>1,5</sup>, on Molecular Epidemiology of *Staphylococcus* and Study Group<sup>3</sup>**

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We are embedded in a microbial world. Naturally occurring microbial communities play fundamental roles in human health and disease. *Staphylococcus aureus*, a leading cause of human bacterial infections worldwide, can cause a wide range of diseases, including superficial skin and soft tissue infections, as well as invasive diseases, such as pneumonia, bacteremia, and endocarditis. Recently, *S. aureus* has been recognized as a pathogen responsible for both, hospital- and community-acquired infections. Although much is known about this pathogen, the efficient sharing of knowledge about its biology and means to prevent its spread are still hampered by the lack of an adequate bioinformatics infrastructure. Therefore, there is the need to incorporate semantic web technologies in the development phase of bioinformatics applications supporting molecular epidemiology research. In this report we exploit how the Simple Sloppy Semantic Database (S3DB), a flexible, distributed and secure information management system, can be used as the basis for a user-oriented bioinformatics infrastructure in the study of *S. aureus* molecular data. Using S3DB, we have created a database linked to a core infrastructure supporting multiple views over the same set of ontologies. In the present work we describe one of these interfaces, the "SPARQL Querying System for *Staphylococcus aureus*". Molecular data regarding isolates from 12,799 patients was used to evaluate our new web interface. Through this multidisciplinary collaboration we devised a user-friendly interface, tested and validated by the domain experts, which allows the user to select the required fields from a selection of relevant parameters for their domain. The query results are automatically presented in a tabular format. These data can be further filtered if a specific parameter is required. The key outcome of this interface is the fact that any biologist expert without programming skills can use this interface for performing semantic queries. In addition, if the user requires an advanced query he/she can do it by creating a SPARQL script, in this way users who want to develop their programming skills can also do it using this interface. It is worth noting that this infrastructure can be reusable and adapted to various molecular domains.

PS4: 3

### Genetic basis underlying drug-resistant *M. tuberculosis*

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Life and Health Sciences Research Institute (ICVS), ICVS/3B's - PT Government Associate Laboratory, Portugal

The implementation in the last decades of therapeutic protocols for Tuberculosis (TB) relying on the combined use of different antibiotics allowed outstanding cure rates with minimal relapses. TB was dramatically decreased in many countries but in the last decades we witnessed the emergence and spread of *M. tuberculosis* strains with acquired resistance to one or more antibiotics. The most recent World Health Organization data estimates about 440 000 new cases of infections every year with multi-drug resistant (MDR) *M. tuberculosis* strains that result in at least 150 000 deaths per year. In addition, infections with extremely-drug-resistant (XDR) strains have now been detected in up to 64 countries presenting mortality rates above 50 %. Thus, antibiotic-resistant *M. tuberculosis* strains are a serious worldwide health concern making research and development in this topic a priority. Recently, whole genome sequencing data from MDR and XDR *M. tuberculosis* strains has become available and sequencing projects of additional strains are underway. As expected, these strains harbor mutations mapped to known drug-resistance targets but also present other mutations with unknown biological relevance that are unique to M/XDR isolates. In this work we used a gene level bioinformatics approach to determine the main sites under positive selection in these M/XDR mutated genes. Results obtained show evidence for positive selection in known drug-resistance genes. In example, site specific analysis in *rpoB* sequences pointed out 4 sites with a high probability for being under positive selection reinforcing the validity of this strategy to identify biologically relevant mutations. Results obtained for a large set of *M.tuberculosis* genes will be presented and discussed. We conclude that the identification of sites under positive selection in *M.tuberculosis* genome might provide relevant insights into the molecular basis underlying the emergence of the M/XDR *M. tuberculosis* strains.

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PS4: 4

### **Modeling plasmid production in recombinant *Escherichia coli* cultivations with Artificial Neural Networks**

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Methodologies for gene therapy and DNA vaccination are progressively favoring use of plasmids as vectors, thereby avoiding some known disadvantages of viral vectors such as limited packaging capacity, high toxicity and lack of stability without integration. However, although considerable attention has been paid to genetic engineering of the plasmid backbone, substantially less attention has been given to the practical challenges of producing large amounts of plasmids. To overcome this limitation, the development of methodologies to model and control the plasmid production processes constitutes a challenge for biotechnology industry. Due to the high complexity and non-linearity of the recombinant *Escherichia coli* cultivation processes used to bioproduce plasmids the present work focus on the development of an 3-layer Multi-layer Perceptron (MLP) model to simulate the plasmid bioproduction using a wide range of *E. coli* cultivation conditions based on different carbon-sources types (glucose and or glycerol) and concentration. Specifically, the model was designed to preview biomass, plasmid, glucose, glycerol and acetate, over a period of 48 hours, based on initial conditions and evolution of some environment variables over time, such as oxygen, stirring speed, and pH. The model was fed with data collected from cultivations with highly different bacteria growth and plasmid production profiles and the sStopping criteria was designed in order to avoid overfitting. The model was tested by measuring Q<sup>2</sup> performance in cross-validation tests with minimum of 0.42 with 8 degrees of freedom in validation subsets, demonstrating the robustness of the model developed.

Work supported by PTDC/BIO/69242/2006(FCT) and IMM/ BD/30-2006 research grants.

PS4: 6

### Comparative genomics of two *Pseudomonas aeruginosa* clinical isolates to elucidate the composition of their mobilomes

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Recently, we have set a collaboration with Hospital de Braga, located in the North of Portugal, that handles over 600 *P. aeruginosa* isolates per year, aiming to rouse a holistic research approach to provide relevant information and tools to the clinicians to circumvent the multi-resistance phenomena in *P. aeruginosa*. Since then, we have set procedures aiming a systematic phenotypic characterization of the clinical isolates and developed strategies for the identification of pathogenicity islands and SNPs among the clinical isolates via comparative genomics. In this context, we have determined the full genome sequence of two clinical isolates using the high-throughput system Illumina Genome Analyzer IIx. These two clinical isolates, named 138244 and 152504, are representatives of allelic sequence types ST175 (widely disseminated and associated with multidrug-resistance) and ST560 (rare allele), respectively. Importantly, under standardized experimental procedures, isolate 138244 did not produce pigments and evidenced an antibiotic pan-resistant phenotype whereas 152504 produced a high amount of pyocyanin pigment and was susceptible to all antibiotics tested. A comparative genomic analysis using the genome sequences of both isolates and of all *P. aeruginosa* strains deposited in Genbank so far, allowed the identification of the accessory genome content of both isolates. Apparently, isolate 152504 harbors in its genome 243 unique genes, often clustered together in the same locus. Based on the genome annotation information, the pool of unique genes mainly encode several virulence factors, chemical stress resistance systems as well as 106 hypothetical proteins, some of which predicted members of the secretome of *P. aeruginosa* 152504. The accessory genome of 138244 mainly includes genes associated with mobile elements (phages, transposases, integrons) and genes encoding for 190 hypothetical proteins. Currently, research approaches are focused on the functional elucidation of sets of genes encoding hypothetical proteins of both isolates and in the description and characterization of their secretomes.

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PS4: 7

### Biogeography of *Xanthophyllomyces dendrorhous*: mapping populations and host associations

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The basidiomycetous red yeast *Xanthophyllomyces dendrorhous* (asexual state: *Phaffia rhodozyma*) has biotechnological relevance due to the production of astaxanthin, a carotenoid with remarkable biological properties. First isolated during the 1960s from exudates of deciduous trees throughout the Northern Hemisphere, this yeast was considered to be restricted to these habitats and latitudes. The discovery of a new *Xanthophyllomyces* habitat in the Southern Hemisphere in 2007 brought a better understanding of the geographic distribution and ecology of this yeast. In Patagonian forests, *X. dendrorhous* was found in association with the fruiting bodies of *Cyttaria*, an ascomycetous parasite of *Nothofagus* trees. Given that the *Cyttaria-Nothofagus* host system is also present in Oceania, sampling of selected localities in Australasia was carried out in 2009 yielding a considerable number of new isolates of *Xanthophyllomyces*. Benefiting from the expanded collection of *Xanthophyllomyces* strains presently available, this work aimed to elucidate the phylogenetic relationships between the different lineages and their possible biogeographic history. To achieve this goal a representative group of isolates was studied using multilocus sequence analysis (MLSA) and karyotyping assays within an ecological and geographic framework. The MLSA results showed that *X. dendrorhous* is composed by four well structured populations distributed in both Hemispheres and also the existence of two new *Xanthophyllomyces* species endemic to Australasia. The population structure of *X. dendrorhous* suggests little contact between the observed populations. However, the existence of an isolate bearing alleles from populations of Australasia and Japan suggests the occurrence of occasional gene flow. *X. dendrorhous* populations have different ecological associations and appear to have diverged first according to host tree and secondly by geography. Moreover no relation between karyotypes and population structure was observed. The extant distribution of the *X. dendrorhous* in the Southern Hemisphere may be related with the biogeographic history of *Nothofagus* trees, while its Northern Hemisphere distribution appears to be coherent with the biogeographic histories of *Betula* and *Cornus* trees. The remarkable diversity observed in Australasia suggests that this region might be related with the origin of the genus *Xanthophyllomyces*.

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PS4: 8

## Evolution and ecology of the specific fructose transport system (Fsy1) in ascomycetous fungi

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The *FSY1* gene codes for a fructose/H<sup>+</sup> symporter and is present only in a subset of species of the *Saccharomyces* genus, namely *S. uvarum*, *S. eubayanus* and two hybrid species (*S. pastorianus* and *S. bayanus*). The Fsy1 transporter has a high affinity for fructose but is unable to transport glucose, which is a rare property among known fungal transporter proteins. The physiological role of the Fsy1 transporter in *Saccharomyces* remains elusive, since it coexists in these yeasts with a large set of hexose transporter genes capable of transporting both glucose and fructose [1]. In order to get some insight into the possible ecological relevance of the Fsy1 transporter in *Saccharomyces*, we set out to identify Fsy1 homologues in other fungi, using whole genome sequence data available for an increasing number of fungal species. In addition to the genes identified in yeasts, this analysis brought to light a plethora of Fsy1 homologues in filamentous ascomycetes. Our survey failed to uncover Fsy1 homologues in other fungal lineages, therefore it seems likely that this transporter appeared early in the evolution of Ascomycota. It is however apparently absent from the earliest derived lineage, the Taphrinomycotina. Curiously, after the whole genome duplication (WGD), an event that took place 100 mln years ago in the lineage leading to *Saccharomyces*, the Fsy1 gene seems to have been lost by the large majority of the species. Our analysis further uncovered several cases of putative horizontal gene transfer well supported by synteny analysis. A comparison of the biochemical properties of four *FSY1* gene homologues, from yeasts with diverse habitats, showed that they are all fructose/H<sup>+</sup> symporters, with similar substrate specificity and a high affinity for fructose. However, when the expression of *FSY1* genes from four yeasts and two filamentous fungi cultivated on different carbon sources was studied, different expression patterns were observed possibly indicative of different physiological roles. This work shows that the fructose transporter encoded by the *FSY1* gene is ubiquitous in the two main lineages of the Ascomycota, exhibiting a complex evolution pattern, with intraspecific duplications and several events of horizontal gene transfer, and that it may possibly play diverse functional roles in different fungi.

[1] Leandro et al. 2009 FEMS Yeast Res 9, 511-525. This work is supported by FCT (project PTDC/AGR-ALI/112802/2009 and grant SFRH/BPD/46471/2008).



PS4: 9

**Multilocus sequence typing of *Pseudomonas aeruginosa*****Nádia Eusébio<sup>1,2</sup>, Tiago Pinheiro<sup>1</sup>, António Amorim<sup>1,2</sup>, Leonor Gusmão<sup>1</sup>, Ricardo Araujo<sup>1</sup>**<sup>1</sup>IPATIMUP, University of Porto, Portugal; <sup>2</sup>Faculty of Sciences, University of Porto, Portugal

*Pseudomonas aeruginosa* is a gram-negative bacterium of the class  $\gamma$ -Proteobacterium. This bacterium can be found, in terrestrial and aquatic environments, as well as in association with plant-, animal- and human-hosts. It can produce serious infections in immunocompromised hosts and can be resistant to antibiotics, which make this bacterium one of the most successful opportunistic pathogen in intensive-care units. Therefore, development of simple and robust methods for distinguish clonal strains from clinical and environmental samples and guide evolutionary lineages may be important in infection control strategies. Multilocus sequence typing (MLST) is a high-resolution genotyping method that allows studying population structure and analyzing epidemiological relationships by comparing sequences of nucleotides between 400 and 500 bp of 5 to 7 housekeeping genes. In this study, we evaluated the genetic diversity of Portuguese populations of *P. aeruginosa*. The strains were characterized by employing 7 loci (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) suggested by Curran *et al.* (2004) in the development of MLST for *P. aeruginosa*. The gene *acsA* was found to be the most variable and *nuoD* the less variable locus in the sequenced Portuguese strains. Some polymorphisms were found exclusively in the Portuguese collection of isolates, supporting the existence of endemic and local microbial populations. Genetic analysis of MLST scheme revealed that with less than 6% (a group of 169 polymorphic positions of the sequence) was possible to distinguish individually the complete group of strains present on online databases and in our collection. We believe that a selection of an even smaller number of polymorphisms can offer similar genotyping information as MLST.

Curran *et al.* (2004), *J Clin Microbiol*, 42, 5644-5649. We thank Fundação Calouste Gulbenkian (grant n.º. 35-9924-S/2009) for financial support. RA is supported by Fundação para a Ciência e a Tecnologia (FCT) Ciência 2007 and by the European Social Fund. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by FCT.

PS4: 10

**Omic characterization of *Salmonella* and *Campylobacter* phages with high therapeutic potential****Silvio Roberto Branco dos Santos<sup>1</sup>, Carla Carvalho<sup>1</sup>, Andrew Kropinski<sup>2</sup>, Eugénio C. Ferreira<sup>1</sup>, Joana Azeredo<sup>1</sup>**<sup>1</sup>Institute for Biotechnology and Bioengineering, Universidade do Minho, Portugal; <sup>2</sup>Laboratory for Foodborne Zoonoses, Public Health Agency of Canada

*Campylobacter* and *Salmonella* have long been recognized as important zoonotic pathogens of economic significance in animals and humans remaining the primary cause of reported food poisoning worldwide. The increased resistance of bacteria toward antimicrobials and the recent legislation restricting the use of antibiotics as growth promoters in animal production requires alternatives to the use of antibiotics in the control of bacterial pathogens. Bacteriophages (phages) are naturally occurring predators of bacteria, self-limiting, self-replicating and ubiquitous in the environment. Their high host-specificity and capacity to evolve to overcome bacterial resistance make them potentially important biocontrol agents. Therefore their use in therapy represents an appealing option to control *Campylobacter* and *Salmonella*. Nevertheless, the effective use of phages as antimicrobials turns important to understand phage biology rendering crucial the analysis of phage genomes and proteomes. The present study aims at describing the genome and proteome of two previously isolated broad lytic spectra *Myoviridae* phages with high potential for therapy: *Campylobacter* phage vB\_CcoM-IBB35 and PVP-SE1 *Salmonella* phage. The genomes of these two phages were obtained by 454 pyrosequencing technology and further annotated. The proteomic analysis was performed by SDS-PAGE and mass spectrometry. The DNA sequence data of vB\_CcoM-IBB\_35 consists of five contigs in a total of 172,065 bp containing 210 identified ORFs. From the 244 genes found in PVP-SE1, approximately 46% encode unique proteins and only 22.1% exhibited homology with known proteins. The mass spectrometric analysis revealed 38 structural proteins as part of the mature *Campylobacter* phage particle and 25 structural proteins encoded by a well-defined gene cluster in the *Salmonella* phage genome. Moreover, the lytic cassette and tail fiber proteins of both phages were identified representing high potential for therapy and diagnosis. The *Salmonella* phage genome presents high homology (145 genes) with the *E. coli* phage rV5, both unrelated to any other known phage, which might suggest that they belong to a new phage genetic group. The analysis of phage vB\_CcoM-IBB\_35 reinforces that *Campylobacter* phages are closely related displaying a distant relationship to the T4-like phages. In conclusion, the omic analysis of these two phages did not reveal any factor which could preclude its therapeutic use.

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PS4: 11

### Phylogenetic characterization of full-length genomes of SIVmnd-2 strains from mandrills (*Mandrillus sphinx*) housed at the Lisbon Zoo.

**Sandra Videira e Castro**<sup>1,2</sup>, **Aida Esteves**<sup>1,2</sup>, **Teresa Venenno**<sup>1,2</sup>, **Teresa Lobo Fernandes**<sup>3</sup>, **Rui Bernardino**<sup>3</sup>, **Ricardo Parreira**<sup>1,2</sup>, **João Piedade**<sup>1,2</sup>

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Since human immunodeficiency viruses (HIV) were shown to originate from simian immunodeficiency viruses (SIV), as the result of zoonotic cross-transmissions from African non-human primates, the characterization of the genetic diversity of SIVs has become a major goal of research. In this scope, the main objective of this study is to obtain the full-length genome sequences of two SIVmnd-2 isolates infecting the mandrills (*Mandrillus sphinx*) housed at the Lisbon Zoo and to characterize their phylogenetic relationships. RNA extracted from plasma was amplified by reverse transcription-nested PCR to originate two overlapping segments, from the primer binding site (PBS) to the end of integrase coding sequence (4426 bp) and from integrase coding sequence to the 3'LTR (U3) (4852 bp). DNA sequences, generated by primer-walking, were edited and submitted to a detailed phylogenetic analysis, using standard electronic algorithms freely available online. The sequences under study (PT.08.ZLX382 and PT.08.ZLX7597) paired with each other in the phylogenetic tree, as expected, forming a robust monophyletic group (bootstrap of 100%) with all the SIVmnd-2 references in the public domain (n=3). The high degree of certainty of phylogenetic analysis pointed to the absence of recombinant breakpoints along both genomes, although this could only be effectively confirmed by bootscanning analysis, in which the two sequences did not reveal a mosaic structure. These results corroborate, with a maximum degree of confidence, that the mandrills under study were infected with a monophyletic lineage of SIVmnd-2, suggesting local transmission of the virus between members of the troop. Finally, based on different pieces of evidence, which will be presented, we could speculate about the natural origin of these Portuguese SIVmnd-2 strains in animals transported from Cameroon, in West Central Africa, to European zoos, most probably during the colonial period.

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PS4: 12

## Phylogeography of the wild yeast *Saccharomyces paradoxus* in Eurasia

**Pedro Miguel Almeida, José Paulo Sampaio**

Centro de Recursos Microbiológicos (CREM), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal

Yeasts of the genus *Saccharomyces* are of major importance for biotechnology and fundamental research. However, the vast wealth of knowledge that has been gathered in different scientific areas contrasts markedly with our ignorance on where and how these important microbes thrive in nature. Moreover, the increasing number of *Saccharomyces* isolates collected from natural habitats has placed this genus at the forefront of population genetic studies. In particular, *Saccharomyces paradoxus*, the wild relative of *S. cerevisiae*, is becoming a model organism for population genetic and genomic approaches. A collection of sixty *S. paradoxus* isolates distributed over different regions in Eurasia was studied in order to expose the partition and the structure of genetic variation of this species. This was achieved employing a phylogeographic rationale over a set of five polymorphic microsatellite data. Estimates of population differentiation were high and suggested the presence of distinct ancestral genetic clusters. Populations from the Iberian and Balkan Peninsulas were assigned to geographically different clusters and seem to have diverged in allopatric putative glacial refugia which resulted in the fixation of molecular signatures of such isolation. By contrast, northern populations were found to share varying contributions from the ancestral clusters and did not exhibit evidence of detectable molecular departures from the refugial populations. Most of the mosaic individuals were from northern latitudes. Moreover, the absence of an "isolation-by-distance" pattern suggested a recent origin for the North European populations. This distribution of genetic variation of *S. paradoxus* in Eurasia is compatible with a scenario in which periods of isolation in southern European refugia during the late Pleistocene glacial cycles were followed by range expansion to northern latitudes during inter-glacial periods.

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PS4: 13

### Refining current knowledge on the genetic landscape of *Mycobacterium bovis*

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Zoonotic animal tuberculosis (TB), caused by *Mycobacterium bovis* and, more rarely, by *Mycobacterium caprae*, is an important disease of livestock, also known to affect more than 40 free-ranging wild species, and representing an important public health issue. The last surveillance reports in Portugal indicate a three-fold increase in animal incidence, suggesting the possibility of a (re)emergent disease. Epidemiological surveys carried out in several countries suggest that test-and-slaughter policies may reduce *M. bovis* strain diversity, favoring clonal expansion as a result of a bovine population bottleneck. A remarkable exception to this observation was the first epidemiological analysis carried out in Portugal, concerning the period 2002 to 2007, which highlighted the apparent high genotype diversity of *M. bovis* based on spoligotyping, possibly due to the low prevalence rates observed in the country and the absence of major epidemics favoring ongoing transmission of the same strain(s). Molecular typing of isolates from TB infection affecting local free-ranging wild animals further emphasized *M. bovis* genotype diversity and disclosed the interaction of several hosts in the epidemiological cycle of TB. In this work, we re-examine the genetic landscape of *M. bovis* isolates from the mainland territory obtained up to 2010 by comparing the direct repeat region (spoligotyping) of 439 isolates from domestic species and 185 isolates from wild hosts. Forty-nine spoligotypes were identified, two of them (SB0121 and SB0119) being markedly prevalent and geographically widespread. Complementary molecular profiling, based on the analysis of *mycobacterial interspersed repetitive-unit-variable-number tandem-repeats*, the chromosomal region RDEu1 and single nucleotide polymorphisms, provides cumulative evidence that most of the strains are, in fact, closely related. A collaborative study involving Portugal and Spain revealed that *Mycobacterium bovis* isolates from the Iberian Peninsula are dominated by strains with spoligotype patterns deleted for spacer 21. Moreover, these strains exhibit a SNP in *guaA* gene that together with the loss of spacer 21 defines a new clonal complex of *M. bovis* that was recently named European 2 (Eu2).

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PS4: 14

### Temporal trends of serotypes included in the novel 13-valent pneumococcal conjugate vaccine carried by young children in Portugal

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*Streptococcus pneumoniae* (pneumococcus) remains a major cause of morbidity and mortality worldwide. In June 2001, a seven-valent pneumococcal conjugate vaccine (PCV7) aimed at preventing invasive pneumococcal disease among young children became available in Portugal. This vaccine included seven capsular types (4, 6B, 9V, 14, 18C, 19F, 23F). In January 2010, PCV7 was replaced by an extended thirteen-valent vaccine (PCV13). This new vaccine includes six additional serotypes (1, 3, 5, 7F, 6A, 19A) compared to PCV7. In the colonization study described in this presentation and conducted in January 2011, we aimed to evaluate the use of PCV13 among children attending day-care centers in Oeiras and determine the prevalence and characterize temporal trends of the six additional serotypes included in PCV13. We also analyzed the recently described serotype 6C, which is structurally close to serotype 6A and for which PCV13 could possibly confer cross-protection. Nasopharyngeal swabs were obtained from 448 children. Pneumococci were isolated by routine procedures and serotyped by PCR and/or by the Quellung reaction. PCV use was evaluated by reviewing the children's immunization bulletins. Serotype proportions were compared to data obtained in similar studies carried out since 2006. Among the children sampled in 2011, 64.9% were pneumococcal carriers and 67.1% were age-appropriately vaccinated; close to one-fifth (18.3%) had received at least one dose of PCV13. The proportion of pneumococci that expressed the six serotypes under study was 5.2% for serotype 19A, 2.4% for serotype 6A, 2.1% for serotype 3 and 0% for serotypes 1, 5 and 7F. Analysis of temporal trends for these serotypes showed a significant decrease of serotypes 19A (from 12.9% to 5.2%) and 6C (from 17.1% to 6.9%) from 2010 to 2011. For serotypes 3 and 6A a decline in prevalence was observed since early 2009 and early 2010, respectively, i.e., before introduction of PCV13. Serotypes 1, 5, and 7F had very low abundances throughout the entire study period. In conclusion, we observed a significant decrease of serotypes 19A and 6C in 2011. Whether such decline is due to secular trends, results from the use of PCV13, or both, is not clear at the moment. Further studies are needed in the years to come to monitor and clearly establish the impact of PCV13 in colonization and improve our understanding of secular variations of serotypes.

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PS4: 15

## A global phylogeographic survey of *Saccharomyces uvarum*

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Yeasts belonging to the genus *Saccharomyces* are recognized as interesting models for evolutionary and population genetics studies because they combine a ubiquitous distribution in nature with well-established laboratorial tractability and the availability of complete genome sequences for all species. Nevertheless, several important aspects of their natural history remain obscure. Like *S. cerevisiae*, that is used for winemaking and bread leavening, *S. uvarum* is a cryotolerant yeast that ferments cider and certain wines whose fermentation is carried out at low temperatures. Since in *S. cerevisiae* molecular signs of domestication have been detected but phylogeographic patterns are minute, we set out to investigate if *S. uvarum* exhibits the same patterns. Specifically, we wanted to investigate if wine and cider strains are phylogenetically derived and less genetically diverse than their wild counterparts and if a geographic imprint is detectable in *S. uvarum*. We performed a phylogeographic study of 50 strains isolated in Eurasia, North and South America and Australasia. Based on concatenated sequences of three nuclear genes three main clades were obtained, two of them including isolates from two well-delimited regions (Patagonia and Australasia) and a third one including strains from all regions except Australasia. However, microsatellite typing did not support the recognition of the Patagonian population as distinct from the population of North Hemisphere. Therefore, and contrary to what has been found for *S. cerevisiae*, no evidence of domestication was detected for *S. uvarum*. Moreover the Australasian population showed genetic and phenotypic divergence from the remaining populations and mating studies showed partial reproductive isolation. This pattern was not observed for *S. cerevisiae*, although *S. paradoxus*, which is only found in the wild, also exhibits partial reproductive isolation between populations of different continents. A possible ancestry of the Australasian population was suggested by the existence of private alleles with high frequencies.

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PS4: 16

### A phylogenetic insight into DNA-signatures to select the fittest markers for microbial detection methods

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“DNA-signatures” is a term that has formally entered the scientific vernacular by Phillippy *et al.* 2007. According to these authors, DNA signatures are nucleotide sequences “that occur in the pathogen and not in any other species” and “that can be used to detect the presence of an organism and to distinguish that organism from all other species.” The term has been coined to Insignia, a database of taxa-specific markers suitable for microbial identification. As in other computational pipelines (reviewed by Albuquerque *et al.* 2009), exhaustive comparative genomics analysis allow the identification of sequences that are exclusively present in a specific taxon. However, these instruments are based on comparative algorithms and dismiss a phylogenetic perspective. From an evolutionary standpoint the *uniqueness* of genomic loci is a contradiction in itself, given that the evolution theory postulates a *continuum* of modifications in which new sequences arise from pre-existent ones. In this work we have studied the evolutionary history of several *Xanthomonas euvesicatoria*-specific DNA regions, aiming to understand how these markers became “unique” within the genome of these phytopathogenic bacteria. We hypothesize that a phylogenetic insight to each taxa-specific sequence should provide valuable information to select the most promising diagnostics markers, as well as the taxonomic groups that should be primarily used for experimental validation trials. The results showed that, in general, the computationally-selected markers are located in chromosomal regions of high genomic plasticity, as revealed by the proximity of mobile genetic elements. Moreover, the low GC content, the CAI (Codon Adaptation Index) values and comparative synteny maps, indicate that most markers were likely obtained by horizontal gene transfer events. Intriguingly, the high E-values obtained by BLAST analyses were inconclusive to track the evolutionary origin of these regions. Nevertheless, the presence of phage related ORF's and tRNA genes in their genomic vicinity evoke a phage-related origin for these DNA-signatures. The experimental validation of these markers by PCR and hybridization methods confirmed their specificity for diagnostic purposes. In conclusion, this work highlights the importance of phylogenetic analyses for the selection of markers with a high discriminatory potential, useful for bacterial detection and eventually genotyping.

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PS4: 17

**The population genetics of AI-2 quorum sensing genes in *Escherichia coli* suggests fitness advantage of cheating during infection****Patricia H. Brito<sup>1</sup>, Isabel Gordo<sup>1</sup>, Karina Xavier<sup>1,2</sup>**<sup>1</sup>Instituto Gulbenkian de Ciencia, Portugal; <sup>2</sup>Instituto de Tecnologia Química e Biológica, Portugal

Although single cell organisms, bacteria can express coordinated multicellular behaviors, such as virulence, biofilm formation, and quorum sensing (QS). Autoinducer-2 (AI-2) represents a nonspecies-specific signal produced to mediate both intra- and interspecies communication in bacterial QS. We analyzed the genetic diversity of AI-2 QS genes that includes the *luxS* gene (the signal synthase) as well as all the genes of the *Isr* regulon that are responsible for the reception, internalization and processing of the signal) across all available genomes of *Escherichia coli*. We found a level of polymorphism in gene content that cannot be explained by neutral evolution alone. Many strains do not hold a complete functional *Isr* operon, and the presence of genes does not correlate with phylogenetic history, pathogenicity, or virotype. The loss of function occurred multiple independent times during the evolution of *E. coli*, and once initiated pseudogenization is fast and does not follow a fixed gene order. Analyses of selection show that two important genes are under balancing selection: the *luxS* (AI-2 synthase) and the *IsrA* the gene that codes for the ATPase enzyme that produces the energy necessary for the internalization of AI-2 into the cell. These signals of balancing selection at the nucleotide level may be the result of evolution on fluctuating environments or alternatively, the consequence of frequency-dependent selection acting on the efficiency of signal production and internalization. The later would support the hypothesis of AI-2 QS as a cooperative behavior vulnerable to the evolution of cheating. In *Pseudomonas aeruginosa* social behaviors are routinely lost from isolates obtained from lung infections indicating a fitness advantage of cheating during infection. We hypothesize that a similar process also occurs with AI-2 system of *E. coli*.

PS4: 18

### **Toxin-antitoxin loci in bacteria: the killers within**

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Toxin-antitoxin (TA) loci are abundant and widely distributed among the genomes of Bacteria and Archea. They are composed by a toxin gene, that encodes a bactericid or bacteriostatic protein, and an antitoxin gene, that can prevent the effect of the toxin. Though these two genes are adjacently located and jointly expressed, their expression, under certain circumstances, can kill or prevent the growth of the cells that harbor them. The biological role of these loci is widely debated: it is known that when they are located in a plasmid, their action stabilizes the plasmid in the cells by post-segregation killing; but their role when located in the chromosome is unclear. Actually, different types of TA systems can have very different roles, from behaving as purely selfish genetic elements, to being “domesticated” and incorporated in important genetic networks. To address the origin and dynamics of TA loci, we are analyzing complete genomes of *Enterobacteriaceae* and studying the number, position and organization of TA loci. Moreover, this comparative approach, coupled with phylogenetic analysis, allow us to study the dynamics of TA loss and gain in the lineages. We will present our current knowledge of the dynamics of a particular set of TA gene families within these genomes.

# Bioprocess engineering

*Poster session thematic symposium 5*

Silva | Cruz | Pereira | Silva | Polizelli | Hekmat |  
Calado | Pereira | Michelin | Monteiro | Pereira |  
Ribeiro | Albergaria | Eusébio | Silva | Fernandes |  
Grilo | Chengalath

PS5: 1

**Efficient and simple purification of plasmid DNA on aqueous two-phase systems with the aid of modified polymers****Nuno Miguel Silva<sup>1</sup>, José António Teixeira<sup>2</sup>, José Alberto Martins<sup>1</sup>, João Carlos Marcos<sup>1</sup>**<sup>1</sup>Centro de Química, Universidade do Minho; <sup>2</sup>Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Portugal

Molecular therapies such as gene therapy and DNA vaccination, requires plasmid DNA molecules (pDNA) with a stringent clearance of impurities. In order to face this challenge several methods had been developed in recent years to be applied in the large-scale purification of these molecules. Aqueous two-phase systems (ATPS) are one of the most promising processes for this purpose. These are formed by the mixture of two polymers or one polymer and a salt in water above some critical concentrations. The distinct physicochemical properties of the two phases allows the separation of components from a complex mixture, like cellular lysates, based on their differential partition between them. Despite their great potential ATPS have some drawbacks like its low selectivity that limits the purification outcome. The addition of modified polymers, ie, compounds with a bio-recognition element that specifically bind to the target biomolecule, allows to circumvent this particular limitation and increase ATPS selectivity. In this work Poly(ethylene glycol)-arginine (PEG-Arg) and poly(ethylene glycol)-amine (PEG-NH<sub>2</sub>), two cationic polymers were studied as affinity ligands for the purification of plasmids from alkaline cell lysates. These ligands are added to ATPS composed of 16,2% (w/w) PEG 600 and 17,4% (w/w) DEX 100 in order to steer the pDNA partition to the phase where less impurities are accumulated (PEG rich phase). In the presence of both polymers the pDNA was quantitatively obtained on the top phase with a slight contamination of RNA and proteins. The subsequent reextraction to an ammonium sulphate phase yields pure plasmid with no measurable presence of RNA and protein. The best results were obtained when it was added 4% of PEG-NH<sub>2</sub> or 0,5% of PEG-Arg in relation to the total PEG of the first system.

PS5: 3

### **Tannase production in several bio-reactors by *A. niger* GH1 in solid fermentation and liquid fermentation.**

**Mario Cruz<sup>1</sup>, Ruth Belmares Cerda<sup>2</sup>, Cristobal Aguilar<sup>2</sup>**

<sup>1</sup>Universidad Autonoma Agraria Antonio Narro, Mexico; <sup>2</sup>Universidad Autónoma de Coahuila, Mexico

The enzymes have an ample application in the modern industry. They are used in processes for the obtaining of foods additives, medicines, biopolymers and chemical substances in general; they are commercialized directly like products (softeners for meat, liquid curdle, among others) and can have applications for bio-remediation. Although the industrial enzyme production initiated in companies that dedicated to the extraction of amylases and proteases of animal and vegetable origin, the enzymes of greater commercial importance are obtained mainly from microbial sources. This enzyme has great importance in the pharmaceutical industry and food industry. The main methods of fermentation of the enzyme tannase are two: liquid culture and solid culture. In the present work 3 different bio-reactors were evaluated: two of liquid culture (bio-reactor anxious tank and bio-reactor Airlift) and one of solid culture (bio-reactor of packed-bed columns). The obtained results demonstrate that the production of tannase is characterized by greater titles in the solid culture, since in liquid culture much production of the intracellular enzyme also exists. The obtained results leave the bases for the design of new reactors to obtain results of greater importance for the industry. In the present work the capacity of degradation of tannic acid firstly was substrate evaluated using a filamentous fungus of genus *Aspergillus* that was reported in the Food Department of the University of Coahuila as able to grow in high tannin concentrations and was characterized like *A. niger* GH1. From kinetic ones made gave like result that the conditions of degradation of the substrate by this fungus were: 35C, 200rpm, pH 5.0. Later the production of the tannase enzyme was evaluated in an anxious tank using the conditions defined in the previous step. An extracellular tannase activity was 200 U/L and the intracellular was greater (500 U/L), whereas in liquid culture in an Airlift bioreactor the values of extracellular tannase activity was 400 U/L and intracellular the 900 U/L, during the production in solid culture the elevated values were: 2000 U/L and 900 U/L for extracellular and intracellular enzyme. Therefore, the contributions of this work are several: Application of 2 fermentation systems for the tannase production the method of activity quantification, as well as the kinetic description of the tannase production in three different bio-reactors.

PS5: 4

### Identification of *Saccharomyces cerevisiae* genes involved in the resistance to multiple stresses during Very-High-Gravity and lignocellulosic biomass industrial fermentations

**Francisco B Pereira<sup>1</sup>, Pedro MR Guimarães<sup>1</sup>, Daniel G Gomes<sup>1</sup>, Nuno P Mira<sup>2</sup>, Miguel C Teixeira<sup>2</sup>, Isabel Sá-Correia<sup>2</sup>, Lucília Domingues<sup>1</sup>**

<sup>1</sup>IBB-Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Portugal; <sup>2</sup>IBB-Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal

Most of the current processes of bioethanol production are based on the use of Very-High-Gravity (VHG) technology and the processing of lignocellulosic biomass hydrolysates. However, inVHG processes the initial high osmotic pressure and the accumulation in the growth medium of high concentrations of ethanol often result in slow and incomplete fermentations. On the other hand, biomass-based fermentations are limited by inhibitors resulting from biomass pre-treatments, including furfurals, weak acids and phenolics.

Aiming the optimization of strains and conditions for industrial bioethanol production a set of *Saccharomyces cerevisiae* genes were identified, in this study, as required for maximal fermentation performance under industrial conditions. The integration of previous chemogenomics data [1,2,3,4,5] was used to identify eight genes whose expression confers simultaneous resistance to high concentrations of glucose, acetic acid and ethanol, chemical stresses relevant for VHG fermentations; and eleven genes conferring simultaneous resistance to stresses relevant during lignocellulosic fermentations. These eleven genes were identified based on two different sets: one with five genes granting simultaneous resistance to ethanol, acetic acid and furfural, and the other with six genes providing simultaneous resistance to ethanol, acetic acid and vanillin. The expression of *BUD31* and *HPR1* was found to lead to the increase of both ethanol yield and fermentation rate, while *PHO85*, *VRP1* and *YGL024w* expression is essentially required for maximal ethanol production in VHG fermentations. Five genes, *ERG2*, *PRS3*, *RAV1*, *RPB4* and *VMA8* were found to contribute to the maintenance of cell viability in wheat straw hydrolysate and/or for maximal fermentation rate of this substrate. The identified genes stand as preferential targets for genetic engineering manipulation in order to generate more robust industrial strains, able to cope with the most significant fermentation stresses and, thus, to increase ethanol production rate and final ethanol titers.

[1] Teixeira et al, 2009, *Appl Environ Microbiol*, 75, 5761; [2] Teixeira et al, 2010, *OMICS*, 14, 201; [3] Mira et al, 2010, *Microb Cell Fact*, 9, 79; [4] Endo et al, 2008, *Biotechnol Biofuels*, 1, 3; [5] Gorsich et al, 2006, *Appl Microbiol Biotechnol*, 71, 339.

PS5: 5

### **Biorefinery: valorization of by-products from the paper industry for single cell protein production**

**Pedro Tiago Santos Silva<sup>1</sup>, Ana Lucia Morgado Ferreira<sup>2</sup>**

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Hardwood Spent Sulphite Liquor (HSSL) is a by-product of the pulp industry obtained from the acid sulphite pulping of wood. HSSL consists, mostly, of lignosulfonates, monosaccharides and acetic acid. Acetic acid and monosaccharides, such as xilose, can be employed, as carbon source, by filamentous fungus *Paecilomyces variotii* for single cell protein (SCP) production. SCP is not pure protein but biomass with a high content of protein produced by bacteria, yeasts, filamentous fungi or algae. It also contains carbohydrates, lipids, nucleic acids, mineral salts and vitamins. In this work, the growth of *P. variotii* was promoted in HSSL without addition of salts, in a sequential batch reactor with three cycles along 16 days. The results obtained were compared to those from another experiment, in which the growth of *P. variotii* was accomplished in HSSL supplemented with salts, in order to study and optimize the process of SCP production. The results also showed that, HSSL without salts was more advantageous process for HSSL detoxification since, *P. variotii* consumed preferentially the acetic acid present instead of the sugar, This fact allows for the use of detoxified HSSL as medium for other microorganisms to produce value added products.. HSSL supplemented with salts led to a higher amount of biomass produced, a higher percentage of protein in dry weight (16.1% compared to 9.1%) and a greater number of amino acids, 14, of which 7 were essential amino acids.

PS5: 6

### Characterization and methanol concentration influence on phytase induction in air-lift bioreactor by mutant *Pichia pastoris*

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<sup>3</sup>Tiradentes University, Brazil

Phytases hydrolyze phytic acid ester bonds releasing inositol and inorganic phosphate. They have important application in animal feed. These enzymes are largely secreted by filamentous fungi, but their production can be increased by heterologous expression in *Pichia pastoris*. Induction effect and the fermentation scale processes have crucial importance in the levels and cost of enzyme production. The aim of this work was to verify the methanol concentration effect on PhyA induction and biomass formation in Air-lift bioreactor by *P. pastoris*. After cloning using vector pPIC9kf1-, the yeast was inoculated in 500 mL of MGY medium (0.34% YNB, 2% glycerol, 4.10<sup>-5</sup>% biotin and 1% ammonium sulfate) and incubated at 30°C under 200 rpm orbital stirring for 24 h, until D.O. 1.3 at 600 nm. After that, the cultures were centrifuged at 6000 g, 4°C for 10 minutes. The pre-inoculum was resuspended in MGY medium and transferred to 6 L Air-lift bioreactor. In order to proceed the expression it was added 0.34% YNB, 4.10<sup>-5</sup>% biotin, 1% ammonium sulfate and 10 mM sodium acetate buffer, pH 6.0, after 24 hour of growth in the reactor and the fermentation was carried out at 30°C and 0.1 vvm O<sub>2</sub>. The PhyA expression was induced by 1% methanol initial addition. This concentration was maintained in all experimental period. The thermal and pH effects were studied incubating the enzyme in several temperatures as well as different pHs. The phytase activity was measured according to the modified method Yin *et al.* (2007) using phytic acid dodecasodium as substrate. An enzymatic unit was defined as the amount of μmols of phosphorus released by mL in the assay conditions. The best methanol concentration for the enzymatic induction was 3% and the biomass decreased to about 1.5 and 1.8 g/L in all fermentation conditions. The PhyA showed maximum activity at the range of pH 5.5-6.0 and at 50°C. This enzyme was stable between 30 and 50°C, with t<sub>50</sub> of 40, 23 and 7 min at 50, 60 and 70°C, respectively. Moreover, the enzyme showed 94 % of residual activity at pH 5.0 and 6.0, and 74% at pH 3.0, after being incubated for 24 h in the substrate absence. The high levels of enzyme expression in bioreactor by *Pichia pastoris* and the results of activity in a wide range of pH as well as its high stability make this promising microorganism to be used in industrial processes.



PS5: 7

### Designing and construction of recombinant pIPI -GFP plasmid expressing HIV-1 virions as a novel system for HIV-1 replication assay

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Safety and targeted utilization of drugs and vaccines are the most important tribulations of the world health for drug discovery and HIV-1 vaccine research. Herein, we designed and constructed the recombinant pIPI-GFP plasmid harboring *gfp* and expressing HIV-1 virions as a novel system for replication assay. We evaluated this simple method as substitution of syncytia formation and ELISA-based p24 assays. pmzNL4-3 plasmid that produce single-cycle replicable HIV-1 virions was already designed by deleting a 2 Kb *M*/sI-digested fragment in reverse transcriptase (RT) and integrase (IN) genes. In order to create a multiple cloning site in addition to *M*/sI restriction and protease cleavage sites, the *gfp* gene and its restriction enzyme recognition sites from pEGFP-N1 plasmid was cloned into the pmzNL4-3 in frame of RT-IN gene. HEK293T cell line was transfected by TurboFect reagent and GFP emission was evaluated under fluorescent microscope. We confirmed and evaluated the GFP emission and virus production by syncytia formation and ELISA-based p24 assays. pIPIGFP plasmid harboring *gfp* with a protease cleavage site in its 3'-end was constructed and sequence-confirmed. Co transfection of HEK 293 cells with pIPIGFP plasmid besides pSPAX2 and pMD2G plasmids, which respectively encode HIV-1 Gag-Pol and vesicular stomatitis virus glycoprotein, not only resulted in the detection of GFP emitting cells, but also produced SCR virions in the culture supernatant which were capable of infecting HEK293T cells and transporting GFP to them. GFP emission and virus production was comparable with syncytia formation and ELISA-based p24 assays. The recombinant pIPI -GFP plasmid harboring *gfp* and expressing HIV-1 virions is a safe system for easy and reliable assay of HIV-1 replication that may be applied for anti-HIV drug discovery studies.

PS5: 8

**High-throughput analysis of *Escherichia coli* cultivation processes by fourier transformed infra red spectroscopy****Teresa Scholz<sup>2</sup>, Gabriel Martins<sup>1</sup>, Vitor V. Lopes<sup>\*2</sup>, Cecília R.C. Calado<sup>\*1</sup>**<sup>1</sup>Catholic University of Portugal, Portugal; <sup>2</sup>Energy Systems Modeling and Optimization Unit, National Laboratory for Energy and Geology, Lisbon, Portugal

The optimization of the production of bio-pharmaceutics by a defined *Escherichia coli* expression system is a complex process, as it depends of multiple factors, and from the complex interrelationship between them, such as the plasmid genetic backbone, strain physiology and cultivation conditions. The small number of experiments per unit of time usually associated with bench scale bioreactor, impelled the development of microbioreactors with the possibility of some on-line analysis as pH and DOC. However, to fully exploit the microbioreactors systems there is an urgent need to develop high-throughput analytical methods to estimate all the critical culture variables of the bioprocess in a rapid and sensible way, at a microliter scale, and using robust calibration methods valid for a wide range of culture conditions. This work focus the development of a high-throughput method based on Fourier Transformed Infra Red (FTIR) spectroscopy associated to chemometrics to analyze all the critical variables, of a recombinant *E. coli* expression system producing plasmids used as DNA vaccine, as biomass production, the consumption of carbon-source glucose and glycerol, the production and consumption of the inhibitory product acetate and the plasmid production along the time. The models developed are valid for a wide range of culture conditions based on complex media, representing a wide range of biomass and plasmid concentrations, and yields of plasmid productions per biomass. To optimize the calibration models (based on partial least square regression), it was evaluated the pre-processing methods of multiplicative scatter correction and the spectra differentiation. For the biomass and plasmid variables, the effect of selecting regions from the whole FTIR spectra on the model prediction was also evaluated. In the case of acetate, and to improve the model prediction it was also necessary to consider the data concerning the acetate production phase apart from the data concerning the acetate consumption phase. In resume, the developed high-throughput method based on FTIR-spectroscopy, using microliter volumes of the culture clarified broth, are able to predict all the critical variables of *the E. coli* culture in a wide range of culture conditions, that may maximize the potential use of parallel multi-microbioreactor platforms used in *E. coli* bioprocess development.

Work supported by PTDC/BIO/69242/2006(FCT) research grant.\* These authors contributed equally

PS5: 9

**Microbial degradation of hydrocarbons and its applications to enhanced oil recovery at lab scale****Jorge F. B. Pereira<sup>1</sup>, Eduardo J. Gudiña<sup>2</sup>, Lúgia R. Rodrigues<sup>2</sup>, José A. Teixeira<sup>2</sup>, João A. P. Coutinho<sup>1</sup>**

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The renewed interest in Enhanced Oil Recovery (EOR) techniques as a consequence of the current oil prices is boosting the development of the Microbial Enhanced Oil Recovery (MEOR). This technique is useful to recover incremental oil from a reservoir beyond primary and secondary recovery operations and can be carried by the injection of exogenous or stimulation of indigenous microorganisms. This last approach is here investigated. In this work we address the isolation and identification of microorganisms from Brazilian heavy oil samples capable of producing biosurfactants and to promote the degradation of heavy oil fractions, in particular long-chain hydrocarbons. Different crude oil samples obtained from an oil reservoir were used to isolate microorganisms for application in MEOR. Most of isolates were *Pseudomonas* and *Bacillus* strains. The growth of different microbial isolates was studied under both aerobic and anaerobic conditions at 40°C. These isolates were found to produce extracellular biosurfactants, reduce surface tension and showed a high emulsifying activity. In addition to these capabilities, we studied the ability of these microorganisms to degrade the heavy oil fraction, in particular long-chain hydrocarbons. Several parameters were studied, such as nutritional conditions, incubation time and paraffinic composition of the mixture. Our results show that some of the isolates displayed a capacity to degrade, under aerobic and anaerobic conditions, the large alkyl chains (18+ carbons in alkyl chains) and to reduce the viscosity of hydrocarbon mixtures. Our results, show the ability of the *Bacillus subtilis* strains to enhance the recovery of paraffinic oil on sand pack columns. These results suggest that the microorganisms' here isolated have interesting characteristics to be applied for MEOR.

PS5: 10

### Pretreatment of brewers' spent grains for cellulases production by *Aspergillus niger* van Tieghem

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Successful utilization of cellulosic materials as a renewable carbon source is dependent on the development of economically feasible process technologies both for the production of biomass-degrading enzymes, and for the enzymatic hydrolysis of cellulosic materials to low molecular weight products. Significant cost reduction is required in order to enhance the commercial viability of cellulase production technology and biomass pretreatment can be an essential processing step for this purpose. Thus, the aim of this work was to evaluate the performance of pretreated brewers' spent grains on the improvement of cellulases production by *A. niger* van Tieghem. For this, brewers' spent grains was submitted to autohydrolysis treatment. Initially, the material was dried, milled and sieved (1.0 mm screen). Water was added to the sample in a closed and pressurized vessel (solid/liquid ratio 1:10 w/v), and the system heated to 180, 190 or 200°C for 10, 35 or 50 min. The liquor obtained (hemicelluloses fraction) was separated from the solids (cellulose/lignin) by filtration and both fractions were used together or not as carbon source on fermentation: 1% (w/v) treated solid fraction; 1% (w/v) solids plus 10% (v/v) liquor, or only liquor. Carboxymethylcellulose, avicel and untreated brewers' spent grains were used as control. The inoculum was done in Mandels medium and the cultivation conditions were 30°C/100 rpm for 6 days. Carboxymethylcellulase (CMCase) and avicelase were assayed by DNS using 1% (w/v) carboxymethylcellulose in sodium acetate buffer, pH 4.0 and 1% (w/v) avicel in the same buffer, pH 5.0, respectively, while  $\beta$ -glucosidase was detected by *p*-nitrophenolate released using 5 mM pnp- $\beta$ -D-glucoside in sodium citrate buffer, pH 4.5. One unit of enzymatic activity was defined as the amount that liberated 1  $\mu$ mol of product per minute on assay conditions. The results showed that the liquor obtained at 190°C/50 min autohydrolysis was quite favorable to CMCase and avicelase production, since the enzyme production was significantly higher than with other sources. However, the effect of the treatment on  $\beta$ -glucosidase production was not as significant as the control. These results show that by using autohydrolysis liquor as an alternative substrate, the performance of the bioprocess for cellulase production can be improved.

PS5: 15

### Design of a liposome-based chromatographic membrane and its use for final plasmid DNA purification from *Escherichia coli* lysate contaminants

**Marina Eduarda Monteiro, Luis Raiado-Pereira, Marília Mateus, D. Miguel F. Prazeres**

IBB – Institute for Biotechnology and Bioengineering; DBE – Department of Bioengineering, IST-UTL, Portugal

DNA vaccines and gene therapy can overcome several clinical pathologies such as infectious diseases and cancer. Pharmaceutical grade plasmid DNA (pDNA) has the potential to deliver the necessary genes for these therapies. Optimal chromatographic techniques and supports are needed to cope with the increasing demand of highly purified pDNA [1]. Reversed-phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) are able to separate major cell culture contaminants from pDNA. These techniques rely on the hydrophobic interactions between patches on the surface of biomolecules and those at the chromatographic matrix. However, the elution in RPC depends on less polar organic solvents like methanol, while in HIC elution succeeds with more polar and less denaturing environments [1,2]. With membrane adsorbers chromatography efficiency increases, as selectivity and productivity improves due to minimal diffusive mass transfer limitations. A specific application on pDNA purification with HIC alkyl ligands was already published [3]. The present work reports the functionalization of a pre-activated commercial membrane support with unilamellar liposomes of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and the testing of the chromatographic performance of the derived membranes on pDNA purification. The DOPE membrane derivatization was based on chloroformate activation which was preceded by membrane preparation steps. The efficiency of lipidic bonding to the precursor membranes was followed by phosphorous analysis. A high phosphorus content was found in derivatized membranes that relates to a high density of DOPE ligands at the membrane surface ( $1.7 \mu\text{eq cm}^{-2}$ ) as compared to the active sites density Sartobind® Aldehyde precursor membrane ( $< 0.1 \mu\text{eq cm}^{-2}$ ). Parallel chromatographic runs over identical DOPE membrane adsorbers allowed comparison between RPC and HIC operations in pDNA purification. Using HIC, the model plasmid pVAX1-LacZ (6050 bp) was completely separated from remaining impurities in clarified *E. coli* cell lysates (specifically RNA), unlike with RPC which rendered a less impressive purification efficiency. This work demonstrates the feasibility of HIC with a new liposome-based membrane adsorber for the pDNA downstream purification process.

[1] Diogo et al (2003) J. Chromatogr. A 1069:3-22 [2] GE Healthcare Handbook 11-0012-69 [3] Raiado-Pereira et al (2010) J. Sep. Sci. 33:1175-1184

PS5: 17

### Insights into the interactions that control the phase behaviour of novel aqueous biphasic systems composed of polyethylene glycols and Ionic Liquids

**Jorge F. B. Pereira<sup>1</sup>, Mara G. Freire<sup>2</sup>, Maria Francisco<sup>3</sup>, Hector Rodriguez<sup>3</sup>, Luis Paulo N. Rebelo<sup>2</sup>, Robin D. Rogers<sup>4</sup>, João A. P. Coutinho<sup>1</sup>**

<sup>1</sup>University of Aveiro, Portugal; <sup>2</sup>University of Nova Lisboa, Portugal; <sup>3</sup>University of Santiago de Compostela, Spain; <sup>4</sup>The University of Alabama, USA

Novel (polyethylene glycol)/(ionic liquid) aqueous biphasic systems (PEG/IL ABS) are presented in this work. Distinct pairs of PEGs and ionic liquids are able to induce phase separation in aqueous media when dissolved at appropriate concentrations. Phase diagrams have been determined for a large array of systems at (298, 308 and 323) K. Comparison of the binodal curves allowed the analysis of the tuneable structural features of the ionic liquid (anion nature, cation core, cationic side alkyl chain length and functionalization, and number of alkyl substituents at the cation), and the influence of the PEG molecular weight, on the ability of these solutes to induce ABS. It was observed that, contrarily to typical ABS based on ionic liquids and inorganic salts, where the phase behaviour is dominated by the formation of the ions' hydration complexes, in the polymer-type ABS here studied, the interactions between PEGs and ionic liquids control the phase demixing. It is shown that both the IL and the PEG can act as the salting-out species – a performance that is dependent on the ionic liquid structural features. For the first time, PEG/IL ABS are reported and insights into the major interactions that govern the polymer/IL phase behaviour in aqueous media are provided. The use of two different non-volatile species (ILs and PEGs) to form ABS allows the tailoring of the phases' polarities [1]. Hence, the development of environmentally friendly separation processes making use of these novel systems is directly envisaged.

[1] Freire et al J.A.P. Chem. – Eur. J.(2011) DOI: 10.1002/chem.200.

PS5: 18

### Delayed fatty acid uptake by a fatty acid secreting yeast strain with modified peroxisomal metabolism

**Gabriela Filipa Ribeiro<sup>1</sup>, Manuela Côrte-Real<sup>1</sup>, Alberto Dias<sup>2</sup>, Björn Johansson<sup>1</sup>**

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Biodiesel, in the form of fatty acid esters, produced by oleaginous microorganisms, could represent an attractive alternative renewable liquid biofuel. In the model yeast *S. cerevisiae* lipid metabolism has been studied extensively and all genes encoding enzymes directly involved in lipid synthesis are known. In this organism exogenous long-chain fatty acids are activated to coenzyme A derivatives prior to metabolic utilization by the fatty acyl-CoA synthetases Faa1p and Faa4p. We use an *FAA1,4* double mutant, that secretes free fatty acids, as a basis for metabolic engineering of this property. However, secreted fatty acids disappear late in the post growth phase, presumably re-metabolized by the cells (1). ScPox1p is the ortholog of the human acyl-CoA oxidase 1, it catalyzes the first metabolic step of fatty acid beta-oxidation and its expression is strongly induced by fatty acids. *S. cerevisiae* ScPox1p null mutants are unable to grow on fatty acids as sole carbon source (2). A triple knock-out mutant *faa1/4-Δ1 pox1-Δ1* studied in this work shows delayed uptake of the secreted fatty acids. The fatty acid production of the modified strains was analysed by optical density of the extracellular medium and gas chromatography and results are discussed.

1. Scharniewski et al *FEBS J.* 275 (2008) 2765–2778. 2. van Roermund et al *EMBO J.* 17(3) (1998) 677–687. This work was supported by FEDER through POFC – COMPETE and by national funds from FCT - project PEst-C/BIA/UI4050/2011. G.R. was the recipient of a FCT fellowship SFRH/BD/42565/2007.

PS5: 21

**Carob pulp as feedstock for bioethanol production****Diana Francisco, Susana Marques, Patricia Branco, Francisco Girio, Helena Albergaria**

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Carob tree (*Ceratonia siliqua*) is an evergreen shrub that is typically grown in the Mediterranean arid areas. Carob seeds are the raw material for the production of carob bean gum which is used in the food industry. The deseeded material or carob pulp represents up to 90% of the fruit dry weight and contains high amounts of easily extractable sugars (40-50% w/w). Thus, carob pulp is a highly suitable raw material to produce fermentable syrups. In addition, carob pulp also contains a lignocellulosic fraction that can be hydrolyzed and fermented. In the present work, we investigated the feasibility of using all carob pulp sugars, i.e. water-soluble sugars (44% w/w) and cellulosic sugars (14% w/w) to produce bioethanol. In a first stage, soluble sugars (i.e. sucrose, glucose and fructose) were extracted from carob pulp by direct contact with water using a liquid/solid (L/S) ratio of 2.0 and different times/temperatures of contact (37°C, overnight and 30°C for 3h). Carob syrups were then collected and 80% of the liquid was recovered after pressing the wet solids with an hydraulic press. The extracted solids were, after extensive washing with water (30 L water/1 kg extracted solids), dried (4% moisture), milled (grid of 0.5 mm) and packaged at vacuum. The lignocellulosic composition of the extracted carob solids (14% cellulose, 7.4% hemicellulose and 24.4% Klason lignin) was determined by quantitative acid hydrolysis. Carob syrups containing 160 g/L of total sugars were then used in batch fermentations (30°C at 150 rpm of agitation) and three *S. cerevisiae* strains were screened for ethanol production. The best fermenting strain produced 63 g/L of ethanol, exhibiting an ethanol yield of 0.45 g/g, a global productivity of 1.86 g/L h and a global yield of 200 L of ethanol *per* ton of carob pulp. Extracted carob solids were hydrolysed using different acids (nitric, sulphuric and chloridric acid) at a concentration of 0.16 N, several concentrations of solids (50, 100 and 200 g/L) and different operating conditions (15 and 30 min at autoclave). The best results were obtained with nitric acid at a solid concentration of 50 g/L which originated hydrolysis yields of 67% for glucan, 81% for xylan and 96% for arabinan. These hydrolysates were tested as fermentation media using the above-mentioned selected strain. Our work showed that carob pulp, likewise sugar cane, can be seen as a promising, economic and non-competing for food feedstock for the production of bioethanol.



## PS5: 23

**Bioaugmentation strategies to enhance long chain fatty acids (LCFA) conversion to methane****Sérgio Silva, Ana Júlia Cavaleiro, Madalena Alves, Diana Sousa**

Institute for Biotechnology and Bioengineering - Centre of Biological Engineering, Portugal

Bioaugmentation of bioreactors with LCFA-degrading bacteria is a possibility for improving methane production from lipid-rich wastes/wastewaters. Cavaleiro et al. [1] has shown that methane production from oleate (unsaturated LCFA) is faster and more efficient in batch tests bioaugmented with *Syntrophomonas zehnderi*, a bacterium that is able to degrade a wide range of both saturated and unsaturated LCFA [2]. In this work, anaerobic sludge bioaugmentation with *S. zehnderi* was studied in order to evaluate: (I) the recovery of bioreactors after an episode of LCFA overload; (II) the potential for decreasing reactor start-up periods. The potential of using *S. zehnderi* for recovering LCFA-overloaded sludge was tested using anaerobic sludge collected from a oleate-fed bioreactor at three different operation times. Bioaugmentation batches were prepared with LCFA loaded biomasses in the presence of *S. zehnderi*. Controls were set using inactivated *S. zehnderi*. Methane yields of 72, 53 and 40% were obtained from the first, second and third collected sludge samples respectively. However, addition of *S. zehnderi* did not significantly improve LCFA conversion loaded-sludges as similar yields were achieved in non-bioaugmented controls. Fed-batch bioreactor start-up, using a non-acclimated sludge, was attempted in the presence of *S. zehnderi*. Assays were conducted in the presence and absence of both a solid microcarrier (sepiolite) and a substoichiometric amount of ferric hydroxide. Blank (no oleate) and control assays (inactivated *S. zehnderi*) were also prepared. Bioaugmentation assays with sepiolite and ferric hydroxide showed the highest methane yield, with an observed methane yield 16% higher than in non-bioaugmented controls. The potential of bioaugmenting *S. zehnderi* as means to recover methanogenic activity of LCFA-loaded biomass was not demonstrated. However faster reactor start-up could be accomplished since higher methane yield was achieved in bioaugmented fed-batch assays in the presence of sepiolite with ferric hydroxide.

[1] Cavaleiro et al 2010. *Water Res* 44:4940-7. [2] Sousa et al 2007. *Int J Syst Evol Microbiol* 57:609-15. This study has been funded by FEDER, through the COMPETE program, and by Portuguese funds, through Portuguese Foundation for Science and Technology (FCT), in the frame of the project FCOMP-01-0124-FEDER-014784.

PS5: 25

### **Microalgae – Sunlight-driven cell factories for biofuel production**

**Bruno Fernandes, Giuliano Dragone, José Teixeira, António Vicente**

University of Minho, Portugal

The biofuel production from photosynthetic microorganisms is considered as a process to produce renewable energy for global warming mitigation. Recently, microalgae have received much attention as a renewable energy resource because the photoautotrophic mechanism can convert atmospheric CO<sub>2</sub> into biomass. Microalgae can provide several different types of renewable biofuels. These include biodiesel from lipids, bioethanol from sugars, methane produced by anaerobic digestion and photobiologically produced biohydrogen. The idea of using microalgae as a source of fuel is not new but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels. Many research works have been conducted to produce microalgae as a source lipids and starch for biofuel production, however, for mass production of bio-fuel, the economic feasibility of microalgal culture greatly depends on the productivity of biomass and lipids. Thus, our research and development activities, in order to maximize the productivity of microalgae cultivation systems, are focused on the design of a new and more efficient photobioareactor and the development of cultivation techniques such as mixotrophic growth and two-step cultivation systems. Our results show that it is possible to achieve an increase of up to eight times the productivity in terms of biomass, lipids and starch using these strategies.

PS5: 26

### Molecular characterization of microbial populations from UASB reactors treating dairy industry wastewaters

**André M Grilo**<sup>1</sup>, **Christian G Ramos**<sup>1</sup>, **Sílvia A Sousa**<sup>1</sup>, **Helena Nadais**<sup>3</sup>, **Jorge H Leitão**<sup>1,2</sup>

<sup>1</sup>IBB- Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, IST, Lisbon, Portugal; <sup>2</sup>Department of Bioengineering, Instituto Superior Técnico (IST), Lisbon, Portugal; <sup>3</sup>CESAM and Department of Environment and Planning, University of Aveiro, Aveiro, Portugal

Up-flow anaerobic sludge bed (UASB) reactors are being increasingly used in wastewater treatment. Methane, a renewable energy source, is produced during the process [1]. The treatment of complex fat containing effluents, as is the case of those originating from dairy industries, presents operational difficulties like scum forming, sludge de-granulation, biomass wash-out and loss of biological activity. The interruption of the reactor feed for pre-determined periods of time, known as intermittent operation, causes a forced adaptation of the biomass towards the degradation of complex substrates [1]. Previous work has shown the occurrence of morphological changes in the microbial community present in samples from UASB reactors when operated intermittently [2]. In order to characterize and compare the microbial populations inside UASB reactors operating either continuous or intermittently, we have adopted a strategy involving the design of 16S-based primers specific for the most relevant microbial phyla, amplification of 16S RNA genes, cloning, sequencing and comparison with sequences deposited in databases. To prevent the sequencing of identical clones, a RFLP-based methodology was adopted to distinguish clones [3]. We also report data on the quantification of the key metabolic groups, assessed based on FISH methodologies using specific probes. Results indicate an increase in the populations of *Syntrophobacter*, Euryarchaeota and Crenarchaeota in the UASB reactor operated intermittently, correlating with an increase in the methane production yield.

[1] Nadais et al (2005) *Water Res* 39:1511-1516 [2] Nadais H, Barbosa M, Capela I, Arroja L, Ramos CG, Grilo AM, Sousa SA, Leitão JH (2011) *Energy* 36:3164- 3168 [3] Ramos CG, Grilo AM, Sousa SA, Barbosa ML, Nadais H, Leitão JH (2010) *Appl Microbiol Biotechnol* 85:801-806. Funding is acknowledge to FCT (contract PTDC/AMB/65025/2006 and Doctoral grant to AMG), and a Doctoral grant to CGR from Fundação Calouste Gulbenkian.

PS5: 27

**Biomethanation of rice straw-pretreatment using *Pleurotus florida* fungi.****Gopinathan Chengalath, Tai Karthikey**

University of Calicut, Kerala, India

Lignocelluloses are often a major or sometimes the sole components of different waste streams from various industries, forestry, agriculture and municipalities. Hydrolysis of these materials is the first step for either digestion to biogas (methane). However, enzymatic hydrolysis of lignocelluloses with no pretreatment is usually not so effective because of high stability of the materials to enzymatic or bacterial attacks. Effective parameters in pretreatment of lignocelluloses, such as crystallinity, accessible surface area, and protection by lignin and hemicellulose are the most important ones. Although several pretreatment methods are available, their effects on improvement in biogas production are not optimised. They include milling, irradiation, microwave, steam explosion, ammonia fiber explosion (AFEX), supercritical CO<sub>2</sub> and its explosion, alkaline hydrolysis, liquid hot-water pretreatment, wet oxidation, ozonolysis, dilute-and concentrated-acid hydrolysis, and biological pretreatments. Rice straw is available in huge quantities, especially in tropical countries, which are at present not properly utilized. Pretreatment of Rice straw exposes the cellulose making it accessible to cellulase enzyme. Both chemical and biological pretreatments are possible. Exposing rice straw to 1 % alkali (sodium hydroxide) and mixing with urea enhances biogas production. But chemical pretreatment causes pollution when used in large scale plants. Growing *pleurotus florida* fungi in the presence of urea, lactose and cupric chloride improves biogas production from rice straw. Urea is used to optimize the carbon:nitrogen ratio of the substrate. Lactose and cupric chloride enhances secretion of laccase enzyme, which delignifies rice straw, without sodium hydroxide.

# Molecular Microbiology and Microbial Physiology

*Poster session thematic symposium 6*

Jorge | Godinho | Pereira | Alarico | Pessoa | Feliciano  
| Azevedo | Pinto | Talaia | Rego | Ferreira | Leandro  
Fernandes | Lima | Correia | Thompson | Trindade |  
Sousa | Maranhã | Tulha | Branco | Pacheco |  
Sanches | Pinto | Pires | Oliveira | Beites | Chung |  
Rajaei | Caetano | Jimenez | Silva | Govender | Pobre  
| Cabrito | Cunha | Pereira | Santos | Francisco |  
Proença | Moita | Franco | Santos | Faria | Costa |  
Aghasadeghi | Silva | Oliveira | Malmierca | Rahimi |  
Santos | Pereira | Espadinha | Santos | Marques |  
Cunha | Salvador | Siadat | Menino | Mourato |  
Henriques | Aguiar | Ferreira | Afonso | Trindade |  
Ministro | Carvalho | Costa | Costa | Morais | Barbosa |  
Teixeira | Arêde | Pessoa

PS6: 1

## Mannosylglycerate, a compatible solute widespread in hyperthermophiles, inhibits $\alpha$ -synuclein aggregation in the cytoplasm of yeast cells

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Many neurodegenerative diseases are associated with protein misfolding, aggregation and deposition in the brain, in the form of amyloid. Parkinson's disease is a fatal, incurable disease and the most prevalent neurodegenerative movement disorder. It is characterized by the presence of intracellular inclusions known as Lewy bodies and Lewy neurites. The major component of these inclusions is  $\alpha$ -synuclein, a protein of unknown function. A promising strategy for preventing amyloid deposition relies on the identification of compounds that interfere with the process of fibril formation. Recently, we observed that mannosylglycerate (MG), a compatible solute accumulated by microorganisms in response to osmotic and/or heat stress, prevented aggregation of model-proteins [1]. However, it remains unclear whether these properties are maintained in the cytoplasmic milieu of living cells. Therefore, we use a yeast model for Parkinson's disease to investigate the effect of MG on  $\alpha$ -synuclein aggregation. The yeast strain VSY72, expressing  $\alpha$ -synuclein tagged to EGFP, was used as the host to construct the parental strain carrying the empty plasmid pRS425, and the MG-producer strain harbouring the plasmid pRS425 containing the gene *mgsD* encoding the bifunctional enzyme involved in MG synthesis [2]. Using fluorescence microscopy we found that 40% of the cells from the parental strain contained fluorescent foci, in comparison with the 12% found in the MG-accumulating cells. The MG content in these cells, as determined by NMR, was 80 micromole/ mg of dry weight. Western blot analysis revealed that the level of  $\alpha$ -synuclein and the levels of chaperones, Hsp 104, Hsp 70 and Hsp 40 were the same regardless the presence or absence of MG synthesis. These results suggest that MG prevents  $\alpha$ -synuclein aggregation via a direct effect, rather than indirectly via induction of natural chaperones or activation of proteolytic processes. Additionally, *in vitro* experiments using fluorescence spectroscopy and transmission electron microscopy showed that MG, at a concentration of 100 mM, was able to inhibit considerably amyloid formation. It is concluded that MG acts as a chemical chaperone *in vivo*, preventing  $\alpha$ -synuclein aggregation. We propose that the accumulation of MG in hyper/thermophiles is part of the cell strategy to protect protein structures at elevated temperatures.

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PS6: 2

## A new translational repression element controls the expression of *araL* within the *Bacillus subtilis* *ara* operon

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AraL from *Bacillus subtilis* is a member of the ubiquitous haloalkanoate dehalogenase, HAD, superfamily. AraL is encoded by the fourth cistron of the arabinose metabolic operon *araABDLMN PQ-abfA* of *B. subtilis*, which is driven by a very strong promoter primarily regulated at the transcriptional level by the regulator AraR in response to the presence of arabinose [1]. Members of the ubiquitous haloalkanoate dehalogenase, HAD, superfamily IIA and IIB, are characterized by broad-range and overlapping specificity [2, 3]. AraL was shown to have low specificity and catalytic activity towards several sugar phosphates, which are metabolic intermediates of the glycolytic and pentose phosphate pathways. On the basis of substrate specificity and gene context within the arabinose metabolic operon, a putative physiological role of AraL in the detoxification of accidental accumulation of phosphorylated metabolites has been proposed [4]. AraL low specificity and catalytic activity towards several sugar phosphates anticipated the need for an additional level of gene regulation within the *ara* operon. In this work, we found a secondary mRNA structure placed at the 5'-end of the *araL* gene that encompasses the ribosome-binding site. Using ectopic *araL-lacZ* translational fusions under the control of the strong *ara* operon promoter we show that this hairpin structure plays an active role in the control of *araL* translation. Furthermore, site directed mutagenesis analysis indicates that the regulatory role of this element is structural and not sequence specific. This second level of regulation within the transcriptional unit will drastically reduce the production of AraL and the details of this control mechanism will be discussed.

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PS6: 3

**Activation of autoinducer-2 internalization and processing in enteric bacteria requires the phosphotransferase system****Catarina S. Pereira<sup>1,2</sup>, António J. Santos<sup>3</sup>, Paulo B. Correia<sup>1,2</sup>, Michal Bejermano-Sagie<sup>4</sup>, João C. Marques<sup>1,2</sup>, Karina B. Xavier<sup>1,2</sup>**<sup>1</sup>Instituto Gulbenkian de Ciência, Oeiras, Portugal; <sup>2</sup>Instituto de Tecnologia Química e Biológica, Oeiras, Portugal; <sup>3</sup>Imperial College, London, UK; <sup>4</sup>Hebrew University-Hadassah Medical School, Jerusalem, Israel

Autoinducer-2 (AI-2) is a signal molecule produced by a wide range of phylogenetically distant microorganisms which enables inter-species cell-cell communication and regulates several bacterial phenotypes. Certain bacteria can interfere with AI-2-regulated behaviors of neighboring species by incorporating extracellular AI-2 using the Lsr transport system (encoded by the *lsr* operon). Once inside the cell AI-2 is phosphorylated by the LsrK kinase. AI-2-phosphate (AI-2-P) is the inducer of the *lsr* operon: it acts by binding the LsrR transcriptional repressor which leads to derepression of the operon. Here we show that the phosphotransferase system (PTS) is required for Lsr activation and is essential for AI-2 internalization. Phosphorylation of Enzyme I (EI) from PTS is necessary for AI-2 incorporation but is not required for AI-2 phosphorylation, as even in the presence of PTS LsrK is essential for this phosphorylation to occur. We also show that the requirement for PTS in the activation of *lsr* transcription is via LsrR and is AI-2-dependent. Overall our results suggest that to initiate AI-2 internalization and intracellular processing, AI-2 has to be first incorporated by a PTS-dependent mechanism, whether directly or indirectly, which relieves *lsr* repression by intracellular AI-2-P. The Lsr transporter is expressed, which starts a positive feedback loop with the consequent fast removal of AI-2 from the extracellular medium. The fact that AI-2 internalization is dependent on both the AI-2-induced Lsr transporter and the PTS could represent a cell strategy to integrate information about its physiological state and, according to that, regulate AI-2 signal incorporation.



PS6: 4

### A new bacterial hydrolase specific for the compatible solutes mannosylglycerate and glucosylglycerate

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Some hyper/thermophilic bacteria and archaea accumulate the compatible solute (CS) mannosylglycerate (MG) during salt stress, while the related solute glucosylglycerate (GG) initially proposed to have a more restricted distribution and an ambiguous role as a true CS, now seems to be widespread among prokaryotes (1). Mannosylglycerate is a major CS in the thermophilic bacteria *Rubrobacter xylanophilus* and *Thermus thermophilus* HB27 (2, 3). Glucosylglycerate is a CS in bacteria and archaea under nitrogen deprivation and it was also found in polysaccharides and glycolipids (1). The limited information on the osmoadaptation mechanisms in these organisms, from the uptake and accumulation of MG or GG to their release to the environment and recycling, led us to search for an enzyme committed to their hydrolysis (4). From the analysis of the genomes of several MG- and GG-accumulating microorganisms we were able to identify a likely candidate gene for such function. We expressed the gene from the MG-accumulating bacteria *Thermus thermophilus* HB27 and *Rubrobacter radiotolerans* RSPS-4, and confirmed that the recombinant enzymes specifically hydrolyzed MG (and GG), to mannose (or glucose) and glycerate. Both enzymes were maximally active at the organisms' optimal growth temperatures and at low pH (4.0 to 4.5); the catalytic efficiencies towards MG and GG were comparable. This is the first report on the identification and characterization of highly specific MG- and GG-hydrolyzing enzymes.

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PS6: 5

### Discovery and initial characterization of members of the new YaaH family of microbial acetate transporters

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The emergence of probiotics and prebiotics has revived the importance of short-chain fatty acids (SCFAs) associated to colonic and systemic health improvement. Although biosynthesis and degradation of SCFAs and other short-chain carboxylic acids, such as lactate, pyruvate or citrate are well understood, the transport of these acids is still a matter of discussion. The presence of SCFAs transporters in cellular membranes is ubiquitous, displaying great level of homology among Bacteria, Archaea and Eukaryotes, indicating the ancient nature of these transporters and their high level of conservation. The mechanism of substrate uptake of these transporters including specificity, kinetics and bioenergetic studies is a field poorly explored. This work constitutes a first approach to establish the mode of action of the SCFA transporters specifically those belonging to the YaaH family. The YaaH family, (TC# 2.A.96, <http://www.tcdb.org/tcdb>) is presumed to be a family of acetate transporters. Its members possess 6 putative transmembrane span domains and are spread by the 3 domains of life: Bacteria, Eukaryotes and Archaea. This work aims at studying the YaaH protein from *E. coli* as well as its homologues from the yeast *Saccharomyces cerevisiae* (ScAdy2) and the fungi *Aspergillus nidulans* (AcpA). We have constructed a disrupted *E. coli* strain in the yaaH gene showing that the yaaH mutant cells are compromised for the uptake of the labelled acetic acid in comparison with the isogenic wt strain. This is the first experimental data that demonstrates the physiological role of the yaaH gene in the transport of acetate in bacteria. Using the yeast and fungi strains we were able to measure the kinetic parameters associated with these transporters and assign a specificity profile to this family. These transporters are specific primarily to acetate and are inhibited by other short chain acids such as benzoic, formic, propionic and butyric acids.

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PS6: 6

### Functional characterization of the BceN protein involved in GDP-D-rhamnose biosynthesis by the *Burkholderia cepacia* complex

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GDP-D-rhamnose is the donor of the D-rhamnose sugar moiety that is mainly found in bacterial structures such as lipopolysaccharides and extracellular polysaccharides. This sugar nucleotide is necessary for the synthesis of the exopolysaccharide Cepacian [1], composed of a branched acetylated heptasaccharide repeating unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, in the ratio 1:1:1:3:1 [2]. Cepacian is produced by environmental and human, animal and plant pathogenic isolates, belonging to several *Burkholderia* species [3]. This EPS has been pointed out as a virulence factor that is involved in persistence of these bacteria in CF lungs, interaction with antimicrobial peptides and formation of biofilms, contributing to the full pathogenicity of Bcc bacteria [4]. The enzyme activities GDP-4-keto-6-deoxy-D-mannose reductase (RMD) and GDP-D-mannose-4,6-dehydratase (GMD) are both required for the conversion of GDP-D-mannose into GDP-D-rhamnose. In Bcc bacteria, these two enzyme activities are performed by the enzymes BceM and BceN, respectively [3]. To perform the kinetic characterization of BceN, the encoding gene was cloned in an expression vector, and the protein overexpressed as a histidine-tagged derivative and purified to homogeneity by affinity chromatography. The GDP-D-mannose-4,6-dehydratase activity was determined by NMR spectroscopy analysis. Results obtained revealed that the BceN protein has GDP-D-mannose-4,6-dehydratase activity and is substrate inhibited. In addition, to understand the role of the BceN protein in *Burkholderia cepacia* physiology, we constructed a *bceN* insertion mutant. The lack of a functional *bceN* gene in the mutant reduced EPS production yield, the ability to form biofilms and the swarming motility. The in silico search for putative *bceN* homologues revealed the presence of 1 to 3 *bceN* orthologues in the *Burkholderia* genomes available.

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PS6: 7

**MAPK signaling pathways mediate acetic acid-induced cell death in *Saccharomyces cerevisiae*****Flávio Azevedo, Susana Rodrigues Chaves, Björn Johansson, Maria João Sousa, Manuela Côrte-Real**

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Mitogenic Activated Protein Kinase (MAPK) cascades are important signaling pathways that allow yeast cells to swiftly adapt to a changing environment. These pathways regulate various important processes, from proliferation and differentiation to cell death. MAPK cascades normally contain three protein kinases that act in sequence: a MAP kinase kinase kinase (MAPKKK, MAP3K, MEKK or MKKK), a MAP kinase kinase (MAPKK, MAP2K, MEK or MKK) and a MAP kinase (MAPK). *Saccharomyces cerevisiae* contains five MAPKs (Fus3p, Kss1p, Hog1p, Mpk1p, Smk1p) on five functionally distinct cascades, associated with mating, invasive growth/pseudohyphal development and cell wall integrity, high osmolarity, and sporulation. It has been shown that deletion of the MAPK Hog1p leads to impaired growth of *S. cerevisiae* on solid medium containing acetic acid. Hog1p directly phosphorylates the aquaglyceroporin channel Fps1p, targeting it for endocytosis and degradation in the vacuole. Accordingly, *fps1Δ* mutant cells grow better than wild type cells on solid medium containing acetic acid. However, it is still not known what role other MAPK pathways play in signaling acetic-acid induced apoptosis. In the present work, we describe the involvement of MAPK pathways in acetic acid-induced cell death. Several mutants deleted for components of these pathways were constructed and screened for altered phenotypes after incubation with acetic acid. We show there is a decreased sensitivity to acetic acid-induced cell death in the *ste20Δ*, *wsc2Δ* and *wsc3Δ* mutant strains. These results correlated with decreased production of reactive oxygen species and increased cell membrane integrity, assessed by flow cytometry. The data obtained suggested a relationship between MAPK pathways and cell death mediated by acetic acid.

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PS6: 8

**Multidrug resistance mediated by active efflux in *Escherichia coli*: new therapeutic strategies using antibiotics and inhibitors of active efflux****Antónia Pinto<sup>1</sup>, Diana Machado<sup>1,2</sup>, Sofia Santos Costa<sup>1,3</sup>, Isabel Couto<sup>1,3</sup>, Leonard Amaral<sup>1,2,4</sup>, Miguel Viveiros<sup>1,4</sup>**<sup>1</sup>Instituto de Higiene e Medicina Tropical, Portugal; <sup>2</sup>UPMM (IHMT, UNL); <sup>3</sup>Centro de Recursos Microbiológicos (CREM, UNL); <sup>4</sup>COST ACTION BM0701 (ATENS)

Multidrug resistance has become a major health threat. Bacteria developed mechanisms that allow overcome the noxious effects caused by a wide variety of compounds and one of these strategies is to increase the activity of efflux pumps (EPs). This work aims to contribute to: (i) a better understanding of efflux inhibitors (EIs) action mechanisms, (ii) study the competition between EPs substrates and, (iii) evaluate, *in vitro*, combinations of antibiotics and EIs that have the capacity to inhibit EPs activity. For that, we used two *E. coli* strains: strain AG100, which has the efflux system AcrAB-TolC active and strain AG100A, with AcrAB-TolC inactivated. The EIs tested were chlorpromazine, thioridazine, sodium orthovanadate and arylpiperazin, putative ATPase inhibitors, and carbonyl cyanide-m-chlorophenylhydrazone, a proton motive force inhibitor. The EIs capacity to inhibit efflux was assayed by a semi-automated method that detects the accumulation and extrusion of ethidium bromide (EtBr), a known broad-range substrate of EPs. This technique was used to evaluate the activity of these EIs in competition assays between EtBr and antibiotics (tetracycline, the  $\beta$ -lactam oxacillin and the fluoroquinolone ofloxacin). For those EIs with significant real-time efflux inhibitory activity, the minimum inhibitory concentration of the antibiotics was determined, in the presence and absence of the EIs. The results demonstrated that chlorpromazine and sodium orthovanadate were the two EIs with more ability to inhibit efflux of EtBr and to decrease the MIC values of the antibiotics tested. Moreover, the results obtained with the semi-automated method further supports the conclusion that combinations of antibiotic plus EIs results in a synergistic effect allowing the retention of more EtBr inside the cells. This study revealed the relation between *E. coli* antibiotic resistance and active efflux, in particular by the AcrAB-TolC system, and its dependence on the energy provided by the hydrolysis of ATP. In conclusion, EIs might be a new potential target for the development of new synergistic therapies to combat multi-drug resistance in *E. coli*.

PS6: 9

### Pinpointing intracellular trafficking determinants in the Jen1 yeast lactate transporter by domain swap

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The intracellular trafficking of plasma membrane proteins, such as receptors and transporters, in eukaryotic cells is a highly regulated process. Changing environment conditions (nutrients, substrates, hormones) can trigger endocytosis of unwanted transporters. The monocarboxylate transporter Jen1p of the yeast *Saccharomyces cerevisiae* has proven to be an excellent model system for genetically dissecting mechanisms that regulate trafficking of a eukaryotic PM protein, according to physiological constraints. Our group has demonstrated that glucose acts as a signal to induce endocytic down-regulation of Jen1 within minutes, a process dependent both on phosphorylation and ubiquitylation (Paiva *et al*, 2002; Paiva *et al*, 2009). In an attempt to identify domains that are important for the subcellular localization, activity and turnover of Jen1p, domain swap experiments were carried out. The hybrid transporter genes also carry a C-terminal fusion with the ORF of the green fluorescent protein (GFP), which enabled the *in vivo* microscopic investigation of the trafficking, membrane localization and turnover of Jen1p protein. This strategy is ideal to identify whether the Jen1 terminals include molecular determinants necessary and sufficient for glucose elicited endocytosis and sorting to endosomes. Studies regarding the rates of endocytosis and/or direct sorting of the chimeric transporters, under various physiological conditions, will be presented. Uptake assays using radiolabeled lactic acid were also employed to measure the activity of the chimeric transporters, under specific conditions.

PS6: 10

**The involvement of sphingolipids in apoptosis induced by acetic acid in yeast****António Rego<sup>1,2</sup>, Ana Costa<sup>1,3</sup>, Susana Chaves<sup>2</sup>, Pedro Moradas-Ferreira<sup>1,3</sup>,  
Manuela Côrte-Real<sup>2</sup>, Vítor Costa<sup>1,3</sup>**

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The yeast *Saccharomyces cerevisiae* can undergo programmed cell death in response to different stimuli, exhibiting typical apoptotic markers such as externalization of phosphatidylserine, DNA fragmentation, chromatin condensation, cytochrome *c* release from mitochondria and production of reactive oxygen species (ROS). Changes in sphingolipid metabolism have been linked to apoptosis and oxidative stress in both yeast and mammalian cells. Indeed, ceramides have been detected in mitochondria and accumulate upon stress treatments, increasing the permeability of the mitochondria to cytochrome *c* and leading to the generation of ROS. We aimed to characterize the relative contribution of biosynthesis versus catabolism of ceramides to the apoptotic cell death induced by acetic acid in yeast. Yeast cells lacking Lag1p, Lac1p (unable to generate ceramide by de novo synthesis), Isc1p (unable to generate ceramide by degradation of inositolphosphosphingolipids), Ydc1p and Ypc1p (unable to breakdown ceramide) were generated by homologous recombination. Our results show that lag1 and isc1 mutant cells exhibited a higher resistance to acetic acid that was correlated with lower levels of mitochondrial ROS production and reduced alterations of the mitochondrial membrane potential. Associated with these events, there was less translocation of cytochrome *c* to the cytosol in response to acetic acid than in the wild-type strain. Our results suggest that ceramide production contributes to cell death induced by acetic acid, especially through the hydrolysis of inositolphosphosphingolipids catalyzed by Isc1p.

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PS6: 11

## Transporters of arabinooligosaccharides in *Bacillus subtilis*: finding missing pumps

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*Bacillus subtilis* is able to breakdown hemicellulosic polysaccharides present in plant cell wall releasing smaller oligosaccharides and monosaccharides, such as arabinooligosaccharides and arabinose, the main products of the extracellular action of two GH43 endo- $\alpha$ -1,5-arabinanases, AbnA and Abn2, on the homopolysaccharide arabinan [1]. Recently, by combining genetic and physiological analyses we characterized the ABC-type importer AraNPQ and identified primary and secondary transporters of *B. subtilis* involved in the uptake of arabinooligosaccharides. We showed that the AraNPQ importer, belonging to the metabolic operon *araABDLMNPQ-abfA*, is involved in the uptake of  $\alpha$ -1,5-arabinooligosaccharides, with up to at least four L-arabinosyl units, and that this system is energized by the ATPase MsmX [2]. Once inside the cell, these arabinooligosaccharides are further degraded by the action of two GH51  $\alpha$ -L-arabinofuranosidases, AbfA and Abf2 [3]. Although AraNPQ-MsmX is the key transporter for  $\alpha$ -1,5-arabinooligosaccharides, we provided evidence for the presence of additional importers implicated in the uptake of  $\alpha$ -1,2- and  $\alpha$ -1,3-arabinooligosaccharides [2]. Here, by insertion-deletion mutagenesis we identify a new MsmX-dependent ABC-type importer involved in the uptake of nonlinear  $\alpha$ -1,2- and  $\alpha$ -1,3-arabinooligosaccharides. The results show that in addition to AraNPQ both transporters are necessary for an efficient utilization of arabinooligosaccharides. Furthermore, the data assign MsmX as a multipurpose *B. subtilis* ATPase required to energize distinct ABC-type importers, playing a critical role in the efficient utilization of several different saccharides.

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PS6: 12

### A new family of yeast sugar transporters phylogenetically unrelated to the Sugar Porter family

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*Zygosaccharomyces rouxii* and *Z. bailii* can spoil foods and beverages with high concentrations of salt or sugar due to their extreme osmotolerance and resistance to weak-acid preservatives. These yeasts are fructophilic, *i.e.* they consume fructose faster than glucose, whereas the main fermentative yeast, *Saccharomyces cerevisiae*, has a glucophilic behavior. In *Z. bailii* a peculiar sugar transporter, Ffz1, was previously characterized as a specific fructose facilitator, with very low protein identity with other sugar transporters of the Sugar Porter family (SP, that includes hexose facilitators, generally, with higher affinity for glucose than for fructose). Since *Z. rouxii* is similar to *Z. bailii*, we searched for putative Ffz proteins in the *Z. rouxii* CBS 732 genome. Two ORFs encoding putative fructose transporters found in *Z. rouxii* were expressed in a *hxt*-null *S. cerevisiae* strain and characterized. Analysis of sugar transport showed that ZrFfz1 is a high capacity fructose-specific facilitator whereas ZrFfz2 is a facilitator transporting both glucose and fructose with similar capacity and affinity. These two proteins, together with the *Z. bailii* Ffz1 fructose-specific transporter, belong to a new family of sugar transport systems mediating the uptake of hexoses by facilitated diffusion, and are more homologous to drug/H(+) antiporters (regarding their primary protein structure) than to other yeast sugar transporters of the SP family. By performing a database search for proteins similar to the Ffz transporters we identified several putative proteins with a high degree of homology to the Ffz (60–64 % identity) in fungi, probably belonging to the same new sugar-transporter family. In all these members, several highly specific conserved sequence motifs could be found. Based on these motifs, the presence of FFZ-like sequences in several other spoilage yeasts (whose genomes have not yet been sequenced) was screened by PCR, unveiling the probable presence of FFZ-like genes in many of these species. These results reinforce the emergence of a new and important family of sugar transporters.

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PS6: 13

### **A novel universal tool for studying virion and host cell molecular requirements for bacteriophage infection: the case of phage SPP1 DNA entry into *Bacillus subtilis***

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The molecular and biophysical mechanisms underlying viral DNA entry into host bacterial cells at the onset of phage infection remain poorly understood. Until now the study of phage genome entry has been hampered by the lack of a suitable and universal tool to follow phage DNA transit from the virus particle into bacteria. Excepting to *E. coli* phage T7, no tool was available that allowed distinguishing the DNA of virions adsorbed to the bacterial surface from the phage DNA inside cells. Here we present a new, universal method based on RT-PCR that allowed us to specifically detect and quantify the presence of different segments of phage SPP1 genome in the *B. subtilis* cytoplasm, in different physiological conditions. This was only possible because we have genetically separated reversible from irreversible SPP1 adsorption. Using this tool we found that SPP1 DNA entry into host cells is a strictly, energy-dependent process, which is completely abolished by gramicidin D, an agent that dissipates both the electrical ( $\Delta\psi$ ) and pH ( $\Delta\text{pH}$ ) gradients across the host cell membrane. When using selective ionophores such as monensin (dissipates  $\Delta\text{pH}$ ) and valinomycin (dissipates  $\Delta\psi$ ) the amount of cell-internalized phage DNA decreased about 95 and 50%, respectively, indicating a major role of  $\Delta\text{pH}$  in the process. Millimolar concentrations of divalent cations such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are for long known to play a key role in bacteriophage infection, although by an unknown mechanism. We show that micromolar concentrations of  $\text{Ca}^{2+}$  are sufficient for SPP1 irreversible adsorption to host cells. However, these  $\text{Ca}^{2+}$  amounts only allow up to 5% of DNA penetration into host cells; efficient DNA entry requires millimolar concentrations of the cation. This result directly links  $\text{Ca}^{2+}$  requirement to the phage DNA translocation process. We will also use this method to study the role of the host cell molecular machinery in phage DNA entry by measuring the number of SPP1 genomes reaching the *B. subtilis* cytoplasm in presence of rifampicin, kanamycin and HPURa, which specifically inhibit transcription, translation and replication, respectively. Preliminary results with rifampicin show an inhibition of 35% of SPP1 DNA entry into the host cell. In conclusion our results indicate that efficient phage DNA translocation in to host cells cannot rely exclusively in the so called capsid pressure or in the widely held idea of the syringe-like mechanism from the classic Hershey and Chase experiment of 1952.

PS6: 14

**Activity of essential oils on growth and sporulation of aflatoxigenic *Aspergillus* spp.****Fernanda Silva<sup>1,2</sup>, Deila Botelho<sup>3</sup>, Virgínia M. Siqueira<sup>1</sup>, Luis R. Batista<sup>2</sup>, Nelson Lima<sup>1</sup>, Sara Chalfoun<sup>3</sup>**<sup>1</sup>IBB/Centre of Biological Engineering, University of Minho, Braga, Portugal; <sup>2</sup>Federal University of Lavras, Lavras, Minas Gerais, Brazil; <sup>3</sup>EPAMIG - Empresa Agropecuária de Minas Gerais, Brazil

The control of fungal growth and potential mycotoxin contaminations are of interest in food mycology. As a matter of consequence, many strategies have been taken to prevent food born fungal contaminations. New search for biological active secondary compounds present in essential oils of plants has been seen as a potential way to control fungal contamination. The aim of this study was to evaluate antifungal activity of essential oils (EOs) of ginger (*Zingiber officinale*), mint (*Mentha* sp.), sage (*Salvia officinalis*), sweet fennel (*Foeniculum vulgare*) and thyme (*Thymus vulgaris*). Essential oils were obtained from Ferquima Industry and Trade Ltda. The EOs were tested against mycotoxin producers *Aspergillus flavus* and *A. parasiticus* isolated from food products bought in a local market in Lavras, MG, Brazil. Minimum inhibitory concentration (MIC) was determined by solid medium diffusion procedure. FUN-1 fluorescent staining for cell viability test, using broth macrodilution, assay was used. High Resolution Gas Chromatography was applied to analyse chemical constituents of essential oils. Effects on mycelial growth and sporulation were determined for each EO at the concentration established in MIC procedure. After 5, 7 and 9 days the extent of inhibition zone of fungal growth and spore counts in a Neubauer chamber were determined. Trans-anethole, zingiberene, menthol, bornyl acetate and thymol are the major component of essential oils of sweet fennel, ginger, mint, sage and thyme, respectively. MIC for sage, ginger, sweet fennel, mint and thyme were 100%, 80%, 50%, 50% and 50% (oil/DMSO: v/v), respectively. The five essential oils under study have showed an antifungal effect on mycelial growth and fungal sporulation capacity. Thyme was the EO with the best inhibitory effect. Additionally, as FUN-1 staining was applied for the first time to broth macrodilution assay it showed to be a rapid and sensitive method for determine viability of hyphal cells under this assay conditions. In conclusion, essential oils can be an alternative to the use of chemical preservatives.

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PS6: 15

**AI-2 quorum-sensing regulation by the phosphotransferase system in enteric bacteria****Paulo B. Correia<sup>1,2</sup>, Catarina S. Pereira<sup>1,2</sup>, Karina B. Xavier<sup>1,2</sup>**<sup>1</sup>Instituto Gulbenkian de Ciência, Portugal; <sup>2</sup>Instituto de Tecnologia Química e Biológica

Quorum sensing is a cell-to-cell signaling mechanism in which bacteria collectively control gene expression and therefore synchronize behaviors that are more productive at a high population density. The autoinducer-2 (AI-2) signal is produced by many bacteria and enables inter-species communication regulating several phenotypes such as bioluminescence and biofilm formation. In *Escherichia coli*, AI-2 is synthesized and secreted, accumulating in the extracellular medium. The signal concentration rises as bacteria divide in exponential phase and decreases rapidly in early stationary phase. This decrease in extracellular AI-2 concentration is partially due to the expression of an ATP-binding cassette transporter, named Lsr (for LuxS-regulated) and encoded by the *lsrACDB* operon, which uptakes AI-2 removing it from the environment. Recent work from our laboratory showed that the phosphoenolpyruvate-dependent phosphotransferase system (PTS) plays an important role in the regulatory network of the Lsr transporter. Specifically, mutants in the *ptsI* gene, encoding for Enzyme I (EI) of the PTS, do not internalize extracellular AI-2 and do not activate the *lsrACDB* operon. Thus, a PTS-dependent mechanism is required for AI-2 import. In this study we performed a genetic screen to determine the molecular mechanism involved in the regulation of AI-2 internalization via EI by identifying suppressors of the *ptsI* mutation capable of internalizing AI-2. We generated a library of single-gene deletions of all nonessential genes in *E. coli* in a *ptsI* deletion background strain carrying a *lsr-lacZ* promoter fusion and screened this library for mutants that showed *lsr-lacZ* expression higher than the parent strain. This screen has proven to be successful in the identification of suppressors of the *ptsI* mutant and we are currently analyzing the most interesting mutants to understand their role in the interaction between the PTS and the Lsr systems. Understanding how the Lsr transport is dependent on the PTS, can reveal the molecular mechanism through which information about the physiological state of bacteria and regulation of AI-2 signal uptake is integrated.

PS6: 16

### **Analysis of the regulation of cell-cell signalling in enteric bacteria and its functional implications in polyspecies communities**

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Bacteria need to perceive, adapt to and exploit the environment. Other bacteria are an integral part of this environment, explaining the existence of mechanisms to sense and respond to both self and non-self, termed intra- and interspecies communication, respectively. This enables bacteria to govern many behaviours including those associated with virulence according to the composition of the polymicrobial community in which they reside. Interspecies communication relies on the signal, autoinducer-2 (AI-2). The enteric bacteria, *Escherichia coli* and *Salmonella typhimurium*, have a unique system, called Lsr, which enables the removal of AI-2 from the environment. This depletion can interfere with the ability of other bacteria to determine population composition and regulate behaviour accordingly. Correct function of the AI-2/Lsr system produces two phenotypic states in these bacteria: synthesis and export lead to extracellular accumulation of AI-2 during early growth; uptake, processing and removal of the signal is most evident during the entry into stationary phase. We are investigating how this phenotypic switching is regulated through expression and activity of three of the major players in this system: LsrR, LsrK and LuxS. Additionally, we are developing an *in vivo* model using the Lsr system to manipulate the AI-2 levels to which the resident microflora of the mouse gut is exposed. Our goal is to determine the impact of interspecies signalling in this multispecies environment during colonization and homeostasis, and on the properties of the gut microbiota as a protective barrier against pathogens.

PS6: 17

**Assessment of oxidative modification of the Aac2p carrier and its contribution to a PTP-like function in yeast mitochondria****Dário Trindade<sup>1,2</sup>, Stéphen Manon<sup>2</sup>, Maria João Sousa<sup>1</sup>, Manuela Côrte-Real<sup>1</sup>**<sup>1</sup>Centro de Biologia Molecular e Ambiental - CBMA, Portugal; <sup>2</sup>Institut de Biochimie et de Génétique Cellulaires du C.N.R.S. - IBGC, France

Mitochondria outer membrane permeabilization (MOMP) and cytochrome *c* (cyt *c*) release have been considered crucial steps of the intrinsic apoptotic pathway. Different mechanisms underlying such mitochondrial events have been suggested involving pro-apoptotic members of the Bcl-2 protein family, putative components of the permeability transition pore (PTP) or both. Recently, the ADP/ATP carrier (AAC), an yeast orthologue of mammalian adenine nucleotide translocator (ANT), has been identified as a necessary element for MOMP and cyt *c* release in yeast cells exposed to acetic acid. Such role of the AAC might be triggered by oxidative modifications mediated by ROS. This work aims to understand whether oxidative modifications to the Aac2p may contribute to MOMP and consequent release of cyt *c*, during acetic acid induced programmed cell death (PCD) in *Saccharomyces cerevisiae*. Since ADP/ATP translocases are known to be sensitive to oxidative stress, especially in the thiol groups formed by cysteine residues, *S. cerevisiae* strain JL1-3, lacking the three forms of the AAC, was transformed with two different copies of the AAC2 gene, a wild-type (*wt*) and a cysteine-less AAC2 (*CL*). Data collected in this work suggests that the cysteines in the Aac2p are not responsible, at least by themselves, for inducing a biochemical change capable of leading the Aac2p to a PTP-like function in yeast mitochondria. Survival assays indicate that there is no significant difference between the *wt* and *CL* strains tolerance to acetic acid. The membrane integrity and the mitochondrial membrane potential were also compared by PI exclusion and DiOC6 staining, respectively. Both experiments show no physiological differences between the death process of the *wt* and *CL* mutants. The levels of ROS, evaluated by DHE staining, are also identical in both cell types. Finally, Western-Blot detection of cytochrome *c* in mitochondria extracts, and the corresponding cytosolic fractions, demonstrated that the *CL* mutation does not prevent MOMP. Our data indicates that the absence of the cysteines in the Aac2p, has no impact in the MOMP of *S. cerevisiae* cells during acetic acid-induced death. However, it is possible that a different set of residues, or a specific region of the ADP/ATP carrier, might be responsible for such process in yeast.

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PS6: 18

### Exploiting the type II phosphomannose isomerase BceA as a target for the development of new antimicrobials against *Burkholderia cepacia* complex bacteria.

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Type II phosphomannose isomerases (PMIs) are bacterial bifunctional enzymes with both PMI (E.C.5.3.1.8) and guanosine diphosphate (GDP)-D-mannose pyrophosphorylase (GMP) (E.C.2.7.7.22) activities [1]. These proteins catalyse two non-contiguous steps in the synthesis of GDP-D-mannose, the donor of the mannose moiety for several bacterial structures, which are essential for survival and pathogenesis, such as glycoproteins, glycolipids, and the cell wall components lipopolysaccharides (LPS) and exopolysaccharides (EPS) [1]. The *bceA* gene, encoding a type II PMI, is the first ORF of a gene cluster encoding enzymes and proteins required for the biosynthesis of the EPS Cepacian by *Burkholderia cepacia* complex(Bcc) [2]. A major concern associated with Bcc infections is their intrinsic resistance to most antimicrobials, rendering their eradication very difficult [3]. Therefore, new strategies for battling these opportunistic pathogens are needed. Similarly to other type II PMIs, BceA exhibits a low amino acid identity to the human PMI. However, limited information is currently available for type II PMIs, limiting the possibility of the rational development of specific inhibitors. Due to the roles played by type II PMIs in bacterial physiology, research work on the rational design of inhibitors was initiated. The kinetic characterization of this protein has been studied by our research group [4]. Ongoing work aims the structural characterization of BceA, with the objective to design specific inhibitors against the protein enzymatic activities. We also report results from site directed mutagenesis experiments of selected amino acid residues thought to be involved in the protein regulation. The enzyme kinetics of these modified proteins will also be presented.

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PS6: 19

### Fungal pellets of *Anthracophyllum discolor* with different formulations to improve biological activities in a biomixture under atrazine application

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White-rot fungi are well known as pesticides degrader due to their ligninolytic enzymes and can be used for bioaugmentation to improve the biodegradation of organic contaminants in the soil. One of the barriers to successful implementation of fungal bioaugmentation is the development of inexpensive and high quality fungal pellets. Furthermore, the effect of fungal pellets on biological activities and microbial communities in biomixture is scarcely known. Biologically active soils (biobeds) are made with a biomixture of lignocellulosic supports, peat and soil to offer an alternative method for pesticide wastes treatment, spraying tank remnants and washates. They are simple to operate and cost effective on-farm systems in which the efficiency is based on their increase capacity to retain and microbially degrade pesticides. Therefore, the aim of this study is evaluate the effect of different formulations of fungal pellets in a biomixture contaminated with the atrazine pesticide. The fungal pellets of *Anthracophyllum discolor* were formulated with 3 different supports, based on lignocellulosic, oligosaccharides and salt (F1, F2, F3) materials. The biomixture was prepared by mixing an allophonic top soil (Andisol), commercial peat, wheat and barley straw in a volumetric proportion of 1:1:1:1 and was inoculated with the three differents formulated fungal pellets of *Anthracophyllum discolor* (10% w/w). The biomixture was contaminated with 60 mg Kg<sup>-1</sup> of atrazine. After 30 days of incubation at 20 °C, the total ligninolytic enzyme activity, fluorescein diacetate activity (FDA) and respiratory activity were studied. The concentration of atrazine was measured by HPLC. The biodegradation of atrazine was 99% for F1 and 95% for F2 and F3 supports, being slightly higher (5%) than the control (biomixture non-inoculated with fungal pellets). At 30<sup>th</sup> day the FDA activity was similar for all supports; the respiration (8866 ± 201.33 mg CO<sub>2</sub> g<sup>-1</sup>) and total ligninolytic enzyme activity (0.78± 0.1 U kg<sup>-1</sup>) were higher for support F1 when compared with F2 (8393±204.2 mg CO<sub>2</sub> g<sup>-1</sup>; 0.54± 0.1 U kg<sup>-1</sup>) and F3 (8371±190.1 mg CO<sub>2</sub> g<sup>-1</sup>; 0.48± 0.1 U kg<sup>-1</sup>). In conclusion, the formulation F1 promotes better performance as support for fungal pellets to be used on biomixtures contaminated with atrazine pesticide.



PS6: 20

**New insights into the biosynthesis of the mycobacterial MGLP arising from the identification of a novel glucosyl-3-phosphoglycerate phosphatase****Vitor Mendes<sup>1</sup>, Ana Maranhã<sup>1</sup>, Susana Alarico<sup>1</sup>, Milton Costa<sup>1,2</sup>, Nuno Empadinhas<sup>1,2</sup>**<sup>1</sup>Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; <sup>2</sup>Department of Life Sciences, University of Coimbra, Coimbra, Portugal

Mycobacteria synthesize unique methylglucose lipopolysaccharides (MGLPs), proposed to modulate fatty acid synthesis (1). Despite the comprehensive characterization of their structure and composition, the identity of most of the genes involved in their biosynthesis remains undeciphered. We have recently identified the gene for the 1<sup>st</sup> step in this pathway and characterized the corresponding enzyme, glucosyl-3-phosphoglycerate synthase (GpgS) (2). Since this gene was deemed essential for the growth of *Mycobacterium tuberculosis* (3), we have determined the 3D structure of the GpgS (4), establishing an experimental scaffold for the design of specific inhibitors to act as anti-mycobacterials. However, a typical glucosyl-3-phosphoglycerate phosphatase (GpgP, EC 3.1.3.70) for the deduced 2<sup>nd</sup> step in the MGLP pathway, the dephosphorylation of glucosyl-3-phosphoglycerate (GPG) into glucosylglycerate (GG), was absent from mycobacterial genomes. To dissect the early steps in MGLP biosynthesis we probed mycobacterial extracts for this activity. We report the purification, identification and characterization of the mycobacterial GpgP, which is highly specific for GPG, but sequence unrelated to any of the homo-functional GpgPs characterized to date. The functional assignment of GpgP in the genome of *M. tuberculosis* furthers our understanding of this intricate metabolic trait and might help us devise new strategies to fight tuberculosis.

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PS6: 21

**Programmed cell death processes in *Saccharomyces cerevisiae* are altered by GUP1 deletion.****Joana Tulha, Fábio Faria-Oliveira, César Costa, Cândida Lucas, Célia Ferreira**

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During the past years, yeast has been successfully established as a model to study mechanisms of programmed cell death regulation. *Saccharomyces cerevisiae* commits to cell death showing typical hallmarks of metazoan apoptosis, in response to different stimuli. Gup1p, an O-acyltransferase, is required for several cellular processes that are related to apoptosis development, such as rafts integrity and stability, lipid metabolism including GPI anchor correct remodeling, proper mitochondrial and vacuole function, bud site selection and actin dynamics. We used two known apoptosis inducing conditions, chronological aging and acetic acid, to assess several apoptotic markers in *gup1Δ* mutant strain. We found that this mutant presents a significantly reduced chronological life span, comparing to Wt and it is also highly sensitive to acetic acid treatment. Although both chronological aging and acetic acid lead to identical effects, the differences between the strains in the levels/types of apoptotic markers are notorious. In addition, ROS levels of *gup1Δ* mutant strain were extremely high. According to our results, cells lacking *GUP1* seem to be incapable of undergoing apoptosis. Instead this mutant appears to be experiencing a necrotic cell death process. Gup1p has been described to have an important function on lipid rafts assembly/integrity as well as on cell lipid profile. On the other hand, in the literature, rafts have been increasingly implicated on apoptotic signaling. The present results reinforce such idea.

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PS6: 22

**Quantification of viable wine yeasts by direct live/dead staining combined with Fluorescence *in situ* Hybridisation****Patrícia Branco, Margarida Monteiro, Diana Francisco, Patrícia Moura, Helena Albergaria**

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Fluorescence *in situ* hybridisation (FISH) has been successfully used to detect and differentiate yeast species involved in wine fermentations [1]. However, molecular methods targeted to DNA (or RNA) are not able to distinguish between viable and dead cells. Viability markers, such as propidium iodide (PI), which are only able to enter on cells with compromised membranes (dead cells), have been frequently used with that purpose. However, combining viability markers with molecular methods to simultaneously assess the viability and the phylogenetic affiliation of target microorganisms is difficult since most molecular methods involve the disruption of the cells. The FISH technique detects intact cells but involves the permeabilisation of the cell membrane *prior* to hybridisation. Nevertheless, Savichtcheva et al. [2] demonstrated that cells previously stained with viability fluorescent dyes and then hybridised with FISH probes could be used to detect and enumerate targeted and non-targeted live and dead cells within a complex microbial environment. The aim of the present work was to apply and validate the direct application of a Live/Dead staining (LDS) procedure combined with FISH probes (LDS-FISH) to quantify dead and viable subpopulations of *Saccharomyces cerevisiae* (Sc) and *Hanseniaspora guilliermondii* (Hg) during mixed fermentations performed in synthetic grape juice. Cells were first stained with PI and then hybridised with the respective species-specific FISH-probes. Total, viable and dead cells were enumerated both by hemacytometry using an epifluorescence microscope and flow cytometry. The effect of the permeabilization FISH-step on the free diffusion of the viability marker (PI) from cells that had been previously stained with PI (dead cells) was evaluated. Results showed that when cells were stained with 5 mg of PI *per*  $10^5$  cells there was no significant difference (less than 2%) between the number of PI-stained cells (dead cells) determined before and after application of the FISH procedure. Thus, our work proved that LDS-FISH method is a powerful tool to quantify, in a fast and reliable way, dead and live cells of specific yeast species involved in complex microbial processes such as wine fermentations.

PS6: 23

**Role of C2-phytoceramide in mediating cell death in yeast****Andreia Pacheco, Flávio Azevedo, Susana Chaves, Manuela Côrte-real, Maria João Sousa**

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Ceramides are naturally occurring sphingolipids that cause several biological effects. It is well established that ceramide induces both apoptosis and autophagy in mammalian cells. However, the molecular mechanisms induced by ceramide in yeast, as well as the signalling systems involved, are poorly understood. It is known that phytoceramides, assumed to be the yeast counterpart of the mammalian ceramides, mediate regulation of cell growth and stress responses in yeast. However, their role has proved difficult to define. In this study, we characterized the effect of exogenous N-Acetyl-phytosphingosine (C2-phytoceramide) on *Saccharomyces cerevisiae* W303 1A cells. We found that C2-phytoceramide induced death in a dose dependent manner (10-40  $\mu$ M), but did not trigger autophagy, since cell survival was not rescued in the absence of Atg5p and C2-phytoceramide did not induce Atg8-GFP cleavage. We then tested whether C2-phytoceramide-induced death was apoptotic in nature. We found that exposure to C2-phytoceramide resulted in increased nuclear condensation (15 %), but not in a significant increase in DNA fragmentation. It did not lead to ROS accumulation and, accordingly, loss of cell viability could not be rescued by treating cells with ROS scavengers. Loss of cell viability could also not be rescued by overexpression of the anti-apoptotic protein Bcl-2. Other apoptotic markers are under characterization. Nonetheless, C2-phytoceramide-induced death was only partially accompanied by an increase in cells with loss of membrane integrity, indicating that it is a regulated process. We determined the involvement of several pathways in signalling C2-phytoceramide-induced death. We found that the mutant strain W303 *isc1* $\Delta$  (unable to generate ceramide by degradation of complex lipids) displayed higher sensitivity to C2-phytoceramide. Several mutant strains lacking components of Mitogen Activated Protein Kinase (MAPK) cascades were also tested. We found that the W303 *hog1* $\Delta$  mutant strain displayed increased sensitivity to C2-phytoceramide, while the *ste20* $\Delta$  strain displayed increased resistance. The overall results suggest that C2-phytoceramide leads to cell death in yeast and points to a crosstalk between ceramide and MAPK signalling pathways in this process.

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PS6: 24

### Molecular epidemiology of group B streptococcal meningitis in children from Angola: evidence of novel genetic signatures

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*Streptococcus agalactiae*, group B Streptococcus (GBS), is the leading cause of neonatal invasive infections in industrialized countries, which are caused by particular clones described in Europe and North America. However, there is a significant lack of information regarding the molecular epidemiology of invasive GBS from Africa. In addition, as GBS rarely infects older children, the present study covers an unusual age group which contrasts with the majority of the published data. To identify and examine the specific causative genetic lineages of GBS childhood meningitis in Angola (children aged 91 days to 12 years), we conducted an extensive phenotypic and molecular analyses of 21 isolates recovered at Paediatric Hospital of Luanda, Angola, during 2004-2005. Antimicrobial susceptibility testing for four antibiotics was done by E-test. Macrolide resistance genes were screened by PCR. Typing was performed by *cps* genotyping, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The identification of alpha-like proteins, the presence of mobile genetic elements in the *scpB-lmb* intergenic region and the allelic variation in the *bibA*, *fbxB* and *sip* virulence genes were also studied. GBS isolates were fully susceptible to all antibiotics tested, except for a single invasive isolate which displayed resistance to erythromycin. A low genetic diversity was observed: the majority of the isolates belonged to capsular type III-2 (86% of cases), and to the clonal complex 17 (CC17), whose carried the genetic element GBSi1. The predominance of single locus variants of sequence type 17, suggested the local diversification of this hypervirulent clone, which presented novel alleles of *fbxB* and *sip* virulence genes. The absence of *scpB-lmb* region in two GBS isolates with Ia/ST23 genotype was not expected since it is more typical of cattle origin. Globally, these data provide novel information about the enhanced invasiveness of the CC-17 genetic lineage in children older than 3 months of age and suggested local diversification of this clone, which may be related with the future emergence of a novel epidemic clone in Angola. Finally, the enrolment of both colonizing and invasive circulating clones is mandatory, as it may have potential implications in the design of a universal GBS vaccine.

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PS6: 25

**Viable but nonculturable state in foodborne pathogens: a quest for resuscitation****Daniela Pinto<sup>1</sup>, Carlos São-José<sup>2</sup>, Mário Almeida Santos<sup>1</sup>, Lélia Chambel<sup>1</sup>**<sup>1</sup>Center for Biodiversity, Functional and Integrative Genomics (BioFIG), Faculty of Sciences, University of Lisbon, Lisbon, Portugal; <sup>2</sup>Center of Molecular Pathogenesis, Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal

Living bacteria are thought as colony formers but viable but nonculturable (VBNC) cells are unable to form colonies when plated on a suitable solid medium. On the other hand, resuscitation, a poorly understood process, leads to the restoration of colony-forming ability. When in VBNC state bacteria can escape detection in food microbiological control and if resuscitation occurs outbreaks can arise with severe human and economic costs. *Listeria monocytogenes* and *Escherichia coli* are two of the main species associated with foodborne outbreaks. Understanding resuscitation process of such pathogens is our main goal. Best conditions for VBNC induction were screened and starvation, high salinity and low pH, combined with low temperature, were selected as the best ones. These experiments lead to the conclusion that induction conditions determine the time needed for cells to achieve the VBNC state and that the induction temperature determines the resuscitation ability. Resuscitation of VBNC cells was achieved by temperature upshift, supernatant of growing cells and amino acid supplementation of growth media. Resuscitation ability is determined by induction conditions and occurs in a period of time beyond which no more resuscitation can be observed. To shed some light on the molecular biology of resuscitation diverse approaches are currently being pursued. Since for *L. monocytogenes* two hypothetically lytic transglycosylases are described as potential resuscitation factors, heterologous expression and purification under native conditions were used as starting point. Assays for lytic activity as well as resuscitation of VBNC cells are under course. For *E. coli*, to test the amino acids as potential resuscitation effectors, mutant strains in a putative amino acid sensor were obtained and VBNC induction of mutants and wild type strains are now in progress. It was observed that a lab strain does not present the same resuscitation phenotype as food isolates and ongoing sequencing of those strains genomes will sustain future studies. VBNC cells induction and resuscitation was already achieved for both pathogens species and some of those conditions were described for the first time. Different strategies are in progress in order to contribute to an understanding of the resuscitation process and, simultaneously, control VBNC cells in food.

PS6: 26

**Clonality and virulence of *Escherichia coli* ST69, ST393 and ST405****João Pires, Ângela Novais, Luísa Peixe**

REQUIMTE, Portugal

The worldwide spread of extended spectrum  $\beta$ -lactamases (ESBLs) has been linked to the expansion of particular extra-intestinal pathogenic *Escherichia coli* (ExPEC) clones. Studies analysing their diversity and virulence profile include mostly temporally and geographically confined isolates. The aim of this work was to characterize a representative collection of phylogenetic group D (ST69, ST393 and ST405) *E. coli* from different origins, geographic regions and temporal frames. Thirty-six representative ESBL and non-ESBL producing isolates (13 ST69, 12 ST393 and 11 ST405) were collected from nine countries/three continents, sources (human infections, healthy volunteers and food-products) during 1980-2010. *E. coli* phylogenetic groups and O antigens were identified by PCR, and clonal relatedness was established by PFGE and MLST. The screening for 38 ExPEC virulence factors (VFs) was performed by PCR. Antibiotic resistance (AbR) profiles to non- $\beta$ -lactams (aminoglycosides, tetracycline, quinolones, sulphonamides, trimethoprim, chloramphenicol and nitrofurantoin) were assessed by disk diffusion tests. Twenty seven (73%) isolates were classified as ExPEC (75% from UTI/bacteremia, 25% from healthy volunteers/food products), whereas 22% of non-ExPEC isolates caused extra-intestinal infections. A high virulence score was observed among ST69 (median 14; range 9-15) and ST393 (median 13; range 3-15) isolates, and lower for ST405 clonal group (median 6; range 2-14), all of them exhibiting high AbR rates. The most common VFs among all STs were *fimH* (82-100%), *fyuA* (62-100%), *iutA* (55-92%), and in a lesser extent *sat* (55-77%), whereas *pap* (69%-92%), *iha* (77%-83%), *kpsMTII-K5* (92%-100%) and *ompT* (50%-92%) were more abundant in ST69 and ST393, and *kpsMT III* (46%), PAI (64%) or *fyuA* (100%) more frequently associated with ST405. Despite the high clonal diversity observed, particular lineages sharing common PFGE-patterns and virulence profiles were identified in different niches and/or geographic regions for several years. The absence of correlation between non-ExPEC and extraintestinal disease suggests the contribution of other non-explored VFs. Although with variable relative contribution, both virulence and AbR profiles seem to be influencing the widespread of ST69, ST393 and ST405 clonal groups. We further demonstrate the spread of particular lineages within different niches and geographic regions throughout time.

PS6: 27

**Evaluation of HPV 16 and 18 viral load, DNA physical status and mRNA expression as molecular markers in clinical practice****Candida de Castro Delgado<sup>1</sup>, Ana Catarina Oliveira<sup>2</sup>, Nuno Filipe Verdasca<sup>2</sup>, Angela Maria Pista<sup>2</sup>**<sup>1</sup>Instituto de Tecnologia Química e Biológica, Oeiras, Portugal; <sup>2</sup>National Institute of Health, Lisboa, Portugal

Infection with high-risk human papillomavirus (HPV) is considered to play a central role in cervical carcinogenesis. Analyses of molecular markers seem to be important to increase HPV specificity and to predict the risk of disease progression. The aim of this study was to evaluate the clinical significance of viral load, integration status and E6/E7 mRNA expression for HPV 16 and 18. Cervical smears from 219 HPV 16 and/or 18 positive women (69 with normal cytology; 46 with ASCUS; 59 with LSIL/CIN1; 40 with HSIL/CIN2+ and 5 with CC) were studied. E6/E7 mRNA transcripts were performed using NucliSENS EasyQ HPV 1.0. Viral load and DNA physical status was determined by a quantitative in-house real time PCR, with TaqMan and SYBRGreen, respectively. Viral load was expressed as HPV DNA copies/cell. All statistical analysis was performed using SPSS software version 16.0, with a  $p$  value of 0.05. Viral load and integration status was assessed in 150 positive cases for HPV 16, 55 for HPV 18 and 14 positive for HPV 16+18. For E6/E7 mRNA transcripts 111 cases were studied (95 positive for HPV 16 and 21 positive for HPV 18). Viral load increased with severity of cervical abnormality for HPV 16 and 18 ( $p=0.001$  and  $p=0.008$ , respectively). These results show an increased risk for HPV persistence, which may be predictive for cervical cancer development. Regarding DNA physical status, the data obtained showed that episomal and integrated forms (concomitant) of HPV DNA were the most prevalent forms observed in HPV 16 and 18 positive cases. It was not observed a significantly association between the severity of cervical lesion and integration status ( $p=0.108$ ). Detection of E6/E7 mRNA transcripts of HPV 16 and 18 increased gradually with the grade of lesions ( $p=0.164$ ;  $\alpha=0.01$ ), suggesting that detection of E6/E7 mRNA transcripts could provide increased specificity of HPV testing. In conclusion, the combination of HPV viral load, integration status and mRNA expression results seem to be important for patient management and cervical cancer prevention in women infected with HPV 16 and 18. Study is ongoing and further data will be presented to better evaluate the clinical significance of these results.

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PS6: 28

**Intracellular H<sub>2</sub>O<sub>2</sub> modulates pimarcin biosynthesis in *Streptomyces natalensis*****Tiago Beites<sup>1,2</sup>, Sílvia D.S. Pires<sup>1,3</sup>, Catarina L. Santos<sup>1</sup>, Antonio Rodríguez-García<sup>4,5</sup>, Pedro Moradas-Ferreira<sup>1,3</sup>, Jesús F. Aparicio<sup>4,5</sup>, Marta V. Mendes<sup>1</sup>**<sup>1</sup>IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto. Porto, Portugal.; <sup>2</sup>FCUP

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Streptomycetes are well-known for their ability to synthesize a wide range of secondary metabolites in a process that is strongly affected by oxygen availability. Although oxygen is essential, its metabolism generates reactive oxygen species (ROS) potentially harmful for the cell. Due to their highly reactive nature ROS intracellular levels are subjected to a strict regulation. When an imbalance in ROS homeostasis occurs, cells have the ability to adapt by activating signalling networks that ultimately affect different cellular processes. In this study we report evidence for a functional crosstalk between the oxidative stress and secondary metabolism regulatory networks in *S. natalensis*. The adaptive physiological response of *S. natalensis* towards the addition of exogenous H<sub>2</sub>O<sub>2</sub> in iron-supplemented cultures, suggested that the modulation of the intracellular ROS levels, through the activation of the H<sub>2</sub>O<sub>2</sub> inducible catalase during the late exponential growth phase, can alter the production of pimarcin. With the construction of defective mutants on the H<sub>2</sub>O<sub>2</sub> related enzymes SodF, AhpCD and KatA1, an effective and enduring modulation of intracellular ROS was achieved. Functional characterization of the knock-out strains revealed different behaviours regarding pimarcin specific production: whilst the superoxide dismutase defective mutant presented low pimarcin production when compared with the wild-type, the mutants defective in the H<sub>2</sub>O<sub>2</sub> detoxifying enzymes, KatA1 and AhpCD, behaved as pimarcin overproducers. Complementation of the *DsodF* and *DahpCD* mutants with *sodF* and *ahpCD* respectively, restored pimarcin production to the wild-type levels. Furthermore, the increase of intracellular H<sub>2</sub>O<sub>2</sub>, either by activation of SodN in the *DsodF* mutant or by the addition of H<sub>2</sub>O<sub>2</sub> to the culture broth in the *DkatA1* and *DahpCD* mutants, enhanced pimarcin specific production. To further unveil this molecular crosstalk we compared mutant and wild type transcriptomes by performing an interspecies microarray experiment. Our results demonstrate that intracellular H<sub>2</sub>O<sub>2</sub> levels modulate the biosynthesis of pimarcin in *S. natalensis*, indicating that the effects of intracellular ROS imbalance extend to secondary metabolism.

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PS6: 29

**Microbial diversity in a uranium contaminated environment assessed by culture dependent and independent techniques: the Urgeiriça mine as a case study****Ana Paula Chung<sup>1</sup>, Alcides Pereira<sup>2</sup>, Luís Neves<sup>2</sup>, Paula V. Morais<sup>1,3</sup>**

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Currently, the Urgeiriça mine is deactivated but it was the most important uranium exploration in Portugal. This uranium contaminated environment is undergoing a program of environmental bioremediation, thus providing the opportunity to develop academic studies with the purpose of finding new methodologies that allow for quick remediation strategies with low costs and a minimum environmental disruption. The aim of the present study was to evaluate the impact of mining activities in the microbial community structure of the former Urgeiriça mine. The bacterial diversity was assessed by culture dependent and independent methods (DGGE and 454 pyrosequencing), based on the 16S rRNA gene. Four different sites were sampled, two of them were in the mine and the other two were from two different stages of the existing water treatment system. All samples, independently of the content in uranium and other metals, showed a high number of cultivable bacteria and uranium resistant populations were also isolated. The bacterial diversity recovered by this traditional culture-dependent methods showed a lower coverage when compared with the high throughput DNA pyrosequencing. However, both approaches showed that the predominant bacterial phylum was the Proteobacteria ( $\beta$ -Proteobacteria) in all the sampled sites, with exception of Poço das Cobras the most contaminated site with uranium (8.2  $\mu$ M), where the vast majority of the isolates belonged to the phylum Actinobacteria. Additionally, the preliminary results of the pyrosequencing method shows that the samples collected in the mine have a higher diversity showing a higher number of OTUs (>1000) when compared with the samples collected in the two different stages of the water treatment plant. These results are also corroborated by the DGGE profile showing a higher number of bands in the samples collected in the mine. The archaeal communities in this former uranium mine were less diverse when compared with the bacterial communities and predominantly belonged to the phylum Euryarchaeota. Members of the phylum Crenarchaeota were only found in the samples from the mine in a low percentage (< 6%).

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PS6: 30

### Molecular detection of antimicrobial resistance gene cassettes associated with Class 2 Integrons in *Salmonella* serovars

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Antimicrobial resistance in *Salmonella* spp. is a major health problem in human and veterinary medicine worldwide. Multiple antibiotic resistances are strongly associated with Integrons, the natural recombination systems that can be transferred in companion with mobile genetic elements and play a major role in spreading antibiotic resistance genes in clinical isolates. Eighty four isolates of *Salmonella* spp. were subjected to antimicrobial susceptibility testing for the following antibiotics: ampicillin, tetracycline, chloramphenicol, trimethoprim, sulfamethoxazole-trimethoprim, streptomycin, nalidixic acid, ciprofloxacin, ofloxacin, levofloxacin, norfloxacin, gatifloxacin, moxifloxacin, cefotaxime, cefixime, ceftriaxone, cefepime, ceftazidime, amikacin, azithromycin, spectinomycin, gentamicin, colistin-sulfate, imipenem. All isolates were screened for the presence of class 2 integrons using primers specific for the *intI2* gene. The gene cassettes inserted in the variable region of class 2 integrons were amplified using primers designed for the conserved segments of integrons. According to the size of IVR amplified, one representative band of each group were sequenced and compared with the GenBank sequences using online BLAST software. Following this analysis, sequences were deposited in the EMBL/GenBank database. Eleven isolates (13.1%) which were resistant to at least 4 groups of antimicrobial agents considered as MDR (multidrug resistant) *Salmonella* serovars. PCR assays detected the *intI2* gene and internal variable regions (IVRs) of class 2 integron in Fourteen (16.7%) and eleven (78.6%) of *Salmonella* clinical isolates respectively. Analysis of the sequence data revealed 3 gene cassette arrays including the *dhfrA1* (0.75kb), *dfrA14-Isp* (1kb), *dhfrA1-sat2-aadA1*(3kb) with three IVR distribution patterns. The nucleotide sequences of IVR in class 2 integrons were deposited in the NCBI GenBank sequence databases under the accession numbers JF264730, JN032741, JN032742, JF264731 which were attributed to the *dfrA14-Isp*, *dhfrA1*, *dhfrA1-sat2-aadA1*, *dfrA14-Isp* gene cassettes respectively. Presence of MDR *Salmonella* serovars demonstrates that antimicrobial selection pressure is widespread in our clinical settings. Detection of class 2 integron carrying gene cassettes which confer resistance to different classes of antibiotics confirms that integron-mediated antimicrobial gene cassettes are prevalent in *Salmonella* serovars isolated in Iran.

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PS6: 31

**Protease specificity in the biosynthesis of a two-component lantibiotic****Tânia Caetano<sup>1</sup>, Raquel Faria<sup>1</sup>, Oldach Florian<sup>2</sup>, Roderich Suessmuth<sup>2</sup>, Sónia Mendo<sup>1</sup>**<sup>1</sup>University of Aveiro, Portugal; <sup>2</sup>Technical University of Berlin, Germany

Lantibiotics are lanthionine-containing antibiotics produced by Gram positive bacteria. They are ribosomally synthesized and post-translationally modified to their active forms by several enzymes. The accomplishment of lantibiotic production in *Escherichia coli* opened new perspectives for the use of easier methodologies enabling their structure re-engineering. However, this manipulation also requires the flexibility of all the other biosynthetic elements. Thus, information regarding the specificity of these elements is of greatest importance. Lichenicidin is a class II lantibiotic produced by *Bacillus licheniformis*, which is constituted by two peptides (Bli $\alpha$  and Bli $\beta$ ). Several class II lantibiotics, including Bli $\beta$ , require a two-step proteolysis. For Bli $\beta$  the first step is performed by the bifunctional transporter/protease LicT. The second is predicted to involve the trimming of 6 N-terminally located amino acids (NDVNPE) by the LicP extracellular protease. The aim of this study was to evaluate the proteolysis specificity of LicP, regarding the NDVNPE-hexapeptide sequence. Also, the ability of LicP to process the modified Bli $\beta$  still attached to the leader sequence was investigated. All the experiments were performed using the lichenicidin *trans* complementation system in *E. coli*. Each one of the hexapeptide amino acids was substituted by Ala using site-directed mutagenesis. The same approach was used to completely remove the entire sequence and substitute the GG-motif, essential for LicT proteolysis. The production of Bli $\alpha$  and Bli $\beta$  by each of the mutants was evaluated by MS experiments. All the performed substitutions did not abolish the proteolytic activity of LicP, since the molecular mass of Bli $\beta$  was identified in all the mutants. Quantification results showed that Bli $\beta$  production in the control strain and in the mutants was not significantly different. This suggested that the LicP proteolytic activity was not decreased due to the introduced alterations. Also, it was shown that LicP processing does not require the removal of the peptide leader sequence by LicT. In conclusion, LicP seems to be a non-specific protease and in the absence of the hexapeptide its activity is completely expendable. Upcoming *in vivo* experiments as SAR studies or the incorporation of non-canonical amino acids can benefit from this study, since the intervention of one less protein in Bli $\beta$  maturation can also reduce the impact of the modifications introduced in its production.

PS6: 32

**Quorum sensing regulation of *Pectobacterium carotovorum*-*Drosophila melanogaster* symbiosis****PoI Nadal Jimenez, Luis Teixeira, Karina Xavier**

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The major virulence factors in *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) are plant cell wall degrading enzymes (PCWDEs), a battery of enzymes that cause maceration and rotting of plant tissue. The production of these enzymes is strongly controlled by both plant signals, that indicate to the bacterium the location of the correct host, and bacterial quorum-sensing signals that enable the expression of bacterial virulence according to population density. The quorum-sensing molecules produced by this bacterium are 3-oxo-C6-HSL and 3-oxo-C8-HSL and belong to the family of *N*-acyl homoserine lactones (AHLs). These AHLs are produced by the synthase *ExpI*, and once they reach a threshold concentration, they bind to the LuxR family of transcriptional regulators *ExpR1* and *ExpR2*, which activates transcription of several virulence factors. In *P. carotovorum*, *ExpR*-AHL regulation is mediated by the global regulator *RsmA*. This regulator is a small RNA-binding protein which inhibits the production of multiple virulence genes including the PCWDEs. The *ExpR1/2*-AHL complex binds to the *RsmA* promoter blocking its expression and thus relieving repression of PCWDEs. We are studying the regulation of *evf* (*Erwinia* virulence factor), a gene recently discovered which allows colonization and persistence of *P. carotovorum* in the midgut of *Drosophila melanogaster*. This symbiosis-promoting gene has the potential to enhance plant infection and transmission during the feeding and reproductive process of *D. melanogaster*. *Evf* expression is controlled by *HOR*, a member of the broad *SlyA* family of transcriptional regulators associated with virulence gene expression in bacteria. Our goal is to investigate whether the virulence factors important for plant and insect infection share common regulatory mechanisms and to define the role of the *P. carotovorum* quorum-sensing network on EVF production and consequent insect-borne disease transmission.

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PS6: 33

**Quorum-sensing and oxidative stress: evidence for cross-regulatory mechanisms in *Streptomyces natalensis*****Sara M Silva<sup>1,2,3</sup>, Catarina L Santos<sup>1</sup>, Sílvia D S Pires<sup>1,3</sup>, Tiago Beites<sup>1,2</sup>, Marta V Mendes<sup>1</sup>**<sup>1</sup>Instituto de Biologia Molecular e Celular, Portugal; <sup>2</sup>Faculdade de Ciências Universidade do Porto; <sup>3</sup>Instituto de Ciências Biomédicas Abel Salazar

*Streptomyces* are soil-dwelling bacteria well-known for their ability to produce a wide range of bioactive compounds. These microorganisms present well-developed quorum-sensing (QS) circuits that promote cell-cell signalling mechanisms based on the production and release of molecular signals. In other models, QS has been shown to have an important role in the intracellular redox homeostasis, which in turn is an important factor in *Streptomyces* secondary metabolism [1]. In this context, we have addressed the influence and putative interactions of quorum-sensing and oxidative stress response in *Streptomyces natalensis*, as well as their effects in the secondary metabolism. *S. natalensis* produces pimarinic acid, an antifungal widely used in food industry. In order to evaluate the effects of a redox imbalance, a knock-out mutant for OxyR was constructed and characterized. OxyR is known to regulate the transcription of the AhpCD system in *Streptomyces* spp. interestingly, the total catalase activity of the  $\Delta oxyR$  along culture growth was significantly higher than that of the wild-type. In fact,  $\Delta oxyR$  presented high levels of intracellular H<sub>2</sub>O<sub>2</sub> during the beginning of the exponential growth phase, which likely led to the early induction of KatA1 (a monofunctional catalase previously shown to be inducible by H<sub>2</sub>O<sub>2</sub> – [1]). Additionally, the high levels of intracellular H<sub>2</sub>O<sub>2</sub> in the early exponential growth phase modulate the secondary metabolism, resulting in a pimarinic acid overproducer phenotype. We analyzed the different inducible properties of the wt and  $\Delta oxyR$  supernatants, based on the rationale that QS signals accumulate in the extracellular medium. The catalase activity profile along growth was differentially affected by the presence of the different supernatants. Moreover, both supernatants appear to confer resistance to paraquat and an increased sensitivity to cumene hydroperoxide. In summary, our results show that, beyond the oxidative stress response, OxyR modifies the external medium inducible properties, which suggests that the oxidative stress response and QS are indeed correlated in *S. natalensis*.

[1] Beites et al.; PLoS One (accepted for publication).

PS6: 36

### The role of Hfq and RNA determinants in the degradation pathways of small non-coding RNAs

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Small non-coding RNAs (sRNAs) affect virtually all genetic pathways in both prokaryotic and eukaryotic cells. Bacterial sRNAs modulate translation and/or mRNA stability by an antisense mechanism parallel to the miRNA degradation mechanism in eukaryotes. sRNAs are diverse in sequence and size. Distinct architecture features can be found in sRNAs and different structural elements can impact their regulatory pathways. In this work we have characterized the RNA determinants important for the stability and function of MicA RNA. Based on MicA sequence and secondary structure we predicted the following MicA domains: a linear 5'end sequence; a structured module harboring two stem loops, an internal A/U-rich sequence and a transcriptional terminator with a U-rich linear 3'end. We constructed several MicA altered variants. The different modules predicted in MicA were found to play distinct roles on MicA stability. Moreover, we have found that different domains of MicA can differentially control mRNA target expression. The 5'end was shown to be the most important MicA binding region for regulation of *ompA* mRNA levels but not for *lamB* mRNA. Surprisingly mutations in the 3' part of MicA were shown to greatly affect MicA ability to down regulate its target mRNAs. The two stem loops present in MicA were found to play distinct roles. Stem1 plays little role in stability and is suggested to be involved in base pairing interactions with target mRNAs while stem2 is critical for MicA stability. Hfq is an RNA chaperone vital for the binding between sRNA and mRNA target. We also identified two Hfq binding sites in MicA, an internal A/U-Rich region and the poly(U) terminator sequence. sRNAs in *E. coli* are rapidly degraded in absence of Hfq, in this study we analysed the degradation pathways of several sRNAs in the presence and in the absence of Hfq. We have found that PNPase is the major enzyme involved in the rapid decay of MicA, GlmY, RyhB and SgrS RNAs namely in Hfq<sup>-</sup> cells. Our data also suggests that upon Hfq depletion the decay of the regulatory MicA occurs mainly in a target-independent pathway in which the endonuclease RNaseIII has a reduced impact. Our work demonstrated that the free pool of small RNAs is preferably degraded by the 3'-5' exonuclease PNPase. Overall, our work highlights the role of 3'end of bacterial sRNAs in sRNA function and stability in an interesting parallel to eukaryotic miRNAs.

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PS6: 37

### The yeast plasma membrane transporter Pdr18 plays a role in plasma membrane sterol composition, conferring multidrug resistance

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The biological role of some multidrug efflux pumps in multidrug resistance (MDR) acquisition has been proposed to be due to the transport of physiological substrates which may indirectly affect drug partition and/or transport across cell membranes (1). This study provides the first functional report on the uncharacterized yeast Pleiotropic Drug Resistance (PDR) transporter, Pdr18, belonging to ATP Binding Cassette (ABC) Superfamily, encoded by ORF *YNR070w* (2). *PDR18* gene was found to be a determinant of yeast resistance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and barban, to the 2,4-D degradation intermediate 2,4-dichlorophenol, to the agricultural fungicide mancozeb, and to the metal cations Zn<sup>2+</sup>, Mn<sup>2+</sup>, Cu<sup>2+</sup> and Cd<sup>2+</sup>. This gene was proved to be required to reduce the intracellular accumulation of the herbicide 2,4-D and up-regulated in yeast cells during 2,4-D induced lag-phase period. The regulation of *PDR18* activation under 2,4-D stress is mediated by the transcription factors Nrg1, controlling carbon source availability and stress response, and, less significantly, Yap1, involved in oxidative stress and MDR, and Pdr3, a key regulator of the yeast PDR network. Since the deletion of *PDR18* was found to lead to the accumulation of the precursors of ergosterol biosynthetic pathway and to decreased content of ergosterol in yeast plasma membrane, the role exerted by Pdr18 in 2,4-D partition between the cell interior and the external environment may be indirect. Indeed, the presence of 2,4-D leads to the reduction of the plasma membrane ergosterol content, this effect being much stronger in  $\Delta pdr18$  background. Altogether, our results suggest that Pdr18 may play a role in plasma membrane sterol incorporation, this physiological trait contributing to the described MDR phenotype (2).

(1) Sá-Correia, I et al. (2009) Trends Microbiol., 17:22-31; (2) Cabrito, TR et al. (in press) Biochem J., DOI: 10.1042/BJ20110876.



PS6: 38

### Yeast as a model system to study mechanisms regulating cisplatin sensitivity and resistance

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Cisplatin is a highly effective chemotherapeutic drug used in the treatment of several tumors. It is a DNA-damaging agent that induces apoptosis of rapidly proliferating cells, an important factor underlying its therapeutic efficacy. Unfortunately, cellular resistance occurs often. A large fraction of tumor cells harbor mutations in p53, contributing to defects in apoptotic pathways and drug resistance. However, cisplatin-induced apoptosis can also occur in p53 deficient cells; thus, elucidation of the molecular mechanism involved will potentially yield new strategies to eliminate tumors that have defects in the p53 pathway. Most of the studies in this field have been conducted in cultured mammalian cells, not amenable to systematic genetic manipulation. Therefore, we aimed to establish a simplified model to study cisplatin-induced apoptosis using the yeast *Saccharomyces cerevisiae*. Our results indicate that cisplatin induces an active form of cell death in yeast, as this process was partially dependent on new protein synthesis and did not lead to loss of membrane integrity. Preliminary studies of apoptotic markers revealed mitochondrial fragmentation and chromatin condensation after cisplatin exposure. Deletion of Yca1p, the yeast metacaspase, did not protect cells from cisplatin-induced cell death, although exposure of yeast cells to cisplatin resulted in an increase in proteolytic activity that was inhibited *in vitro* by MG132, a commonly used proteasome inhibitor. *In vivo*, co-incubation with MG132 increased resistance to cisplatin and, accordingly, yeast strains deficient in proteasome proteolytic activity were more resistant to cisplatin than wild type strains. Proteasome inhibitors induce apoptosis in various cell types, whereas in others they prevent apoptosis induced by different stimuli. Our results indicate inhibition of the proteasome protects from cisplatin-induced apoptosis in budding yeast. The molecular mechanism involved is under characterization.

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PS6: 39

### **A test strain for arabinose and xylose transport**

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D-Xylose and L-arabinose are two important aldopentose sugars present in lignocellulosic hydrolysates, which is a major feedstock for 2<sup>nd</sup> generation bioethanol production. *Saccharomyces cerevisiae* is the preferred ethanol production organism but lack efficient pentose transport and metabolism. Heterologous xylose and arabinose metabolic genes have been successfully expressed in *S. cerevisiae*, enabling growth on these carbon sources. A practical way of cloning transporter genes is by functional complementation where enhanced transport can be scored as higher growth rate. This is efficient if growth rate is limited only transport. The growth rate on arabinose for recombinant *S. cerevisiae* is very slow, even at very high substrate concentrations, preventing functional complementation for transport. We have designed test strain that utilize glucose, but is limited by aldopentose consumption for growth. Proof of concept will be presented and examples of the use of this strain in the search for arabinose and xylose transporters will be discussed.

PS6: 40

**Antibiotic resistance genes detected in *Enterobacteriaceae* colonizing hospital inanimate surfaces****Cátia Santos<sup>1,2</sup>, Raquel Diaz<sup>2</sup>, Elmano Ramalheira<sup>2</sup>, Tânia Caetano<sup>1</sup>, Sónia Mendo<sup>1</sup>**<sup>1</sup>University of Aveiro, Portugal; <sup>2</sup>Hospital Infante D. Pedro, Aveiro, Portugal

Background: Within the hospital multidrug resistant (MDR) strains are not only confined to the patient, but they can also colonize and survive in the surrounding environment. Objectives: We aimed to compare the prevalence of different antibiotic resistance genes in Gram-negative bacteria collected from the furniture in a female ward in Hospital Infante D. Pedro, Aveiro. Methods: Sterile swabs were rubbed in inanimate surfaces, placed in rich medium (TSB), at 37°C, overnight. Serial dilutions were plated in MacConkey agar. Phenotypically different colonies were selected and their clonal relationship determined by rep-PCR and analysed with the GelComparII 5.0 (Applied Maths, Kortrijk, Belgium). Identification to the species level was determined by 16S amplification. Antibiotic susceptibilities were determined according to guidelines of CLSI standards. Presence and further characterization of integrons, beta-lactamases, *ISCR1*, *sul3* and *qnr* genes was performed by PCR. Nucleotide and deduced aminoacid sequences were analyzed with Blast and ClustalW programs and compared to others deposited in the GenBank database. Results: The inanimate surfaces are colonized with different Gram-negative species, mainly *Klebsiella spp.*, *Escherichia coli*, *Enterobacter cloacae* and *Citrobacter freundii*. The clonal relationship among all the isolates (264) showed the presence of different clonal groups. Only one isolate was selected from each group. 190 isolates were analyzed: class 1 integrons were found in 36,8%; different arrays were characterized and some are associated to *ISCR1* element (60%) or to *sul3* gene (12,1%); class 2 and class 3 integrons were not detected. Different Extended Spectrum Beta-Lactamases (ESBL), carbapenemases and cephalosporinases were investigated: TEM-1 and OXA-30 (28%), follow by SHV-12 (22%) and CTX-M-15 (17%). KPC-3 carbapenemases were found in two species, *K. pneumoniae* and *K. oxytoca*, and VIM and IMP was not detected. Cephalosporinases DHA and FOX were the more frequent (21%). *qnr* genes were detected in 14% of the isolates. Conclusion: The prevalence of MDR bacteria to different classes of antibiotics, surviving in inanimate surfaces was demonstrated. The resistance genes are associated with mobile genetic elements showing their ability to disseminate to other bacteria. Furthermore this study suggests that surface sanitation should be improved as an important measure to the control and prevent dissemination of multidrug resistant strains.

PS6: 41

**Application of molecular methods for the direct detection of MDR-TB****Samuel Francisco<sup>1</sup>, Diana Machado<sup>1,2</sup>, Miguel Viveiros<sup>1</sup>, Jorge Ramos<sup>1</sup>, Teresa Pacheco<sup>3</sup>, José Melo-Cristino<sup>4</sup>, Luís Marques Lito<sup>4</sup>, Isabel Portugal<sup>5</sup>, Leonard Amaral<sup>1,2</sup>, Isabel Couto<sup>1,6</sup>**

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Methods that allow the rapid detection of *M. tuberculosis* complex (MTBC) and occurrence of mutations leading to drug resistance are key tools to diagnose as early as possible patients with multidrug resistant tuberculosis (MDR-TB). This has proven to be the most efficient way to control the spread of MDR-TB and provide to the patient the most adequate therapy in the shortest time possible. We compared two molecular systems (INNO-LiPA Rif. TB, Innogenetics and Genotype MTBDRplus, Hain Lifescience), for their performance to detect directly MTBC and mutations related to resistance to rifampicin (RIF) (INNO-LiPA Rif. TB, MTBDRplus) and isoniazid (INH) (MTBDRplus). Both systems were run in parallel on 33 acid-fast bacilli (AFB) positive respiratory samples, according to the protocol established by each manufacturer. The results were compared to the ones obtained by conventional isolation in culture followed by AccuProbe MTBC identification (Gen-Probe) and susceptibility testing to first-line antibiotics by BACTEC MGIT 960 (Becton Dickinson). INNO-LiPA Rif. TB showed 100% sensitivity and specificity in the identification of MTBC, with 100% sensitivity and 96.7% specificity for the detection of mutations associated with RIF resistance. MTBDRplus system showed 87.1% sensitivity and 100% specificity for MTBC identification. For samples that were correctly identified as MTBC, the system showed 100% specificity for detection of mutations that confer RIF or INH resistance, but failed to identify an INH resistant sample. These results indicate that INNO-LiPA Rif. TB would be the better choice to be used directly in AFB+ respiratory samples for the early diagnosis of tuberculosis and the detection of mutations associated with RIF resistance, as an early predictor of MDR-TB. The MTBDRplus system provided better results with cultures, being a useful system to complement the results of INNO-LiPA Rif TB.

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PS6: 42

### Bacteria and Archaea: complexity of endophytic microbial community in pine trees in areas subject to Pine Wilt Disease

**Diogo N. Proença<sup>1</sup>, Romeu Francisco<sup>1</sup>, Gabriel Paiva<sup>1</sup>, Susana S. Santos<sup>1</sup>, Isabel M. O. Abrantes<sup>1,2</sup>, Paula V. Morais<sup>1,2</sup>**

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Devastating impact of tree diseases was recognized early in the 20<sup>th</sup> century with severe epidemics associated with the introduction of new pathogens to native forest ecosystems. Pine wilt disease (PWD), native to North-America, has spread into Asia and recently into Europe (Portugal and Spain). The first report in Portugal was in 1999 but in 2008 spread to the Center-North of the country. *Bursaphelenchus xylophilus*, the pinewood nematode (PWN), has been thought to be the only causal agent of PWD. However, bacteria have been reported as play a role on PWD. This study aimed to characterize the endophytic microbial community structure from infected and non-infected pine trees, *Pinus pinaster*, based on culture isolates and molecular profiling. Pine trees samples from Arganil and Oliveira do Hospital, consisted of pinewood cross-sections or wood obtained by drilling under aseptic conditions. All samples were screened for nematodes. The endophytic microbial community was studied by cultivable (R2A at 25°C) and molecular methods (Denaturing gradient gel electrophoresis -DGGE) and 293 endophytic strains (representing all RAPD groups) were identified by 16S rRNA gene sequencing. The classes Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Gammaproteobacteria and Sphingobacteria were found in both sampling areas, and Flavobacteria was only found in Arganil. The relative percentage of each class in the endophytic community was different according to sampling area. The most abundant bacteria were identified as belonging to Gammaproteobacteria in both sampling areas (34% in Arganil and 59% in Oliveira do Hospital). In Oliveira do Hospital, the Gammaproteobacteria included mostly strains from the families *Xanthomonadaceae* and *Enterobacteriaceae*. DGGE profiles were not able to produce a specific pattern for PWD. From DGGE gels, 85 bands were cloned and sequenced. Compared to the cultivable methods, DGGE detected the presence of endophytes belonging to 4 additional classes: Acidobacteria, Bacteroidia, Deinococci and Spirochaetes. Furthermore, *Archaea* were found as part of the endophytic community (DGGE) and were identified as belonging to "Methanomicrobia", Thermococci, Thermoprotei or Crenarchaeota. This is the first report of *Archaea* as endophytes in trees. *P. pinaster* showed to have a diverse endophytic community composed by Bacteria and Archaea which biodiversity could potentiate the ability to adapt to changing in the habitat.

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PS6: 44

**Development of a novel fusion system for antibiotic-free *Escherichia coli* recombinant protein production****Lara Oliveira Franco<sup>1,2</sup>, Eduardo Coelho<sup>1,2</sup>, Sofia Judite Costa<sup>1,2</sup>, André Almeida<sup>3</sup>, António Castro<sup>2</sup>, Lucília Domingues<sup>1</sup>**<sup>1</sup>IBB-Centre of Biological Engineering, Portugal; <sup>2</sup>INSARJ - Instituto Nacional de Saúde Dr. Ricardo Jorge, Porto, Portugal; <sup>3</sup>Hitag Biotechnology, Lda., Portugal

*Escherichia coli* is one of the most widely host systems used for production of recombinant proteins. Antibiotics are commonly used during bacterial fermentation, and the vast majority of expression vectors, particularly for *E. coli*, contain antibiotic resistance genes as selection markers. Despite being a powerful selection tool, their use has been considered unacceptable in many areas of biotechnology, as the recombinant protein production for therapeutic use and vaccines, by regulatory authorities. In fact, this selection system has several disadvantages, namely, the risks for human health due to the spread of the resistance genes, loss of selective pressure as a result of antibiotic degradation and the excessive metabolic burden exerted by the constitutive expression of the antibiotic resistance gene on the host cell. Those drawbacks can be stopped by using antibiotic-free expression systems, which represent lower production costs and, in many cases, permit to obtain highly desirable characteristics, such as the sharp increase in recombinant protein production. The novel fusion system presented in this study is composed by one low molecular weight moiety, the Fh8 fusion partner. Previous studies have demonstrated the ability of this fusion partner to improve soluble protein expression using antibiotic selection systems. Taking into account the advantages of producing recombinant proteins without antibiotics, the novel fusion partner was applied to an antibiotic-free system from Delphi Genetics in order to evaluate its contribution, as a solubility tag, on recombinant protein production. Using DNA recombinant technology, the sequence of the Fh8 partner was inserted into pStaby plasmids and the codifying genes for three target proteins were genetically fused to the novel partner. The bio-productions were carried out on cultures of 100 mL and the resulting fusion proteins were analysed by SDS-PAGE and compared to the non-fused protein controls. Fusion proteins used in this work were purified using nickel affinity resins and quantified by Bradford. All fusion proteins presented higher soluble expression than the non-fused ones. Results from this work showed that the Fh8 fusion system can be used in an antibiotic-free expression system, offering an effective and safe methodology to produce soluble recombinant proteins in *E. coli*.

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PS6: 45

### **Development of a *Tams1*-based real time PCR approach for detecting *Theileria annulata* in bovines**

**Marcos André Santos, Ricardo Fernando Soares, Pedro Miguel Costa, Ana Amaro, João Inácio, Jacinto Gomes**

LNIV, Portugal

Tropical theileriosis is a tick-borne hemoprotozoan disease which poses important health problems in cattle. The agent is *Theileria annulata*, occurring around Mediterranean basin, Middle East and Southern Asia. Traditionally, detection of *Theileria* pathogens in infected animals is accomplished by microscopic examination of stained-blood smears, which has low sensitivity for the assessment of carrier animals, in which low numbers of erythrocytes remain infected. The aim of this study was to develop a real time PCR assay able to detect *T. annulata* parasites in bovine blood samples. Novel primers were designed targeting the *T. annulata* species-specific *Tams1*-encoding gene. Genomic DNA was extracted from blood samples, using a robotized commercial method, and used as template for SYBR green chemistry real time PCR reactions. These samples were previously characterized for the presence of *Theileria/Babesia* parasites using a standardized reverse line hybridization (RLB) assay. The developed real time PCR assay presented 100% specificity and nearly 80% sensitivity for the detection of *T. annulata* in blood samples, using the RLB assay as gold-standard. False negative samples usually corresponded to extremely low levels of *T. annulata* parasitemia and mixed infections with other *Theileria/Babesia* species. Efforts regarding the improvement of theilerial DNA extraction approaches are currently underway towards the development of more effective molecular diagnostic assays for tropical theileriosis.

PS6: 46

### Ecology of staphylococci in the humans' anterior nares: methicillin resistance and population diversity

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Coagulase negative staphylococci (CoNS) and *Staphylococcus aureus*, are major causes of nosocomial infections, especially those strains resistant to  $\beta$ -lactam antibiotics (e.g. methicillin). The colonization of the human's nasal cavity by *S. aureus* is a risk factor for ulterior development of infections. However, the ecology of staphylococci in the nasal cavity is poorly understood; in particular is not known if transfer of SCCmec - the central element for methicillin resistance (MR) - actually occurs in this ecological niche. In order to study the staphylococci nasal ecology, 101 isolates were collected from the anterior nares of 67 Danish patient volunteers corresponding to all observed colony morphologies on selective media (SaSelect, chromID™ MRSA). Species identification was performed by VITEK2 system, ITS-PCR and 16S RNA sequencing. Susceptibility was tested for a panel of 8 antibiotics by disc diffusion. PFGE was performed for all MR-CoNS isolates and *spa* typing was performed for all *S. aureus* isolates. SCCmec types were assigned by the determination of *mec* complex class and the *ccr* allele. Staphylococci were found in 54/67 volunteers (81%). Overall, 9 different staphylococci species were identified, but *S. epidermidis* and *S. aureus* represented 80% of the total collection. Six volunteers (9%) carried MRSA and 29 (43%) carried MRCoNS. Different staphylococci species (up to five) were identified simultaneously in 18 volunteers (27%), with *S. aureus* and *S. epidermidis* co-colonization the most common association (12 cases). *S. epidermidis* was the species most frequently associated to other species (16 cases), and was rarely isolated as the single species. Interestingly, in 34 (calculated as 6+29-1) individuals MR was only found once and in particular, MRSA and MRCoNS were never co-isolated from the same host. We found MR *S. hominis* and MR *S. haemolyticus* in one patient. The results suggest that staphylococci ecology in the human anterior nares is complex and variable. *S. epidermidis* and *S. aureus* are the prevalent colonizers and therefore the species that have more opportunities for genetic exchange. But no evidence for intra species transfer of SCCmec was found, suggesting that this should be a rare event. However, mutual exclusion of species upon MR acquisition cannot be disregarded.

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PS6: 47

### Evaluation of IS6110-targeted conventional and real-time PCR assays for detecting *Mycobacterium tuberculosis* Complex in paraffin embedded bovine tissues

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Bovine tuberculosis is an infectious disease caused by *Mycobacterium bovis*, a species belonging to the *Mycobacterium tuberculosis* complex (MTC). This disease is a serious problem for health authorities not only for its zoonotic nature, but also by the high economic losses in animal production and to restrictions imposed on the trade of animals and their products. The routine laboratory diagnosis of bovine tuberculosis is usually performed using two exams, histopathological and bacteriological, made in the INRB, I.P.LNIV according to procedures developed and optimized based on the recommendations of the World Organization for Animal Health (OIE). However, this diagnosis is difficult and time consuming, primarily due to the fastidious growth of the mycobacteria. The aim of this study was to evaluate two IS6110-targeted conventional and real-time PCR (TaqMan) approaches to detect the presence of MTC in histological sections of tissues from cattle with suspected tuberculosis. Both assays were evaluated in a retrospective study of 32 paraffin blocks of tissue samples from the repository of INRB, I.P.LNIV. The paraffin embedded tissues used were previously characterized using both histopathological and bacteriological techniques: 22 samples were considered positive for tuberculosis and 10 negative samples. After extraction of mycobacterial DNA the solutions were used as template for the amplification of the insertion sequence IS6110, specific of MTC species. The PCR amplification of the bovine constitutive gene of b-actin was performed as internal control amplification. The detection of MTC members in samples revealed a sensitivity of 55% and 45% for conventional and real-time PCR assays, respectively (with a specificity of 80% and 100%). Both methods proved to be potentially useful to assist histopathological diagnosis of bovine tuberculosis, contributing to shorten the laboratory diagnosis. However, efforts are needed towards the optimization of more efficient methods for the extraction and concentration of mycobacterial DNA and removal of reaction inhibitors from infected bovine tissue samples.

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PS6: 49

**HSP90: endogenous modulators of yeast acetic acid-induced apoptotic cell death****Alexandra Silva<sup>1,2</sup>, Belém Sampaio-Marques<sup>1,2</sup>, Manuel Santos<sup>3</sup>, Paula Ludovico<sup>1,2</sup>**

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Heat shock protein 90 (HSP90) are highly conserved proteins with crucial functions in protein folding/refolding, playing a crucial role in cell proliferation, apoptosis and necrosis [1, 2]. Proteomic analysis [3] of *Saccharomyces cerevisiae* acetic acid-apoptosing cells revealed a decreased expression of the translation factors eIF4A, eEF1A, eEF2 and eEF3A [3] that associated to the phosphorylation of eIF2 $\alpha$  show a global translation impairment. However, in the same conditions there is an increase in the protein levels of HSP90 isoforms. Consistently, transcriptomic analysis of mRNAs associated to polysomes showed an increase in HSP90 isoforms expression levels, indicating that in spite of global translation impairment HSP90 isoforms are being actively translated during acetic acid-induced cell death. These evidence claims for a crucial role of HSP90 during acetic acid-induced cell death. Genetic abrogation of HSP90 isoforms indicate divergent roles for HSP90 isoforms, Hsc82 a pro-survival and Hsp82 a pro-death, upon acetic acid-induced cell death. These undisclosed functions of yeast HSP90 isoforms were confirmed through pharmacological inhibition of HSP90 activity, using 17-allylaminogeldanamycin (17AGG), and by the overexpression of each HSP90 isoform during acetic acid-induced cell death. The data highlight the importance of HSP90 isoforms for the execution of yeast apoptotic cell death induced by acetic acid.

[1] Sreedhar et al (2004) FEBS Lett, 562:11-15. [2] Lanneau et (2008) J Cell Mol Med, 12:743-761. [3] Almeida et al (2009) Proteomics. 9(3):720-732. This work was supported by a grant from FCT (PTDC/BIA-MIC/114116/2009). A.S. and B.S.M have fellowships from FCT (SFRH/BD/33125/2007 and SFRH/BD/41674/2007, respectively).

PS6: 50

**Identification and fingerprinting of cork fungi: a phenetic approach****Beatriz Reis Oliveira<sup>1</sup>, Maria Vitória San Romão<sup>1,2</sup>, Rogério Tenreiro<sup>3</sup>**<sup>1</sup>IBET, Oeiras, Portugal; <sup>2</sup>ITQB - UNL, Oeiras, Portugal; <sup>3</sup>BioFIG, Lisboa, Portugal

The industry of cork stoppers is extremely important for Portuguese economy so there has been always a huge investment in this market row. The manufacture process of cork stoppers includes a series of stages that can favor molds development on cork planks. As a result, some compounds can be produced being a source of unpleasant flavors (such as cork taint of wine). At IBET exists a collection of fungi isolated along the years from different stages of cork stoppers manufacture process. A sample composed by 198 fungi was randomly chosen from that culture collection and was analyzed phenotypic and molecularly. Macroscopically, features like diameter, texture and color have been observed. For color coding an optimization was made in order to minimize the subjectivity associated to this parameter. Microscopic slides were prepared trying to describe the different reproductive structures of each isolate. Molecularly, the core sequence of phage M13 and [HVH(GACA)4] were used as primers for DNA fingerprinting. 157 isolates from the genus *Penicillium* were identified and 26 fungi from the genus *Aspergillus*. Moreover, in a fewer proportion, it were also identified 2 fungi from the genus *Chrysonilia*, 3 fungi from *Cladosporium* genus, 5 from genus *Mucor* and 4 from genus *Trichoderma*. Although DNA fingerprinting did not reveal full diagnostic power to separate the isolates at species level, the utilization of other primers in association with M13 and HVH(GACA)4 may improve this analysis. Moreover, it was observed that phenotypic characterization allowed the identification of fungi up to genus level.

PS6: 51

### Identification of a trichothecene gene cluster and description of the harzianum A biosynthesis pathway in the fungus *Trichoderma arundinaceum*.

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Trichothecenes are sesquiterpenes that act like mycotoxins. Their biosynthesis has been mainly studied in the fungal genera *Fusarium*, where most of the biosynthetic genes (*tri*) are grouped in a cluster regulated by ambient conditions and regulatory genes. Unexpectedly, few studies are available about trichothecene production by other fungal genera like *Myrothecium* or *Trichoderma*. The aim of this work is to determine the presence of a *tri* gene cluster in *Trichoderma arundinaceum* (produces harzianum A) and to functionally characterize some of the genes involved in the harzianum A pathway. A genomic library was constructed and positive clones were selected by hybridization with *tri* gene probes from *Fusarium* and *Myrothecium*. The cluster region was sequenced using a genome walking strategy. By precursor feeding experiments and heterologous expression in yeast of *tri5*, *tri4* and *tri11* genes and the later identification of the final compound by GC-MS, we propose the harzianum A biosynthesis pathway. Thus, we describe a *tri* gene cluster in *T. arundinaceum* that contains 4 structural genes (*tri3*, *tri4*, *tri5* and *tri11*), 2 regulatory genes (*tri6* and *tri10*) and a gene that encodes a trichothecene efflux pump (*tri12*). The harzianum A biosynthesis starts with the cyclization of farnesyl pyrophosphate to trichodiene by TRI5, this compound is then hydroxylated at 3 different positions by TRI4 and once more by TRI11 to get trichodermol. The putative final step is the C-4 acetylation of trichodermol by TRI3. Thus, we demonstrate the presence of a *tri* gene cluster in *Trichoderma* but, unexpectedly, gene organization and orientation are very different from those described for *Fusarium*. The main differences are 1) *tri5* is located outside the main cluster; 2) TRI4 hydroxylates the trichodiene 3 times (4 in *Fusarium*) and 3) TRI11 hydroxylates the trichothecene skeleton in C-4 (C-15 in *Fusarium*).

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PS6: 52

### Identification of human Parechoviruses involved in viral meningitis and sepsis –like illness in Iran.

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Human parechoviruses (HPeV), are members of the *Parechovirus* genus of *Picornaviridae*. Although they are highly prevalent pathogens among very young children, they have been poorly studied, and little is known of their diversity, evolution, and molecular biology. It has been revealed that there are at least 12 genotypes of these viruses according to the sequence comparisons of complete genome or the capsid gene, VP1. HPeVs can cause a variety of clinical symptoms, including diarrhea, and respiratory infection. HPeV may constitute the second most frequent causative virus after enterovirus in severe conditions, such as meningitis and infant sepsis. During the years 2009-2011, 150 Cerebral Spinal Fluid specimens were collected from hospitalized children younger than 8 years with primary diagnosis of viral meningitis or sepsis-like illness in Tehran. CSF samples were transferred on ice to the department of virology, Pasteur Institute of Iran, and were stored in -80°C. Genomic RNA was extracted and converted to cDNA. Then enteroviruses were investigated using the Pan-EV primers. EN negative samples were considered for detection of human parechoviruses. HPeV was detected using the specific primer pairs for the 5'-NTR by PCR method, and Real-Time TaqMan probe that both of them were set up properly for the first time in Iran. 120 CSF samples were EN negative, and HPeV genome was detected in 69 (57.5%) of them with the frequency of 18.84% (13 out of 69) HPeV in sepsis-like illness patients, and 81.1% (56 out of 69) in meningitis cases. There were 75% male and 25% female. Despite the current evidence for a significant role of HPeV in several severe diseases, routine diagnostic assays are rarely available in clinical diagnostic laboratories. This results in underestimation of the actual involvement of HPeV in clinical illnesses. Early and rapid diagnosis of these viruses in hospitalized children may lead to reduced admission and antibiotics treatment. New generation of sensitive and specific molecular methods for HPeV detection and type identification, is improving and providing a greater understanding its molecular epidemiology. This report on HPeVs from Iran confirms their global distribution.

PS6: 53

**In37 – a complex class 1 integron found in different species from hospital furniture****Cátia Santos<sup>1,2</sup>, Tânia Caetano<sup>1</sup>, Elmano Ramalheira<sup>2</sup>, Sónia Mendo<sup>1</sup>**<sup>1</sup>University of Aveiro, Portugal; <sup>2</sup>Hospital Infante D. Pedro, Aveiro, Portugal

**Background:** In37 is frequently reported in different countries. Since this integron is plasmid located and encodes resistance to different antibiotics its horizontal gene transfer is a serious problem. **Objectives:** From a control study, the presence of In37 complex class 1 integron was detected in isolates recovered from inanimate surfaces from a female ward in Hospital Infante D. Pedro, Aveiro. **Methods:** Sterile swabs were rubbed in inanimate surfaces, placed in rich medium (TSB), at 37°C, overnight. Serial dilutions were plated in MacConkey agar. The clonal relationship of the isolates was evaluated by rep-PCR. Identification to the species was determined using the automatic VITEK2 system and Advanced Expert System (VITEK2 AES) (BioMérieux, Marcy L'Étoile, France), and confirmed by 16S amplification. Antibiotic susceptibilities were determined according to guidelines of CLSI standards. Presence and characterization of class 1 integrons, beta-lactamases enzymes and *qnr* genes were performed by PCR. Nucleotide and deduced aminoacid sequences were analyzed with Blast and ClustalW programs. PFGE of S1 digested total DNA followed by Southern blotting hybridization using *int1* DIG labelled probe was performed to locate the class 1 integrons. Plasmids incompatibility groups were determined using specific primers. **Results:** An array corresponding to In37 complex class 1 integron associated with the ISCR1 element was found in *Klebsiella pneumoniae* and *Enterobacter cloacae* collected from furniture of the female ward. S1 digested genomic DNA and PFGE analysis showed the presence of a plasmid of approximately 200 Kb. Plasmid characterization (incompatibility groups) and conjugation studies were performed. Hybridization with *int1* DIG labelled probe confirmed the plasmid location of In37. Both isolates contain other resistance determinants such as TEM-1 and OXA-30 beta-lactamases and *qnrA1* gene. *qnrB4*, SHV-12, DHA and FOX genes were present only in *K. pneumoniae*. **Conclusion:** This is the first description of In37 complex class 1 integron in *E. cloacae* and in bacteria colonizing inanimate surfaces. These results reinforce the ability of bacteria to survive in inanimate surfaces and more importantly to be able to transfer resistance genes to other bacteria, since these genes are associated to mobile genetic elements.

PS6: 54

### Is MALDI-TOF ICMS better than molecular biology in the identification of *Trichophyton rubrum*?

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Dermatophytoses are the most common fungal infection worldwide with a non-despicable impact in health-care costs. *Trichophyton rubrum* is an antropophilic dermatophyte species very well adapted to human host causing chronic and slowly progressing disease on keratinized tissues. It is the causative agents of about 70% of all human dermatophytoses. Besides their distribution all over the world this species is by far, the most frequently isolated species on onychomycosis and *tinea pedis*. Identification of individual species remains important from an epidemiological point of view and also for therapy administration. Molecular Biology tools have been widely used for accurate organism identification, solving problems concerning morphology-based identification of dermatophytes and improving knowledge on the epidemiology of dermatophytes. Recently a spectral technique analysis by Matrix Assisted Laser Desorption Ionization Time of Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) has been increasingly used as a rapid technique in the identification and classification of microorganisms. It has proved also to be a valuable tool being incorporated in the polyphasic approach to improve the accuracy of the microbial identification issue. In this study, eighteen clinical isolates of *T. rubrum* from human nails were analysed using primers M13, (GACA)<sub>4</sub>, (AC)<sub>10</sub> and ITS 1-4 sequencing data and MALDI-TOF ICMS analyses. *Trichophyton interdigitale* and *Aspergillus flavus* were used as two outgrouping isolates. All *T. rubrum* identifications were confirmed by molecular and spectral techniques. For molecular approach 16 isolates were clustered in a single group with 100% of genotypic homology of the ITS1-4 region, except for isolate MUM 08.11 and MUM 10.133 that were found having 98% of homology in this region. Same clusters were found with M13 and (GACA)<sub>4</sub>. MALDI-TOF ICMS analysis corroborated molecular identification. Moreover nine isolates were clustered on a single group by evidencing 100% similarity and remaining isolates were distributed in the MALDI dendrogram showing phenotypic variability. Both approaches have shown the same accuracy, however spectral analysis suggested a better discriminating tool for intraspecific variability determination. MALDI-TOF ICMS is faster and inexpensive when compared with molecular biology techniques. As a consequence, it is suitable as point-of-care diagnostic for dermatophytoses.

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PS6: 55

### Massive clonal replacement and increase in MRSA prevalence in a major Portuguese central hospital

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The prevalence of methicillin resistant *S. aureus* (MRSA) in a major hospital in Lisbon has increased in the last years. A previous study (Couto, I. et al., JCM 1995) reported a prevalence of MRSA around 30% in 1993, and the presence of two dominant nosocomial clones: the Portuguese clone (ST239-IIIvariant) and the Iberian clone (ST247-IA). Although no significant changes were made in hospital clinical practices during the last two decades, a steep increase to 49% in MRSA prevalence was observed in 2010. In order to understand the reasons lying behind this increase in MRSA prevalence, 533 MRSA isolates were recovered between January and December 2010. A representative collection (n=180), isolated from pus, urine and blood, was characterized by several molecular typing techniques. All isolates were characterized by pulsed-field gel electrophoresis (PFGE) and staphylococcal cassette chromosome *mec* (SCC*mec*) typing and subtyping and a selection of isolates were further characterized by *spa* typing and multilocus sequence typing. Moreover, the Panton-Valentine Leukocidin (PVL) and the arginine catabolic mobile element (ACME) were detected by PCR in all isolates. We observed that the great majority of the isolates (70%) were related to the EMRSA-15 clone (PFGE K, ST22-IVh, t2357, t910, t025, t032, t1302, and t1467). Two additional clones, both belonging to CC5 and PFGE I, were also well represented, namely ST105-II, with *spa* type t002 and ST125-IVc, with *spa* type t067 that together accounted for 22% of the isolates. The remaining isolates belonged to ST36-II, with *spa* type t018, PFGE B (n=4) and ST239-III, with *spa* type t037, PFGE F (n=3). None of the isolates tested carried PVL or ACME. Our study showed that a massive clonal replacement has occurred over the last 17 years, wherein the pandemic EMRSA-15 clone replaced the epidemic Portuguese and Iberian clones. In Portugal like in other countries worldwide, the EMRSA-15 clone is well established in hospitals and is also known to have conquered the community. We hypothesize that the increase in MRSA prevalence observed in this hospital may be associated to epidemic characteristics specific of EMRSA-15 clone.

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PS6: 56

**Microbial community of the migratory shorebirds that uses Tagus estuary as stopover habitats****Susana S. Santos<sup>1</sup>, Sara Pardal<sup>1</sup>, Diogo N. Proença<sup>1</sup>, Jaime A. Ramos<sup>1,2</sup>, Luísa Mendes<sup>1</sup>, Paula V. Morais<sup>1,2</sup>**<sup>1</sup>IMAR-CMA, Coimbra, Portugal; <sup>2</sup>Department of Life Sciences, FCTUC, University of Coimbra, Coimbra, Portugal

Migratory shorebirds travel some of the greatest distances of all migratory birds. Tagus estuary, Portugal is a stopover site, where the shorebirds feed to accumulate fat and protein to fuel their migrations. The relationship between microorganisms and birds has received increases attention recently. However the state of knowledge of this relationship, is based largely on examination of sick or dead birds. Information on the prevalence, community structure and function of microbes in healthy wild populations is limited. The diversity of the cloacae microbial community from 3 populations of migratory shorebirds (common redshank, black-winged stilt and nominate and Icelandic populations of black-tailed godwit) was assessed by cultivation and molecular methods. Culture with R2A medium and denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes amplicons followed by cloning and sequencing was employed to assess the bacterial populations present in the cloacal swabs. A total of 26 DGGE bands and 240 isolates of cloacal samples were analyzed. Most isolates were members of the Firmicutes and Actinobacteria and only a small portion belonged to the Proteobacteria and Deinococcus-Thermus Phyla. The diversity of the cultivable bacteria was high for the strains of the Firmicutes phylum (*Bacillus*, *Enterococcus*, *Leuconostoc* and *Staphylococcus*) while the number of bacteria belonging to the classes Actinobacteria (*Corynebacterium*, *Isoptericola*) and Proteobacteria (*Psychrobacter*, *Paracoccus*) was low. Pathogenic bacteria from *Clostridium* and *Campylobacter* species were only detected by molecular approach. Among the strains potentially pathogenic carried by the birds were found strains from *Campylobacter* and *Psychrobacter* in common redshank and black-winged stilts, *Clostridium*, *Mycobacterium* and *Rhodococcus* in black-winged stilts, *Corynebacterium*, *Escherichia* and *Paracoccus* in black-winged stilts, and *Staphylococcus* in all the bird species. Bacteria from the phylum Deinococcus-Thermus were detected in all the bird species especially in the Icelandic black tail godwit. Since they have not been reported in bird species before or estuarine environments, strains from the genus *Deinococcus* could function as a genetic marker for shorebirds, however further investigation is needed to confirm the potential of these results. This study provides an important and valuable base for the design and development of bacterial-bird studies.

PS6: 57

### Mitophagy is contributing to alpha-synuclein, wild-type and A53T mutant form, toxicity in aged yeast cells

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Autophagy, the major pathway involved in the degradation of proteins and organelles, has been recognized as a central player in the pathogenesis of several neurodegenerative disorders, like Parkinson's disease (PD). Apparently, deficient autophagy activation would impair the clearance of proteins aggregates, nevertheless, the role of excessive autophagic activation linked to PD remains unclear. Using a simple model, the yeast *Saccharomyces cerevisiae*, we studied the toxicity induced by the heterologous expression of the human wild-type a-synuclein (a-syn) and the mutant form A53T, during chronological life span (CLS). Heterologous expression of a-syn wild type and the mutant form A53T resulted in a shorter CLS compared with cells harboring the empty vector or expressing the non toxic mutant form A30P. The CLS reduction was accompanied by an increase of both the autophagy and mitophagy activity, revealed by the ALP assay, and an accumulation of intracellular reactive oxygen species. Impairment of mitophagy by deletion of *ATG32* resulted in a CLS extension, implicating mitophagy in the a-syn toxicity. Additionally, *SIR2*, which has been described as a regulator of autophagy, is also controlling mitophagy and thus contributing to a-syn toxicity. Evidence gathered in this work highlights that increased mitophagy activity is an important phenomenon mediating the a-syn toxicity.

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PS6: 58

**Molecular analysis of *Campylobacter jejuni* and *Campylobacter coli* from a nationwide survey performed on broilers****Ana Cláudia Carreira, Teresa Rocha, Margarida Galdes, Maria José Barahona, Luendes Clemente, Ana Botelho, Mónica V. Cunha**

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Campylobacteriosis is the leading cause of gastrointestinal illness in developed countries, *Campylobacter jejuni* being the most frequent etiological agent. Food producing animals, particularly poultry, are common sources for pathogenic strains. Although the disease is usually self-limiting, it is also the most common antecedent infection leading to the development of inflammatory neuropathies including Guillain Barré (GBS) and Miller Fisher syndromes (MFS). The pathway from *C. jejuni* infection to the development of GBS and MFS is still poorly understood. Cumulative evidence points to an important role of certain *C. jejuni* lipooligosaccharide (LOS) subtypes and sialyltransferases involved in LOS synthesis that may induce autoimmune pathogenesis via molecular mimicry. Particularly, class A LOS strains are significantly more common in GBS patients as compared to those with uncomplicated campylobacteriosis. In this work, we report results from the first nationwide study of *Campylobacter* prevalence in broilers at slaughter performed in the framework of the Commission Decision 2007/516/EC. Samples from broilers belonging to more than 200 farms were collected at 14 poultry abattoirs geographically widespread. *Campylobacter* species were recovered from 82% of fecal samples, being *C. coli* the most prevalent (61%). One-hundred and seventy seven isolates of *C. coli* and *C. jejuni* were genotyped based on the restriction fragment length polymorphism analysis of the *flaA* gene (*flaA*-RFLP) encoding a sub-unit of the polar flagellum. Genotyping with *Hinf*I yielded 11 *flaA*-RFLP patterns (Hunter index,  $h=0.62$ ), the most frequent being present in 56% of surveyed isolates. Restriction with *Dde*I generated 48 profiles ( $h=0.89$ ), the most common being prevalent in 28% of the isolates. The predominant genotypes were widespread. The high genetic variability of *Campylobacter* isolates under study based on the *flaA* gene is in agreement with reports from other countries. The prevalence of potentially neuropathic strains among the characterized isolates is currently being investigated using a LOS locus typing scheme. To our knowledge, this is the first report on the molecular analysis of *C. jejuni* and *C. coli* field isolates from broilers on a national scale and on the presence of bacterial genetic polymorphisms conferring increased risk for neuropathy.

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PS6: 59

### Molecular characterization of *Amanita ponderosa* mushrooms from Alentejo (Portugal) and fungal microflora which lives in association

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*Amanita ponderosa* are wild edible mushrooms much appreciated in terms of gastronomy and high export potential in Portugal. These mushrooms grow only in some microclimates, particularly in the Southwest of the Iberian Peninsula, particularly in the Alentejo and Andalusia. Due to the immense diversity of mushrooms in nature, it is essential to differentiate and identify the edible species. Genetic profile characterization and polymorphic sequences analysis are important tools for an effective characterization, namely in the wild mushrooms certification process. M13-PCR fingerprinting is a simple technique for evaluation of genetic variability, which does not require a detailed knowledge of the genome. The electrophoretic patterns of fragments generated by DNA amplification with M13 primer can be used as a DNA fingerprinting to evaluate the diversity of isolated species [1]. In this study, genetic profile of *A. ponderosa* mushrooms and fungal strains that live in association with them in their natural habitat were characterized and compared by M13-PCR amplification of genomic DNA. The main fungal species isolated in association with studied *A. ponderosa* mushrooms were *Aspergillus* spp., *Penicillium* spp. and *Mucor* spp.. Molecular analysis showed different genetic profiles obtained with M13-PCR for fungi isolates and *A. ponderosa* and allow differentiate, fast and reproductively, the different strains and grouped them according to their genera. This fingerprinting also distinguishes different *A. ponderosa* mushrooms collected from different regions and others basidiomycetes, displaying a very discriminatory approach.

[1] Caldeira et al (2009) Annals of Microbiology, 59 (3) pp. 1-6. This work was supported by FCT, SFRH/BD/61184/2009.

PS6: 60

**Molecular detection of *qnrA*, *qnrB* and *qnrS* resistance genes among *Salmonella* spp. in Iran.**

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*Salmonella* resistance to antimicrobial agents such as quinolones and fluoroquinolones has emerged worldwide in recent years. The molecular epidemiology of resistance plasmids has been major scientists became aware of plasmids role in the spread of antimicrobial drug resistance. The purpose of this study was to investigate the presence and dissemination of *qnr* gene among *salmonella* spp isolates from different provinces in Iran. A total of 85 isolates of *Salmonella*. spp were collected from several hospitals in different provinces of Iran during the years of 2008 to 2010. The Antimicrobial susceptibility test was carried out by disk diffusion method. The antimicrobial disks have been used were Nalidixic acid (30µg/ ml) and Ciprofloxacin (30µg/ ml). Minimum inhibitory concentration (MIC) of selected antimicrobial agents were determined by broth microdilution method. The *qnrA*, *qnrB* and *qnrS* genes were detected by PCR. Disk diffusion susceptibility patterns of *qnrA*, *qnrB* and *qnrS* isolates were as follow: 49 (57.6%) isolates exhibited resistance to Nalidixic acid and none isolates to Ciprofloxacin. As expected MIC assay showed these results. Plasmid extraction and PCR assays detect that 22 (25.8%) of 85 *Salmonella* spp clinical isolates have the 700 bp amplicons of *qnrA* gene, 1 ( 1.17%) isolate have *qnrB* gene with the 120 bp amplicons, 1 ( 1.17%) isolate have the 280 amplicons of *qnrS* gene and 1 of 85 isolates carried all of *qnrA*, *qnrB* and *qnrS* genes. Resistance to quinolones and fluoroquinolones by these genes have been confirmed by disk diffusion method. The presence of *qnr* genes has been associated with an increase in the quinolone MIC values and treatment incomplete when quinolone are used to treat quinolone- susceptible *Salmonella* spp. In conclusion we found that drug resistance genes from different types of *qnr* genes are increasing among clinical isolates. According to the fact that quinolones and fluoroquinolones are drugs choices for treatment of Salmonellosis, the effective surveillance programme to monitor the antimicrobial resistance mechanisms are needed. Therefore future studies on the transfer mechanism of the *qnr* genes among *Enterobacteriaceae* are suggested.

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PS6: 61

**Morphological heterogeneity of *Paracoccidioides brasiliensis*: relevance of the Rho-like GTPase PbCDC42****João Filipe Menino<sup>1</sup>, Nuno Osorio<sup>1</sup>, Mark Sturme<sup>1</sup>, Diana Barros<sup>1</sup>, Ana Alves<sup>1</sup>, Agostinho Almeida<sup>1</sup>, Paula Ludovico<sup>1</sup>, Patricio Costa<sup>1</sup>, Gustavo Goldman<sup>2</sup>, Fernando Rodrigues<sup>1</sup>**<sup>1</sup>Life and Health Sciences Research Institute, Portugal; <sup>2</sup>Laboratory of Molecular Biology of the Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brazil

*Paracoccidioides brasiliensis*, the etiological agent of paracoccidioidomycosis, is a thermodimorphic fungus characterized by its distinctive budding and cell division pattern and polymorphic growth, leading to mother and bud yeast cells with extreme variations in shape and area. Its budding pattern and polymorphic growth were previously shown to be closely linked to the expression of *PbCDC42* and to influence the pathogenesis of this fungus. In this work, a detailed morphogenetic evaluation was carried out in the yeast-form of 11 different clinical and environmental *P. brasiliensis* isolates from the 4 different lineages (S1, PS2, PS3 and Pb01-like) as well as in a *PbCDC42* knock-down strain. High variations in the shape and area of mother and bud cells from each isolate were found not revealing characteristic morphologic profiles for each phylogenetic group. In all studied isolates bud area and shape was demonstrated to be highly dependent on the mother cell, indicating a high level of conservation of these traits throughout cell progeny. Importantly, we found strong correlations between *PbCDC42* expression and both the shape of mother and bud cells and the area of the buds in the evaluated isolates and knock-down strain. Our results revealed that *PbCDC42* can explain approximately 80 % of both mother and bud cell shape and 19 % of bud cell area. This further supports that *PbCDC42* expression is a relevant predictor of *P. brasiliensis* morphology. Altogether, these findings explore the polymorphic nature of *P. brasiliensis* and provide additional evidences on how this fungus' morphology is controlled, supporting the hypothesis of CDC42p as a drug target against paracoccidioidomycosis.

PS6: 62

### **Multidrug resistance in a methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate mediated by efflux pump QacA**

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Drug efflux has become an important cause of multidrug resistance (MDR) in bacteria. Plasmid-encoded MDR efflux pumps such as QacA, are implicated in resistance to biocides, generally used in the prevention and control of nosocomial infections, including the ones caused by MRSA. In this study, we evaluated the importance and contribution of QacA to the resistance phenotype of the clinical MRSA isolate SM39, by comparing SM39 (*qacA*) with the plasmid cured derivative, SM39-cured. QacA efflux activity was characterized by two methods: (1) determination of minimum inhibitory concentration (MIC) for biocides, dyes and fluoroquinolone and beta-lactam antibiotics, in the absence and presence of the efflux inhibitors thioridazine, chlorpromazine, verapamil and reserpine; and (2) real-time fluorometry. MIC determination showed that QacA-mediated efflux was involved in the resistance phenotype to the biocides and dyes tested, which included hexadecyltrimethylammonium bromide, cetrimide, benzalkonium chloride, berberine, chlorhexidine and ethidium bromide, since SM39-cured showed lower MIC values for these compounds than SM39. Fluorometric assays also confirmed the higher efflux activity of SM39, harbouring QacA. Both SM39 and SM39-cured strains presented resistance to beta-lactam antibiotics, although with lower values for SM39-cured. Nitrocefin test confirmed the presence of a beta-lactamase in the SM39 plasmid. This work highlights the relevance of QacA in the resistance to biocides, and consequently to the survival and maintenance of MRSA isolates in the hospital environment and in the community. Furthermore, the presence of a beta-lactamase and *qacA* determinants in the same plasmid rises the question of the potential biocide/antibiotic cross-resistance in MRSA strains.

PS6: 63

**Multiplex PCR for the discrimination of vaginal bacterial population****Ana Margarida Magalhães, Joana Castro, Ana Filipa Henriques, Nuno Cerca**

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Bacterial vaginosis is one of the most common disorders in women of reproductive age worldwide and while not life-threatening, it leads to increased risk of more serious gynecologic infections and pre-term labor. It is a pathology which has been recognized to possess several microorganisms involved such as *Lactobacillus* species, *Gardnerella vaginalis*, *Atopobium vaginae*, and other anaerobic bacteria of difficult identification, whose fragile balance may determine the appearance or not the pathology. Currently diagnosis is made mostly based on symptoms and is variable dependent on the symptoms which the physician judge to be most important for diagnosis, and laboratory culture techniques for the identification of bacterial strains present is very time consuming and not always successful in identifying most microorganisms present. In order to have a better understanding of the aetiology of BV and thus be able to identify better tools for the correct diagnosis of this pathology, more effective tools for identification of bacteria commonly associated with the vaginal flora are needed. The use of new molecular biology techniques, such as PCR, has begun to be extensively developed for the study of pathologies such as BV. Besides conventional PCR there have been key developments made to this techniques such as multiplex PCR (allowing the detection of the presence of several strains of organisms in a single reaction) and qPCR which allows the fast quantification of organism which would otherwise take many days to quantify using traditional microbiology techniques. Here we describe the development of a multiplex PCR assay for the identification of 3 of the most commonly studied bacterial strains for the diagnosis of BV: *Lactobacillus* spp., *G. vaginalis* and *A. vaginae*.

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PS6: 64

**N-glycan profiling of proteins secreted by *Ashbya gossypii* in different culture conditions****Tatiana Quinta Aguiar<sup>1</sup>, Merja Penttilä<sup>2</sup>, Lucília Domingues<sup>1</sup>**<sup>1</sup>IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Portugal; <sup>2</sup>VTT Technical Research Centre of Finland, Espoo, Finland

The filamentous hemiascomycete *Ashbya gossypii*, used for the industrial production of riboflavin (vitamin B<sub>2</sub>), has recently been explored as a new fungal host system for the expression and secretion of proteins. Therefore, the interest in the carbohydrate structures (glycans) attached to the glycoproteins produced by this fungus has been raised. Currently almost no information is available on the size distribution and structural characteristics of the *N*-glycans of *A. gossypii* derived glycoproteins. From a phylogenetic point a view, *A. gossypii* is more closely related to yeast than to other filamentous fungi, nevertheless, expression of heterologous glycoproteins in *A. gossypii* has indicated that the recombinant glycoproteins obtained appear to be less extensively glycosylated than those from *Saccharomyces cerevisiae*. A better understanding of the *N*-glycosylation profile of *A. gossypii*'s native proteins may possibly be of practical impact in the production of heterologous proteins. Therefore, in this study, using MALDI-TOF mass spectrometric profiling, we have examined the *N*-glycans present in *A. gossypii*'s native glycoproteins secreted under different culture conditions. *N*-glycan profiling revealed that the major glycan species derived from *A. gossypii*'s secreted proteins are high-mannose type glycans containing core-type structures with eight to eleven mannoses (Man<sub>8-11</sub>GlucNac<sub>2</sub>). Smaller structures (Man<sub>5-7</sub>GlucNac<sub>2</sub>) were also present in lower abundance and only minor relative amount of glycans were shown to have structures larger than Man<sub>12</sub>GlucNac<sub>2</sub>. Growth in defined minimal medium also resulted in charged glycan structures that were slightly bigger (Man<sub>13-15</sub>GlucNac<sub>2</sub>) and phosphorylated. In contrast, no charged glycans could be detected when complex rich medium was used, implying that small glycan species (Man<sub>5-12</sub>GlucNac<sub>2</sub>) may be the general feature of *N*-glycans of the secretory pathway in *A. gossypii* when growing in these conditions.

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PS6: 65

### Novel TaqMan-based multiplex real-time PCR assays to detect and discriminate pathogenic *Leptospira* genomospecies

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Leptospirosis is an underestimated zoonotic disease caused by pathogenic species of *Leptospira*. Serological approaches are commonly used for diagnosing the disease and the isolation of *Leptospira* from animals showing clinical signs suggestive of acute leptospirosis constitutes the definitive diagnosis. However, these culture-based procedures are technically demanding and highly susceptible to failure, essentially due to the fastidious growth of the agent in artificial media and ephemeral bacteraemias. The detection of the leptospires in kidney or urine samples is also necessary to identify animal carriers. The aim of this study was to develop improved real-time PCR-based assays able to detect and discriminate the most relevant pathogenic species of *Leptospira* in animal biological samples, particularly in bovine kidney tissues. Improved sets of species-specific TaqManprobes, and respective flanking primers, were designed “*in silico*” with complementary target sequences in 16S rRNA, *Ompl1*, *RpoB* and *SecY* genes. Genomic DNA was extracted from reference *Leptospira* strains using both commercially available kits and an “*in house*” phenol chloroform extraction. One week-old cultures of *Leptospira interrogans* (strain Autumnalis), *L. kirschneri* (strain Portugal 1990), *L. borgpetersenii* (strain Mitis Johnson) and *L. noguchii* (strain CZ 214 K) in EMJH medium were used for artificially infecting bovine kidney samples and used for assay optimization. Real-time PCR assays were optimized for critical variables such as template DNA and primer/probe concentrations and standard curves were analyzed. The assays developed could efficiently detect and identify the most relevant pathogenic genetic species of *Leptospira*, *L. interrogans* sensu stricto, *L. borgpetersenii*, *L. kirschneri* and *L. noguchii*, in a multiplex real-time PCR format. Efforts towards the implementation and validation of the real-time PCR assays to detect these species in naturally infected bovine samples, such as blood and urine, are currently underway.

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PS6: 66

## Protective effect of acetic acid against ethanol-induced cell death in *Saccharomyces cerevisiae*

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Ethanol is a well-known end product of alcoholic fermentation carried out by *Saccharomyces cerevisiae*. At high concentrations it is responsible for the reduction of cell viability and inhibition of fermentation. Furthermore, during fermentation some weak acids such as acetic, butyric and pyruvic acids, produced by yeast metabolism, may accumulate in the growth medium, and enhance ethanol toxicity which results in a higher inhibition of growth and fermentation. However, previous data obtained in our lab showed that *S. cerevisiae* cells treated simultaneously with toxic concentrations of ethanol and low concentrations of acetic acid displayed higher survival (measured either by CFU or by propidium iodide staining) when compared to cells treated only with ethanol. These results indicated that acetic acid induces a cellular response that provides protection against the cytotoxic effect of ethanol (Vieira et al unpublished results). Subsequently we showed that the Mitogen-Activated Protein Kinase (MAPK) Hog1p and the aquoglyceroporin Fps1p, involved in acetic acid resistance, did not mediate this protective effect of the acid<sup>1</sup>. The aim of the present work was to study the role of other signaling pathways and the mechanisms conferring protection of acetic acid against death induced by ethanol (13% v/v, pH 3.5). We found that this effect is dose-dependent and optimal for 0.1% (v/v) of acetic acid. Propionic, but not lactic or formic acids, also confer protection. Deficiency in the MAPK *Slit2/Mpk1*, known to be activated in the presence of acetic acid<sup>2</sup>, partially revert the acetic acid protective effect, especially regarding the preservation of plasma membrane integrity. The presence of trehalose in the culture medium promoted the protective effect of acetic acid. Trehalose transport and accumulation seem to be necessary for protection by acetic acid against loss of the plasma membrane integrity, but not of cell viability, induced by ethanol. A respiratory deficient mutant (*r<sub>0</sub>*) does not display higher cell viability in the presence of acetic acid, when compared with ethanol treatment alone, suggesting that mitochondria may be involved in the protective effect.

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PS6: 67

**Role of ceramides and ceramides-formed pores in the process of mitochondria permeabilization and yeast programmed cell death****Dário Trindade<sup>1,2</sup>, Manuela Côrte-Real<sup>1</sup>, Maria João Sousa<sup>1</sup>, Stephen Manon<sup>2</sup>**<sup>1</sup>Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, Portugal; <sup>2</sup>Institut de Biochimie et Génétique Cellulaires (IBGC), UMR5095 CNRS & Université de Bordeaux 2, Bordeaux, France

Ceramides are second messengers of different physiological processes. Some ceramides species can have a pro-apoptotic function involving mitochondria. Indeed, ceramides are able to form pores in artificial lipid bilayers and mitochondria. Furthermore, it has been recently demonstrated that the disassembly of these pores is stimulated by anti-apoptotic proteins of the Bcl-2 family. Ceramides signaling is generally conserved, from yeast to human. Though *Saccharomyces cerevisiae* only possess a homolog of a BH3-only protein, it exhibits several features similar to mammalian apoptotic programmed cell death (PCD). Ours and others reports suggest the involvement of these sphingolipids in the modulation of yeast PCD during cell ageing and in response to different death stimuli. The yeast cell death program is associated to the permeabilization of the outer mitochondrial membrane (MOMP) that can be followed by the relocalization of cytochrome c to the cytosol. We observed this relocalization process during acetic acid-induced cell death and during the death induced by the heterologous expression of human pro-apoptotic protein Bax. We have shown that the permeabilization process was dependent on the presence of the adenylic nucleotides carrier (ANC) for acetic acid-induced death and was independent from ANC for Bax-induced death. While Bax-induced permeability is likely dependent on the formation of a protein channel essentially formed of Bax itself, we still do not know on which type of mechanism acetic acid-induced permeability relies on. This study aims to precise the potential role of ceramides and ceramides-formed pores in the process of MOMP and yeast programmed cell death. For this purpose, we tested the response of several *S. cerevisiae* BY4742 mutants on sphingolipid metabolism to Bax- and acetic acid-induced cell death. Yeast cells lacking Isc1p (unable to generate ceramide by degradation of inositolphosphosphingolipids), exhibited a higher resistance to Bax- and, consistently with data obtained with another genetic background (W303 strain), to acetic acid-induced cell death. We will discuss whether this resistance phenotype is related with a reduction in mitochondrial dysfunction induced by these death stimuli.

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PS6: 68

## Role of $\beta$ -lactamase operon on the *mecA* expression in methicillin-resistant *Staphylococcus aureus* (MRSA)

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*Staphylococcus aureus* is one of the leading causes of nosocomial infections worldwide. This microorganism can develop  $\beta$ -lactam resistance through two main mechanisms: production of  $\beta$ -lactamase, encoded by *blaZ* gene, and production of penicillin binding protein 2a (PBP2a), encoded by *mecA* gene. The production of  $\beta$ -lactamase and PBP2a is regulated by similar signal transducing pathways, which include the sensor/inducers BlaR1 and MecR1, and the repressors Blal and Mecl, respectively. Moreover, coregulation of *mecA* and *blaZ* by both systems has been demonstrated, although with remarkable differences in the induction kinetics. In fact, the induction of *mecA* by *mecI-mecR1* is so slow that it has been assumed that high-level  $\beta$ -lactam resistance implies non-functional *mecI-mecR1* regulatory system. However, in a recent study, we have demonstrated that there is no correlation between the presence of a functional *mecI* gene and the level of  $\beta$ -lactam resistance and that for most MRSA strains there were no significant changes on the resistance expression upon the overexpression in *trans* of *mecI*, suggesting the influence of other regulatory mechanisms. Since  $\beta$ -lactamase locus plays an important role in the stabilization and control of *mecA* gene and that the great majority of clinical MRSA strains are positive for this locus, we hypothesized that the  $\beta$ -lactamase locus would account for those puzzling observations. In this study we aimed to test the role of the  $\beta$ -lactamase operon in the regulation of the *mecA* expression, specifically, the putative interference with the Mecl-mediated repression. To this purpose, the native  $\beta$ -lactamase plasmid was introduced, via bacteriophage-mediated horizontal gene transfer, into three strains with no  $\beta$ -lactamase locus and non-functional *mecI-mecR1* system. Remarkably, the presence of  $\beta$ -lactamase genes caused an increase in the resistant phenotype when compared to the parental strains, leading to an high and homogenous resistance level to oxacillin. Furthermore, it disrupted the *mecI*-mediated repression expressed in *trans*. These experiments demonstrated the important influence of the  $\beta$ -lactamase plasmid on the *mecA* mediated oxacillin resistance. In summary, the results provided new insights into the regulatory mechanism of *mecA* expression and presented new perspectives for the important role of the  $\beta$ -lactamase operon in the MRSA phenotype.

PS6: 69

**Staining pattern and potential targets of a benzo[a]phenoxazinium chloride with antiproliferative activity****Ricardo Emanuel Silva Carvalho, Maria Sameiro Gonçalves, Maria João Sousa**  
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Fluorescent markers are widely used in numerous applications. Despite their great variety, in the last years, research has been conducted focusing on the design and synthesis of new fluorophores based on small organic molecules.<sup>1</sup> It was recently demonstrated antimicrobial activity of *N*-(5-((4-ethoxy-4-oxobutyl)amino)-10-methyl-9*H*-benzo[*a*]phenoxazin-9-ylidene)ethanaminium chloride, an heterocyclic compound with an extended planar aromatic system and different substituent groups.<sup>2</sup> However, from our knowledge, no studies to elucidate the intracellular distribution and the actual mechanism of action of this compound have been performed. In the present work, fluorescent probes or fusion proteins linked to green fluorescent protein (GFP), specific for different intracellular compartments were used to evaluate by fluorescence microscopy the cellular distribution of the above mentioned benzo[*a*]phenoxazinium chloride. In order to study the efficiency of the staining as a function of concentration over time, cells incubated with this compound were also analyzed by flow cytometry. The results showed that the benzo[*a*]phenoxazinium chloride in levels close to the minimum inhibitory concentration (MIC), accumulates at the membranes of the endoplasmic reticulum and the vacuole. For concentrations above the MIC, there was a marked disruption of the sub-cellular structures and it was possible to observe some apoptotic nuclei by staining with the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI). It was also found that the fluorescence intensity decreases over time and also when cells are incubated with 10 mM HEPES buffer pH 7.4, 5% glucose, pointing to the possible involvement of efflux pumps of the PDR (pleiotropic drug resistance) family. The results suggest that vacuole and endoplasmic reticulum stress/damage may be triggering of the cell death process.

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PS6: 70

**The novel Fh8 fusion technology for protein expression in *Escherichia coli*: a comparison with the traditionally used fusion systems****Sofia Judite Costa<sup>1,2</sup>, André Almeida<sup>3</sup>, António Castro<sup>2</sup>, Hüseyin Besir<sup>4</sup>, Lucília Domingues<sup>1</sup>**

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The recombinant expression of natural proteins in *Escherichia coli* is limited by the lack of efficient methods for soluble production. Several efforts have been made to overcome this limitation, including the genetic fusion of highly soluble protein domains (fusion partners) to the target proteins. Among the available fusion partners, the *E. coli* maltose-binding protein (MBP), glutathione S-transferase (GST) and N-utilization substance A (NusA) are often used to enhance protein solubility. However, due to their large size, these partners can be problematic for structural and functional analyses, requiring their removal using specific proteases. The removal of the fusion partner is not always successful and the resulting cleaved target protein may also precipitate into insoluble aggregates. In recent years, new fusion partners, as the SUMO, have emerged competing directly with the traditional used ones at producing and purifying soluble proteins. This work aims at the comparison between a novel fusion partner, the small-size Fh8 tag, and the traditionally used ones for soluble protein production in *E. coli*. Six target proteins were fused to eight fusion tags including the well-known NusA, Trx, GST, MBP and SUMO. A high throughput small-scale analysis was initially performed using 10 mL cultures to select the best strain per target protein and to compare both the total expression and solubility effects promoted by the different fusion partners on the used target proteins. The NusA, Trx and MBP fusions resulted in the best *E. coli* expressions, followed by the Fh8 fusions and the SUMO fusions. In spite of the expression results in *E. coli*, the recombinant Fh8-fused proteins achieved similar solubility levels as the NusA and Trx fused ones. The Fh8 fusions presented similar or higher solubility than the GST fusions and higher solubility than the SUMO fusions. These results were also observed after performing a protein production in 500 mL cultures. The tag removal process was compared in both small-scale analysis and in the 500 mL production using the Tobacco Etch Virus (TEV) protease. After Fh8 tag removal, the cleaved target proteins remained soluble and presented similar or higher production yields than the cleaved proteins from the Trx and NusA fusions. Results from this study revealed the Fh8 as a robust fusion tag standing among the best fusion tags for protein expression and solubility in *E. coli*.

PS6: 71

**The role of NorA in the adaptative response of *Staphylococcus aureus* to stress stimuli****Sofia Santos Costa<sup>1,2</sup>, Miguel Viveiros<sup>1,3</sup>, Leonard Amaral<sup>1,3,4</sup>, Isabel Couto<sup>1,2</sup>**

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Efflux of antimicrobial compounds is a first-line defence mechanism to chemical stress. We previously adapted the antibiotic pan-susceptible *S. aureus* ATCC25923 (ATCC) to ethidium bromide (EtBr), yielding the progeny strain ATCC-EtBr, which showed overexpression of the efflux pump (EP) gene *norA*. We now continued this study by reversing the EtBr adaptation process and comparing the parental and derivative strains. Reversion of ATCC-EtBr was done by serial passages in EtBr-free media, resulting in ATCC-Rev. Susceptibility profile of the parental and derivative strains was assessed by MIC determination. Efflux activity was evaluated by real-time fluorometry and RT-qPCR used to analyze gene expression of the global regulator *mgrA*, *norA* and other EP genes. The *norA* gene was sequenced and its transcription initiation site determined by 5'-RACE PCR. *norA* mRNA half-life was determined for ATCC and ATCC-EtBr. The ATCC-EtBr overexpressed *norA* presented a new transcription initiation site and a mRNA half-life comparable to the one of ATCC. ATCC-EtBr *norA* also carried a mutation leading to the substitution Phe303Tyr. The regulator *mgrA* was also found to be overexpressed in ATCC-EtBr. ATCC-Rev showed complete reversion of the increased resistance to EB, fluoroquinolones and biocides presented by ATCC-EtBr and the loss of efflux activity. This was accompanied by decrease of both *norA* and *mgrA* expression levels and the introduction of a stop codon in *norA*, originating a NorA truncated at position 274. This work highlights the importance of EPs in the response of *S. aureus* to stress stimuli. The presence/withdrawal of EtBr led to an increase/decrease in the expression of *norA* and the regulator *mgrA*. However, we found that other alterations at the transcription initiation site or in the aminoacid sequence are also to be considered when analyzing differences in NorA expression and/or activity. All these factors interplay in an intricate network of *S. aureus* cellular responses to chemical stress.

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PS6: 72

## The yeast *Saccharomyces cerevisiae* is sensitive to colorectal cancer routine treatment EGFR antibody Cetuximab

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Cetuximab/Erbitux® (Merk Sereno), a drug used in routine treatment of colorectal cancer and other malignant pathologies, is a monoclonal antibody against the Epidermal Growth Factor Receptor (EGFR). A frequent problem affecting the clinical use of Cetuximab is the lack of effectiveness deriving from frequent mutations in K-ras (1-3). A set of mutations in K-ras gene, KRAS c.35G>A (G12D), KRAS c.38G>A (G13D) in exon 2, and KRAS c.183A>T (Q61H) in exon 3, all implicated in the development of colorectal cancer, have been recognized as impeding Cetuximab's EGFR inhibitory action in human (1). Ras human genes have recognized counterparts in yeast, RAS1 and RAS2. The corresponding proteins belong to the PKA/cAMP MAPK pathway are involved in cell proliferation, in differentiation into hyphae and spores, in response to nitrogen starvation, and in carbon source regulation (4, 5). In opposition to Ras, yeasts do not have a recognized ortholog of EGFR. Nevertheless, yeast is sensitive to Imatinib, another drug that targets specifically EGFR in human cells (6). We generated recombinant yeast strains expressing human wild-type (wt) and mutated open reading frames (ORFs) of K-ras to use in the optimization of phenotypic tests appropriate for the assessment of cell sensitivity to Cetuximab. We observed that *Saccharomyces cerevisiae*, is sensitive to the treatment with this drug at identical concentration as human cell cultures. Moreover, the complementation of yeast deletions in RAS1 and/or RAS2 with wt or the above mentioned mutated forms of human K-RAS did not alter the response of the cells to the treatment. This suggests that the sensitivity of *S. cerevisiae* to Cetuximab is independent of the Ras/cAMP pathway. These results further indicate the existence of a paralog of EGFR protein in yeast cell surface. In view of these results, research focused on identifying the EGFR yeast counterpart, downstream effectors and target genes, and determining the correspondent Cetuximab/Erbitux® mode of action.

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PS6: 73

### Molecular detection of AmpC $\beta$ -Lactamase resistance gene among *Salmonella* spp. in Iran

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The present study extended our knowledge of the prevalence and evolution of AmpC in Iran. Attention should be drawn to this serious situation of AmpC producing *Salmonella* evolution. The emergence of resistance to extended-spectrum Cephalosporins (ESC), the main therapeutic choice for treatment of *Salmonella* infections, has caused a global problem. The extended spectrum cephalosporin (ESC) resistance is attributed to a plasmid-borne *bla*<sub>CMY-2</sub>  $\beta$ -lactamase which can spread horizontally to different serotypes carrying multiple resistance determinants. A total of 85 isolates of *Salmonella* spp. were collected from several provinces of Iran during the years of 2008 to 2010. Antimicrobial susceptibility testing and PCR assays were carried out to detect *bla*<sub>CMY-2</sub> gene, a class C plasmid-encoded AmpC  $\beta$ -lactamase. PCR assays detected 24 (28.2%) *Salmonella* isolates carrying *bla*<sub>CMY-2</sub> gene, a class C plasmid-encoded AmpC  $\beta$ -lactamase confers resistance or decreased susceptibility to Extended Spectrum Cephalosporins (ESCs). Disk diffusion susceptibility patterns and MIC profile of *bla*<sub>CMY-2</sub> positive isolates were as follow: 9 (10.5%) isolates exhibited resistance to ceftazidime, 6 (7.05%) isolates to ceftriaxone, 9 (10.5%) isolates to cefotaxime, 9 (10.5%) isolates to cefexime, 5 (5.88%) isolates to cefepime, 4 (4.7%) isolates to cefpodoxime. Only one isolate exhibits an ESBL phenotype. A surprisingly prevalence of AmpC producers was detected in human isolates of *Salmonella* in Iran with the *bla*<sub>CMY-2</sub> type enzymes being most predominant. This study showed the distribution of the extended-spectrum cephalosporin (ESC) resistance among *Salmonella* spp. Isolates in Iran. The MDR *bla*<sub>CMY-2</sub> isolates may disseminate by horizontal transfer and also further spread of the resistance clone is possible. In this study the *bla* gene was detected in the majority of sensitive isolates. Despite possessing the gene encoding cephalosporinase (*ampC*), wild strains of *Enterobacteriaceae* such as *Salmonella* are susceptible to most  $\beta$ -lactams because of the absence of an efficient *ampC* promoter region. Some of the genes associated with  $\beta$ -lactamase enzymes harbored on the same plasmid which leads to their over expression. But the extensive use of  $\beta$ -lactam antimicrobial drugs has led to the emergence of resistant strains worldwide. It is necessary to examine the factors contributing to ESC dissemination.

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PS6: 74

**Activation of the Hog1p kinase in *Isc1p*-deficient yeast cells is associated with mitochondrial dysfunction, oxidative stress sensitivity and premature aging****António Daniel Barbosa<sup>1,2</sup>, João Graça<sup>1</sup>, Vanda Mendes<sup>1</sup>, Maria Amélia Amorim<sup>1,2</sup>, Marta Vaz Mendes<sup>1</sup>, Pedro Moradas-Ferreira<sup>1,2</sup>, Vítor Costa<sup>1,2</sup>**<sup>1</sup>IBMC, Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal; <sup>2</sup>ICBAS, Instituto de Ciências Biomédicas Abel Salazar, Departamento de Biologia Molecular, Universidade do Porto, Porto, Portugal

The sphingolipid metabolite ceramide is an important second messenger that regulates processes such as stress responses, cell cycle arrest, apoptosis and senescence. In *Saccharomyces cerevisiae*, it can be produced from *de novo* biosynthesis or by the hydrolysis of complex sphingolipids mediated by *Isc1p*, the yeast orthologue of mammalian neutral sphingomyelinase 2. *Isc1p* plays a key role in mitochondria function, oxidative stress and chronological aging, regulating redox homeostasis, iron levels and apoptosis. Interestingly, loss of *Isc1p* was previously correlated with an increase of long chain ceramides during chronological aging. This work aims to disclose cell signaling pathways controlled by *Isc1p* that affect mitochondrial function, oxidative stress resistance and chronological lifespan. Here we show that both ceramide and loss of *Isc1p* lead to the activation of Hog1p, the MAPK of the high osmolarity glycerol (HOG) pathway that is functionally related with mammalian p38 and JNK. The hydrogen peroxide sensitivity and premature aging of *isc1Δ* cells is partially suppressed by *HOG1* deletion. Notably, Hog1p activation mediates the mitochondrial dysfunction and catalase A deficiency associated with oxidative stress sensitivity and premature aging of *isc1Δ* cells. Downstream of Hog1p, *Isc1p* deficiency activates the cell wall integrity (CWI) pathway. Deletion of *SLT2* gene, which encodes for the MAPK of the CWI pathway, is lethal in *isc1Δ* cells and this mutant strain is hypersensitive to cell wall stress. However, the phenotypes of *isc1Δ* cells are not associated with cell wall defects. Our results support a role for Hog1p in the regulation of mitochondria function and suggest that the constitutive activation of Hog1p is deleterious for *isc1Δ* cells under oxidative stress conditions and during chronological aging.

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PS6: 75

**Description of a novel staphylococcal pC194-Family plasmid carrying the *smr* efflux pump gene****Sofia Santos Costa<sup>1,2</sup>, Cláudia Mourato<sup>1</sup>, Miguel Viveiros<sup>1,3</sup>, José Melo-Cristino<sup>4</sup>, Leonard Amaral<sup>1,3,5</sup>, Isabel Couto<sup>1,2</sup>**

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Increased resistance to biocides with antiseptic and disinfectant properties bestowed by efflux pumps (EPs) is an important mechanism for bacteria to persist in hostile environments. We have previously isolated a methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate, SM52, carrying the plasmid pSM52 with the *smr* gene, which encodes the efflux pump Smr. A thorough study was conducted to assess the contribution of Smr to resistance towards antimicrobial compounds in this human clinical isolate of *S. aureus*. Smr efflux activity was evaluated in SM52 and RN4220:pSM52 by MIC determination for a panel of twenty-three antimicrobial compounds in the absence and presence of efflux inhibitors (EIs) and characterized by real-time fluorometry. Gene expression analysis was performed by RT-qPCR assays using ethidium bromide (EtBr) and ciprofloxacin as efflux inducing agents. pSM52 was isolated and sequenced by primer-walking. pSM52 corresponded to a 2.8 kb plasmid containing 7 putative ORFs, including the *smr* gene and a partial gene duplicate  $\Delta smr$ , as well as a replication initiation protein (Rep) and truncated forms of Cop proteins. Sequence analysis showed that pSM52 is a new member of the pC194-family plasmid. Smr efflux activity was correlated with increased resistance to quaternary ammonium compounds (EtBr, cetrimide, CTAB and cetylpyridinium chloride) in both SM52 and RN4220:pSM52 by MIC determination in the presence of the EIs thioridazine and chlorpromazine together with real-time fluorometry assays. RT-qPCR assays detected *smr* expression induction only by EtBr. The finding of a plasmid in a human MRSA isolate harbouring a single antiseptic resistance gene, *smr*, strengthens the importance of plasmid-encoded EPs in the maintenance of strains in the hospital environment as well as in the community. Also, the general use of antiseptics and disinfectants for the prevention and control of bacterial infections could potentiate the dissemination of isolates carrying biocide-resistance plasmids.

PS6: 76

**Role of Isc1 in the regulation of unfolded protein response: effect on cell death induced by oxidative stress and ageing in *Saccharomyces cerevisiae*****Vitor Hugo Teixeira<sup>1,2</sup>, Pedro Moradas-Ferreira<sup>1,2</sup>, Vitor Costa<sup>1,2</sup>**<sup>1</sup>IBMC, Portugal; <sup>2</sup>ICBAS, Portugal

The sphingolipid ceramide plays key roles on cellular processes, such as redox homeostasis, apoptosis and ageing. In *Saccharomyces cerevisiae*, it can be produced by the *novo* synthesis or generated through hydrolysis of complex sphingolipids, catalysed by the inositol phosphosphingolipid phospholipase C protein, Isc1p (the yeast orthologue of the mammalian neutral sphingomyelinase 2) [1]. Cells lacking Isc1p display shortened chronological lifespan (CLS), oxidative stress sensitivity and mitochondrial dysfunctions [2]. Isc1 is an upstream regulator of Sit4, the catalytic subunit of the type 2A ceramide-activated protein phosphatase, and *SIT4* deletion suppresses *isc1Δ* phenotypes [3]. The endoplasmic reticulum (ER) plays a key role in the regulation of the aging process. Upon an ER stress condition, cells trigger an adaptive response called unfolded protein response (UPR) [4]. This study aimed to evaluate changes in ER function of *isc1Δ* cells and its correlation with oxidative stress sensitivity and premature ageing. For this purpose, UPR induction was assessed by using a UPR-*lacZ* reporter. For overexpression of an intron-less form of the *HAC1* gene, which encodes for the transcription factor that regulates UPR, cells were transformed with *pHAC1*<sup>1</sup>. In parental cells, UPR was transiently induced during chronological ageing. Loss of Isc1p decreased UPR basal levels and its induction during cell ageing, an effect that was suppressed by deletion of *SIT4* gene. The overexpression of *HAC1* partially suppressed the shortened CLS of *isc1Δ* cells but did not affect the oxidative stress sensitivity or mitochondrial dysfunction of this strain. These results suggest that Isc1 deficiency decreases UPR by a Sit4-dependent mechanism but the deregulation of UPR does not seem to be associated with the premature ageing of *isc1Δ* cells.

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PS6: 77

**Characterization at the molecular level of a new regulator of the expression of methicillin-resistance in *Staphylococcus aureus*****Pedro Arêde<sup>1</sup>, Hermínia de Lencastre<sup>2</sup>, Duarte C. Oliveira<sup>3</sup>**

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a very important human pathogen both in hospitals and in the community. The *mecA* gene is the central genetic element of methicillin resistance and its transcription can be regulated by a repressor (*mecI*) and a sensor-inducer (*mecR1*). In spite of the central role of *mecA* for the methicillin resistance phenotype, its transcriptional control remains not fully understood. Previously (Micro'09, Oral session S-1, 184), we have provided evidence for the existence of a new regulator – *mecR2* – of the expression of methicillin-resistance by constructing a null mutant in a prototype MSRA strain. The main goals of the current work consist on the validation of the *mecR2* null mutant by genetic complementation and the evaluation of the effect of *mecR2* deletion on the transcription levels of *mecA*. The *mecR2* null mutant was complemented *in trans* using a plasmid containing a wild type copy of the *mecR2* gene under the control of an inducible promoter (*Pspac*). Northern blot analysis and Real-Time PCR were used to assess the effect of the *mecR2* deletion in the induction of *mecA* upon exposure to the antibiotic in the parental, *mecR2* null mutant and complemented strains. The complementation studies showed that the phenotypic expression of  $\beta$ -lactam resistance of the parental strain was fully restored in the presence of optimal concentration of the inducer. Whereas, using 10-fold excess of the inducer, the phenotype of the parental strain could not be restored, suggesting a requirement for a delicate balance of MecR2 cellular levels. Northern blot and RT-PCR analysis demonstrated that *mecA* transcription levels decreased in the *mecR2* null mutant but that were not fully restored in the complemented mutant, suggesting that the effect of *mecR2* is not only restricted to the cellular amount of *mecA* transcripts. Based on these results we can conclude that, in the presence of *mecI-mecR1*, *mecR2* gene is essential for optimal expression of  $\beta$ -lactam resistance in MRSA strains, however, the effect of *mecR2* does not correlate directly with the cellular amount of *mecA* transcripts.

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PS6: 78

### Identification of amino acid residues critical for the substrate translocation in lactate permease Jen1p of *Saccharomyces cerevisiae*

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Lactic, acetic and propionic acids have been used for many years in industrial and pharmaceutical companies. In *Saccharomyces cerevisiae*, Jen1p is a major monocarboxylate: H<sup>+</sup> symporter specific primarily for lactate, pyruvate and for acetate (TC # 2.A.1.12.2) (Casal et al., 1999). A phylogenetic tree of ScJen1p homologues (Casal et al., 2008) showed the existence of two main clusters: a Jen1 group (monocarboxylate transporters) and a Jen2-like (dicarboxylate transporters). Structure-function relationships in Jen1p have been approached by using a rational mutational analysis of conserved amino acid residues (Soares-Silva et al., 2007). Analysis of the conserved sequence 379NXX[S/T]HX[S/T]QDXXT391, located in transmembrane segment seven (TMS-VII), showed that residues N379, H383 or D387 are necessary for function and specificity, while Q386 is important for the kinetics of Jen1p-mediated transport. In this work, we rationally designed and analyzed novel mutations in conserved regions located in TMS-II, TMS-V and TMS-XI of Jen1p, which we predicted to affect Jen1p specificity (distinction between mono and dicarboxylates) and function. Among the residues studied, F270 (TMS-V) and Q498 (TMS-XI) are specificity determinants for the distinction of mono- from dicarboxylates, and N501 (TMS-XI) is critical for function. Using a model based on Jen1p similarity with the GlpT permease, we show that all polar residues critical for function within TMS-VII and TMS-XI are aligned along the protein pore and substrate docking studies reveal a potential substrate translocation trajectory consisting mostly of the polar residues genetically identified as important for function. Overall, our results constitute a first step towards the genetic manipulation of substrate specificity in the lactate/pyruvate:H<sup>+</sup> symporter subfamily and a tool for the in silico prediction of the function of Jen1p homologues in other fungi (Soares-Silva et al., 2011).

Casal, M., et al. (1999) *J. Bacteriol.* 181, 2620-2623. Casal, M., et al. (2008) *FEMS Microbiol Rev.* 32, 974-994. Soares-Silva, I., et al. (2007) *Mol. Membr. Biol.* 24, 464-474. Soares-Silva, I. et al. (2011) *Mol Microbiol.* 81, 805-817. I.S.S. (SFRH/BPD/22976/2005) and J.S.P. (SFRH/BD/61530/2009) received fellowships from FCT, co-funding by FSE in the scope of QREN/POPH

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# Cell and Tissue Engineering and Biomaterials

*Poster session thematic symposium 7*

Azevedo | Mendes | Silva | Ferreira | Pereira | Breia |  
Barbosa



PS7: 1

## **Biomimetic extracellular matrices as artificial environments for cell culture**

**Helena S. Azevedo**<sup>1,2</sup>, **Rui C. Pereira**<sup>1,2</sup>, **Ana C. Mendes**<sup>1,2</sup>, **Daniela S. Ferreira**<sup>1,2</sup>, **Rui L. Reis**<sup>1,2</sup>

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The development of 3D culture models that allows studying cell behavior in physiological and pathological conditions in the context of the extracellular matrix (ECM) would be extremely useful to investigate specific cell-cell and cell-ECM interactions, as well as the effects of specific factors. Compared with culturing cells on plastic, cell culture in 3D environment provides another dimension for external mechanical and chemical inputs, which dramatically affects integrin ligation, cell contraction and associated intracellular signaling. By binding to a wide variety of soluble growth factors and other signaling molecules, native ECM provides their controlled diffusion and serves to regulate their local concentrations and gradients. The diversity of the (often unknown) cues that are present in native ECM, strongly contributes for the difficulty to realize such complexity *in vitro*. Naturally-derived ECMs provide a wide spectrum of chemical and physical cues that are known to influence the function of many cells, but their variability in composition and mechanical properties are clearly a disadvantage when trying to isolate the effects of specific factors. Advances in synthetic biomaterials are converging to allow the creation of *in vitro* models that capture some complex features of the *in vivo* environment. Synthetic matrices, that are tailored to mimic specific ECM properties, are being implemented to provide well controlled and reproducible cellular environments. Moving to well-defined synthetic 3D systems is however more challenging and requires control not only of cell adhesion sites and matrix viscoelasticity, but of nano and microporosity (which regulates cell motility and the transport of soluble molecules), growth-factor binding and matrix degradation. Consequently, the need for matrices that combine the benefit of natural and synthetic materials has become apparent. This talk will focus on our efforts to develop bioactive matrices for applications in tissue regeneration strategies, in particular for cartilage and skin, but also as *in vitro* model systems for studying cell behavior and discover new therapies. These matrices are formed by self-assembly when combining designed peptide structures with natural polymers, and can capture many aspects of the structure and function of natural ECM.

PS7: 2

## **Fabrication of self-assembled xanthan-peptide microcapsules as 3D environments for cell culture and therapy**

**Ana Carina Mendes<sup>1,2</sup>, Erkan Turker Baran<sup>1,2</sup>, Rui Luís Reis<sup>1,2</sup>, Helena Sepulveda Azevedo<sup>1,2</sup>**

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Self assembling peptides are excellent structural units to form complex nanostructures that can recreate some of the architectural features of the natural extracellular matrix, as they can self-assemble into fibril nanostructures. We report here a mild cell encapsulation method based on triggering the self-assembly of a multidomain peptide in presence of xanthan gum polysaccharide, which has been investigated in our group as artificial matrix for the encapsulation of chondrocytic cell [1]. The self-assembling peptide K<sub>2</sub>(QL)<sub>6</sub>K<sub>2</sub> has a central block of glutamine-leucine (QL) repeats, and two flanking positively charged lysine (K) to bind to the negatively charged xanthan. Using a microfluidic device we were able to produce microcapsules with homogenous size by forming a water-in-oil multiphase. This technology allows a control over the properties of the microcapsules in terms of size and morphology, being a low stress inducing method suited for cell encapsulation. The properties and performance of xanthan-peptide microcapsules were optimized by changing peptide/polysaccharide ratio and their effects on the microcapsules properties, such as permeability and mechanical stability were analyzed. Moreover, the effect of microcapsule formulation on viability and proliferation of encapsulated chondrogenic cells were also investigated. The encapsulated ATDC5 cells were metabolically active, showing an increased viability and proliferation over 21 days of *in vitro* culture demonstrating the long-term stability of the developed microcapsules and their ability to support and enhance the survival of encapsulated cells over prolonged time. Combining self-assembling materials with microfluidics processing proved to be innovative approach to fabricate suitable matrices for cell encapsulation, culture and delivery.

[1] A. C. Mendes, E. T. Baran, C. Nunes, M. A. Coimbra, H. S. Azevedo and R. L. Reis, *Soft Matter* 2011. DOI: 10.1039/c1sm05594a. Acknowledgments: ACM acknowledges to FCT for the financial support (PhD grant SFRH/BD/42161/2007)

PS7: 3

## Gellan gum – hyaluronan hydrogels encapsulating human adipose derived stem cells for skin tissue engineering

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Tissue engineering (TE) relies on the use of biomaterials for providing cells mechanical support and the correct microenvironment to interact with each other and form new tissue. In the particular case of skin TE, the tissue soft nature, supports the use of hydrogels as vehicles for cell delivery and tissue ingrowth. Hydrogels, having a high water absorption capability, facilitate the transport of oxygen, nutrients and waste products. Human adipose derived stem cells (hASCs) isolated from the adipose tissue present immunological features very attractive in the context of TE. Indeed, this work attempted to prepare and optimize glycosaminoglycans (GAGs)-based hydrogels constituted of hyaluronic acid (HA) and gellan gum (GG) to support hASCs encapsulation for skin tissue engineering purposes. Different GG-HA hydrogels formulations, ranging from 0.75 to 1.25% (m/V) of GG and from 0.25 to 0.75% (m/V) of HA were prepared. The *in vitro* enzymatic degradation was evaluated by incubating the hydrogels with hyaluronidase solutions (0, 50 and 150 U/ml) and by quantifying the weight loss of the degraded hydrogels and the resultant fragments using the DNS assay. The swelling capacity, as well as the mechanical properties of the developed hydrogels were analysed by compression tests. Finally, the hydrogel morphology was visualized by SEM. The best formulations were selected for further biological assays. hASCs were encapsulated in the different hydrogels while the polymerization process occurred. The viability of the encapsulated hASCs was followed, along 3, 7 and 14 days, after Calcein-AM and Propidium Iodide staining. Hydrogels with different mechanical properties were obtained by altering the % (m/V) of the GG-HA formulations. Hydrogels with high percentage of GG were stiffer, while increasing concentrations of HA promoted hydrogel flexibility and higher degradation rates. Moreover, the hydrogels showed an intermediate degradation rate compared to the currently used photocrosslinkable HA-methacrylated hydrogels that rapidly degraded in PBS at 37°C. Hydrogels effectively encapsulated hASCs and cell viability, after 14 days, was not compromised. Finally, this work allowed to obtain innovative GG-HA based hydrogels with tuned properties according to the different compositions. More importantly, their capacity to support cell encapsulation makes these innovative formulations very appealing for skin TE applications.

PS7: 4

## **Biomimetic membranes as *in vitro* models for cell culture and wound healing therapies**

**Daniela S. Ferreira<sup>1,2</sup>, Alexandra P. Marques<sup>1,2</sup>, Rui L. Reis<sup>1,2</sup>, Helena S. Azevedo<sup>1,2</sup>**

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The extracellular matrix (ECM) is a highly complex network that serves as a reservoir of signaling molecules and growth factors and it's known to regulate cellular processes in wound healing, such as adhesion, growth and migration. Biomaterials that mimic multiple aspects of native cellular environment during the wound healing process, including presenting adhesion sites and displaying growth factors in the context of a matrix would be of great interest for application in skin regeneration. Here we present a biomimetic matrix that incorporates structural components of skin extracellular matrix (hyaluronan) and biochemical signals (RGDS epitope) to recreate some aspects of skin tissue niche. The RGDS sequence is present in cell binding domains of extracellular proteins (such as fibronectin) and is known to promote integrin-mediated cell adhesion. These matrices result from the self-assembly between peptide amphiphiles and hyaluronic acid (HA), a major component of skin ECM. The RGDS sequence was incorporated in the peptide structure to provide the matrices with cell-adhesive properties. To investigate the potential of 2D biodegradable membranes as supportive bioactive matrix for wound healing, human dermal fibroblasts were cultured on these matrices to study the effect of the RGDS epitope on the adhesion, morphology and proliferation processes. Cell responses to RGDS matrices were compared to matrices containing DGSR (scrambled sequence that does not promote cell adhesion). When cultured on membranes without the cell recognition epitope RGDS, human dermal fibroblasts showed lower adhesion to the matrices when compared to the ones containing RGDS. SEM analysis showed adherent cells on the RGDS matrices and the presence of filopodia which are known to be involved in the regulation of cell migration. We expect that the proposed biodegradable bioactive matrices could offer significant potential in skin regeneration strategies and also as model systems for fundamental mechanistic studies in wound remodeling.

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PS7: 5

## Culture of primary human articular chondrocytes within a 3D injectable matrix for cell delivery in cartilage repair strategies

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In the last decades, the biology of articular cartilage and its exclusive cellular component – chondrocytes – have been under focus of an increasing interest by the tissue engineering and regenerative medicine community. Embedded in a dense extracellular matrix (ECM), chondrocytes show a limited capacity to regenerate articular cartilage after being damaged [1]. The aim of this study was to investigate the potential of a new biodegradable injectable gel, based on carrageenan/fibrinogen/thrombin/hyaluronic acid polymers, for encapsulation and delivery of human articular chondrocytes (HACs). *In vitro* genotype and phenotype and *in vivo* deposition of cartilage markers by 3D encapsulated HACs was investigated. Cells harvested from articular cartilage biopsies were isolated and expanded in 2D. Encapsulated cells within the injectable gel were maintained *in vitro* for a period of 4 weeks. Over the course of time, samples were analyzed via light microscope and optical sections acquire with APOTOME. Images acquired by APOTOME of encapsulated HACs labelled with Hoechst showed homogeneous viable cell dispersion within the gel over time. The MTT assay showed a constant and small increasing in cell viability. Histological and immunohistochemical analysis were made to verify the deposition of cartilage ECM. RT-PCR was performed to assess the levels of transcripts for chondrocyte markers. These analyses, demonstrated the strong chondrogenic phenotype of encapsulated cells. *In vivo* organ culture was performed using portions of bovine articular cartilage where defects were drilled, injected with the gel and implanted subcutaneously in nu/nu mice. *In vivo* results revealed the formation of a healthy cartilaginous tissue within the drilled defect by delivered HACs. Our results demonstrated that encapsulated HACs within this hydrogel, remained viable, proliferated, maintained the expression of the typical chondrogenic marker genes and deposited cartilage ECM. With the *in vivo* model, we showed the formation of a new cartilage tissue well integrated with the surrounding pre-existing one. We anticipate that the developed hydrogel may be used with success to deliver *ex vivo* expanded HACs by arthroscopy injection into the articular cartilage lesions.

PS7: 6

### **In vitro cultures of grape tissues: new possibilities to study grape berry physiology**

**Richard Breia<sup>1</sup>, João Serôdio<sup>2</sup>, Hernâni Gerós<sup>1,3</sup>, Ana Cunha<sup>1,3</sup>**

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Grape berries suffer important morphological, biochemical and physiological changes during its development and maturation. It is known that photoassimilates translocated from leaves serve as the major source of carbon and energy to support fruit needs, but recent findings revealed that, at least in the green phase, grape berries show high photosynthetic activity especially in the exocarp. The contribution of fruit photosynthesis for fruit growth and production of organic compounds is far from being understood. In this study photomixotrophic cell suspensions were established as an *in vitro* model to complement the study grape berry photosynthesis. *Calli* of CSB (Cabernet Sauvignon Berry) cells derived from the inner tissues of the grape berry were sub-cultured in liquid modified MS medium supplemented with 2% sucrose and different hormonal combinations, one auxin (NAA) and three cytokinins (BAP, ZEA and KIN) at two different final concentrations (0.5 and 1 mg mL<sup>-1</sup>). Two different growth light intensities (45- 60 and 80-105 mmol m<sup>-1</sup> s<sup>-1</sup>) were also tested. Chlorophyll fluorescence PAM fluorometry was used to evaluate the photochemical efficiency ( $F_v/F_m$ ) of all suspensions and chlorophyll content was also determined. Results showed that the cytokinin type was crucial to induce the photosynthetic phenotype, but  $F_v/F_m$  was low when compared to the value exhibited by grape berry skin. To further study the photoautotrophy of the grape berry tissues new *callus* cultures were established from the exocarp tissues of the fruit, harvested at the green stage (Alvarinho cv). Portions of detached exocarp were cultured on B5 solid medium supplemented with different hormonal combinations (NAA or 2,4-D with BAP or ZEA) at different concentrations (0.1 or 0.2 mg mL<sup>-1</sup> for auxins and 0.2 or 0.4 mg mL<sup>-1</sup> for cytokinins). Only two combinations were responsive but both induced pale green *calli* with higher growth rates. To our knowledge, this is a pioneer study on *calli* production from the exocarp of the grape berry from the Portuguese variety Alvarinho. This approach opens good perspectives to study in more detail the physiology of these cells, namely the role of photosynthesis on cell growth and metabolite production. Moreover, it will allow to investigate the impact of several environmental factors on fruit photosynthesis, such as temperature, light and water potential.

PS7: 7

## A factorial design approach for modeling mouse embryonic stem cell self-renewal at different O<sub>2</sub> levels

**Hélder S.C. Barbosa**, Tiago Fernandes, Tiago Dias, Margarida M. Diogo, Joaquim Cabral

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Mouse embryonic stem (mES) cells can self-renewal in feeder-free culture conditions via activation of STAT3 signaling by the leukemia inhibitor factor (LIF) and via Smad pathway activation by the bone morphogenetic protein (BMP-4). Alternatively, undifferentiated mES cells can be expanded via inhibition of the mitogen-activated protein kinase (MAPK, MEK/ERK signaling pathway) and inhibition of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) using the small molecules PD0325901 and CHIR99021, respectively. Work previously performed in our group showed that culturing mES cells under different oxygen tensions gave rise to different cell proliferation patterns and commitment stages dependent on which signaling pathways are activated/inhibited to support mES cells self-renewal. These results show that mES cell self-renewal and pluripotency can be differently influenced by O<sub>2</sub> tension levels according to the effect exerted by this culture parameter towards the different signaling pathways. To further investigate these effects, the influence of each one of the small molecules used for the inhibition of MEK/ERK pathway, activation of Wnt/ $\beta$ -Catenin pathway through inhibition of GSK-3 $\beta$  and activation of STAT3 signaling, was evaluated for the *ex vivo* expansion of 46C mES cells under 2% and 20% oxygen tensions. Cell proliferation was analyzed using cumulative fold increase after 5 consecutive passages (10 days) and apoptose levels were measured; pluripotency was analyzed using colony forming efficiency, immunofluorescence microscopy (Nanog) and real-time PCR (Nanog, Oct4, Dax1 and Rex1). GSK-3 $\beta$  phosphorylation level was analyzed by flow cytometry. In the presence of CHIR99021, mES cell proliferation and pluripotency were enhanced in both oxygen tensions, especially under hypoxia. With LIF supplementation only, low oxygen tensions decrease cell proliferation and increase differentiation of mES cells. In addition, the conjugation of the two chemical inhibitors resulted in higher mES cell proliferation and pluripotency in all conditions studied, suggesting a synergy between the two molecules. Hypoxia appeared to modulate GSK-3 $\beta$  phosphorylation level, promoting GSK-3 $\beta$  activation and, consequently, Wnt/ $\beta$ -Catenin pathway downregulation. However, CHIR99021 supplementation can fully-revert hypoxia effects, resulting in higher mES cell self-renewal, suggesting a central role on Wnt/ $\beta$ -Catenin pathway activation in the maintenance of mES cell pluripotency at hypoxia under the studied conditions.

# Functional Genomics and Systems and Synthetic Biology

*Poster session from thematic symposium 8*

Gil | Lima | Soares-Castro | Moshiri | Machado |  
Jarzab | Gomes



PS8: 1

## Genome-wide screening of molecular biomarkers in *Saccharomyces cerevisiae* relevant for predicting alachlor toxicity

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Alachlor (ALA) has been a commonly applied herbicide and is one substance of ecotoxicological concern. We aimed to identify molecular biomarkers in the eukaryotic model *Saccharomyces cerevisiae* that may be used to predict potential cytotoxic effects of ALA while providing mechanistic clues. Transcriptional profiling in a yeast population in response to two exposure scenarios exerting effects of slight to moderate magnitude at phenotypic level (namely, inhibition of yeast growth) was carried out. In particular, 100 and 264 genes were found as differentially expressed upon a 2 h exposure of yeast cells to, respectively, the lowest observed effect concentration (LOEC; 110 mg/L) and the 20% -inhibitory concentration (IC<sub>20</sub>; 200 mg/L) of ALA, in comparison with cells not exposed to the herbicide [1]. Of particular interest is the detection of expression induction > 2-fold of a significant number of genes at ALA concentrations causing a comparatively minor phenotypic effect in the yeast cells populations (< 8% growth inhibition). The datasets of ALA-responsive genes showed functional enrichment in diverse metabolic pathways, as well as in transmembrane transport, cell-defense and detoxification categories [1]. About 16% of the yeast proteins encoded by ALA-differentially expressed genes were found to share significant homology with proteins from ecologically relevant eukaryotic species [1]. In general, the modifications in transcript levels of selected candidate biomarkers, assessed by qRT-PCR, varied in a consistent manner with increasing ALA concentrations either in the parental strain BY4741 or in the strain with a single mutation in gene *ERG6* known to confer increased cells permeability to xenobiotics [2]. Significantly, the *Derg6* mutant was significantly more susceptible to ALA than the parental strain BY4741, and a drop from 20-70 mg/L (parental) to 1 mg/L (*Derg6* mutant) in the concentration of ALA leading to an increased expression of several candidate biomarkers above a threshold value of approximately 2-fold, was observed. Results thus suggest a significant improvement in sensitivity when using the *Derg6* mutant as test organism, thus appearing as better suited to monitor ALA toxicity.

[1] Gil et al. 2011. *Environ Toxicol Chem.* DOI: 10.1002/etc.640 [2] Mukhopadhyay et al. 2002. *Antimicrob Agents Chemoter* 46:3695-3705. Acknowledgments to FEDER and FCT (contract PTDC/AMB/64230/2006, a PhD fellowship to FNG SFRH/BD/60933/2009, and a BII fellowship to MJJ)

PS8: 2

## Isolation and molecular cloning of $\gamma$ -terpinene synthase gene from *Thymus caespititius*

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*Thymus caespititius* Brot., commonly known as 'tormentelo' or 'erva-úrsula', is a *Lamiaceae* aromatic specie endemic of the NW Iberian Peninsula, and of the Azores and Madeira archipelagos characterized for high essential oil chemical variability [1, 2]. As part of an ongoing effort to isolate genes involved in scent production on different chemotypes, genomic characterization of exon and intron numbers, sizes and placement, of a putative gene encoding a monoterpene synthase,  $\gamma$ -terpinene synthase (*TcTPS2*), was performed on chemically distinct *T. caespititius* accessions collected at Azorean islands and in the Mainland Portugal. In *Origanum vulgare* *TPS2* is responsible for the first step of the 'cymyl'-pathway, giving rise to phenolic terpene isomers thymol and carvacrol and related compounds [3]. Being these terpenes the main components in two of the chemotypes of *T. caespititius*, the present work aims at showing the expression of *TcTPS2* in *Thymus. T. caespititius* mRNA was isolated from aerial parts collected during the flowering stage and a homology based RT-PCR strategy was used to clone the *TcTPS2* gene. One cDNA clone (*TcTPS2-D1*) was chosen to perform the heterologous expression in *Escherichia coli* for further characterization. A BLASTP search on GenBank revealed 27 to 93% of similarity of the cloned *TcTPS2* gene to other known terpene synthases genes from different members of other *Lamiaceae* species. Full-length His-Tag *TcTPS2-D1* cDNA was ligated to the vector pET-29a(+) for protein expression. Recombinant *TPS2* was detected in *E. coli* cultures by SDS-PAGE with the predicted molecular weight (67 kDa). The best soluble protein production was obtained for cultures induced with 0.2mM of isopropyl-1-thio-b-d-galactopyranoside (IPTG) for 19h at 20°C in a rotary shaker. Scale-up protein production is in progress, and further purification as well as enzymatic assays will be performed. Herewith reported for the first time for the genus *Thymus*, these cloning and expression approaches will contribute to elucidate the function of these *TPS* genes.

1. Figueiredo A.C. et al. (2010) *Nat. Prod. Commun.* 5: 1465-1476, 2. Figueiredo A.C. et al. (2008). *Cur. Pharm. Design* 14: 3120-3140, 3. Crocoll C. et al. (2010). *Plant Mol. Biol.* 73: 587-603. **Acknowledgments:** This study was partially funded by the Fundação para a Ciência e Tecnologia (FCT) under research contract PTDC/AGR-GPL/101334/2008.

PS8: 3

## An integrated omic approach towards the metabolic engineering of myrcene pathway of *Pseudomonas* sp. M1

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*Pseudomonas* sp. M1 is able to utilize a large variety of toxic and/or recalcitrant compounds as sole carbon and energy sources, including phenols, benzene and monoterpenes like myrcene [1-3]. Therefore, M1 strain holds great potential as a source of novel biomolecules and cell factories for various biotechnological applications namely in biocatalysis, biosensors, bioremediation and biomedicine. However, the full exploitation of its enzymatic repertoire requires detailed and integrated information about the biomolecular catalog of M1 strain, including genes, proteins and metabolites. The genome of M1 strain was sequenced by NGS technologies, using Illumina GA IIx and Roche 454 FLX. Resulting raw data was assembled and annotated using different pipelines. The current genome draft of *Pseudomonas* sp. M1 has an estimated GC content of 67%, a size of about 7.1 Mbps and includes 6276 CDS. Importantly, *in silico* genome analysis predicted metabolic pathways involved in utilization/biotransformation of several unusual carbon sources (e.g. biphenyls, halophenols, different monoterpenes). Proteomic and transcriptomic approaches have been setup envisaging the elucidation of the myrcene stimulus. In 2009, a set of myrcene-dependent proteins was described using subproteome analysis of the cytoplasmic fraction [3]. In this work, a RNA-seq transcriptome analysis led to the identification of a 28kb genomic island of key importance in myrcene catabolism. This island includes genes involved in: i) myrcene oxidation and bioconversion of myrcene derivatives via a beta-oxidation like pathway; ii) regulation of myrcene pathway; iii) myrcene sensing. In addition several other gene clusters spread in the genome of M1 have been found to be myrcene-dependently expressed and are under investigation. Integration of genomic, transcriptomic, proteomic and metabolic data will deliver a very solid and detailed description of the myrcene catabolism (and other monoterpenes), and on the associated molecular mechanisms of adaptation, providing the adequate support for the application of M1 as a biocatalyst in whole-cell biotransformations of plant-derived volatiles.

[1] Santos P.M., Sá-Correia I. (2007) *J. Biotechnol* 131:371-378, [2] Santos P.M., et al (2007) *OMICS* 11:233-251, [3] Santos P.M., Sá-Correia I. (2009) *Proteomics* 9:5101-11. This work was supported by FEDER through POFC – COMPETE, by national funds from FCT - project PEST-C/BIA/UI4050/2011 and by the project PTDC/EBB-BIO/104980/2008 (FCT).

PS8: 4

## Designing and construction of bicistronic plasmid capable of simultaneous expression of Hepatitis C virus NS3 protein and IL18

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Hepatitis C virus (HCV) infection is the main cause of chronic liver disease throughout the world, and may progress to cirrhosis and hepatocellular carcinoma (HCC). The NS3 protease/helicase is one of the most well conserved proteins of HCV. This protein is known to induce transcription factors promoting HCV replication. IL18 is a novel cytokine that plays an important role in the T-cell-helper type 1 (Th1) response and hence, plasmid-encoded IL18 is considered as a potent genetic adjuvant for DNA vaccine studies. Herein, a bicistronic plasmid was constructed which is capable to express Hepatitis C virus NS3 protein and IL18 simultaneously. cDNA corresponding to NS3 fragment was constructed by RT-PCR on RNA purified from HCV positive sera. Cloning of NS3 fragment into *Bst*xl/*Not*I sites of pIRES-IL18 plasmid and removal of GFP fragment led to the construction of pIRES- IL18-NS3 plasmid, which was transfected to HEK293T cell line by lipofection method. Expression of NS3 was evaluated by SDS-PAGE and Western blotting methods. Double digestion of pIRES-IL18-NS3 plasmid with the enzymes that were applied for cloning led to the isolation of fragments with expected sizes. This plasmid was also confirmed following sequencing reactions. Moreover, expression and secretion of NS3 to the medium was evidenced in transfected HEK293T cell line, compared to non-transfected controls. pIRES-IL18-NS3 plasmid possesses the capacity of the cloning and expression of Hepatitis C virus NS3 protein under the direction of IRES sequence, while at the same time has the potential of secretion of IL18 adjuvant. In vitro biological activity of the expressed IL18 and immune responses of NS3 with enhanced adjuvant effect of the constructed plasmid in animal models will remain to be tested.

420

PS8: 5

## High level expression and facile purification of recombinant silk-elastin-like polymers in auto-induction shake flask cultures

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Silk-elastin-like polymers (SELPs) are protein-based polymers which composition is based on the repetition of amino acid sequence motifs present in silk fibroin (GAGAGS) and in mammalian elastin (VPGVG). These copolymers have proven its importance for several biomedical applications and in the fabrication of micro- and nano-structures. Previously, SELPs were purified by immobilized metal affinity chromatography with volumetric productivities of 25-30 mg/l. With advances in recombinant DNA technology it is possible to design and produce tailored synthetic genes, creating multifunctional complex polymers, with total control on its composition and structure. In this work we report the construction of four new SELPs which composition is based on silk fibroin crystalline blocks and elastin-like thermoplastic (VPAVG) blocks. All the copolymers were constructed on the basis of different number of silk/elastin blocks and silk:elastin block ratios. The rationale is that by modifying the polymer structure it will exhibit different properties that will be further explored. Recombinant protein expression was achieved in *Escherichia coli* strain BL21(DE3) and purified by a chromatographic and non-chromatographic method. High levels of protein expression were obtained in bacterial cells cultured in terrific broth supplemented with lactose to allow auto-induction. The auto-induction cultures showed optimal recombinant expression in shake flask cultures with oxygen-limited conditions as determined by protein analysis in baffled and non-baffled flasks. Purification of recombinant copolymers was easily achieved by an acidic pre-treatment followed by ammonium sulphate precipitation. Adjustment of the soluble crude lysate to pH 3 – 3.5 demonstrated to be very effective in the removal of endogenous *E. coli* proteins, especially in the case of SELP1, where the acidic treatment allowed obtaining a reasonable pure polymer fraction. The copolymers precipitated with 20% of ammonium sulphate with excellent recovery rates. A volumetric productivity of more than 150 mg/l was obtained for all the copolymers.

*This work was supported by FEDER through POFC – COMPETE and by national funds from FCT - project PEst-C/BIA/UI4050/2011 and by the European Commission via the 7th Framework Programme project EcoPlast (FP7-NMP-2009-SME-3, collaborative project number 246176). R.M. was the receiver of a FCT fellowship (SFRH/BD/36754/2007), co-funding by FSE through QREN/POPH*

PS8: 6

## OMP-38 protein from *Shigella flexneri* 3a as a potential antigen for conjugate vaccine

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The *Enterobacteriaceae* family, Gram-negative rod-shaped bacteria are the main cause of diarrheal diseases. Annually an approximate 1.9 million people die of diarrheal diseases, mainly in developing countries among infants and young children under 5 years of age [1]. The development of vaccines against enteric pathogens represents a serious challenge because of raising antibiotic-resistance and no access to quick diagnostic tests providing pathogen identification and treatment [2,3]. The emergence of multiple drug resistance in bacteria has necessitated a search for alternative therapeutic strategies. In our earlier studies on an animal model we observed protective properties of the 38-kDa Outer Membrane Protein (OMP) isolated from *Shigella flexneri* 3a. The 38-kDa protein interacted in immunoblotting with human umbilical cord plasma [4]. This important observation indicates that cord plasma antibodies interacting with the 38-kDa OMP may be protective, because antibacterial protection is transferred from the mother to the fetus. We think the OMP-38 may be a potential antigen for conjugate vaccine as an immunogenic and protective component mainly exposed on bacterial cell wall [4]. In our present studies we estimated the OMP-38 epitopes involved in antibody binding process using bioinformatics. We also determined the epitope present on the protein surface by experimental methods using modern Fmoc chemistry techniques and standard ELISA test. The results obtained for the purified OMP-38 that cause protective immune response allowed us to assume that the epitope represented by a peptide can cause similar immune response and can be used as a potential antigen for vaccine design. The results obtained indicate that a short peptide may be a potential antigen as an immunogenic and safe component for conjugate vaccine.

1) Jarzab A., Górka-Frączek S., Rybka J., Witkowska D.; *Postepy Hig Med Dosw* 2011; 65: 55-72, 2) Woods C.R.; *Paed Resp Rev* 2006; 7s: s128– s129, 3) Gómez-Duarte O.G., Bai J., Newel E.; *Diagn Microbiol Infect Dis.* 2009 ; 63(1): 1–9, 4) Witkowska D., Masłowska E., Staniszevska M., Szostko B., Jankowski A., Gamian A.; *FEMS Immunol Med Microbiol* 2006; 48: 205–214. "The research was supported by Wrocław Research Center EIT+ under the project "Biotechnologies and advanced medical technologies – BioMed" (POIG 01.01.02-02-003/08-00) financed from the European Regional Development Fund (Operational Programme Innovative Economy, 1.1.2)"

PS8: 7

## Tunable morphology and structural properties of recombinant silk-elastin biopolymers by electrospinning

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Silk-elastin-like proteins (SELPs) are a new class of bioinspired, biologically synthesized block copolymers, consisting in silk fibroin (GAGAGS) and elastin (VPGVG) repeating units. With the aim of developing new high performance protein polymers, we report the electrospinning of two new SELP copolymers based on silk fibroin crystalline blocks and elastin-like thermoplastic (VPAVG) blocks. These new copolymers, named SELP1 with composition S10E20 and SELP3 with composition S5E10, where S corresponds to the number of repetitions of the silk block and E to the number of elastin blocks, were chemically synthesized by recombinant DNA technology and biologically produced by *Escherichia coli*. Due to its easy implementation, electrospinning has received a lot of attention as a technique to produce nanofibers. Electrospinning of different SELPs concentrations (5, 9, 13, 17 and 21 wt%) was performed in aqueous solution or in formic acid without addition of external agents. Electrospun structures were analyzed by scanning electron microscopy and the average diameter was calculated. The effect of methanol in the electrospun mat was also evaluated, morphologically by scanning electron microscopy and structurally by analyzing the secondary structure by FTIR. By varying the concentration, the morphology and size of the electrospun structures, can be customized to tailored applications. Polymer concentration and solvent, either water or formic acid, showed to play an important and determinant role in the process of electrospinning. While low concentrations of polymer solution lead to the formation of nano-microsized spherical structures, higher polymer concentrations produced electrospun fibers with increasing diameter and size distribution, ranging from the nano to the sub-microscale. Comparing the solvents, electrospun fibers in aqueous solution led to the formation of fibers with higher diameter and size distribution.

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PS8: 8

## Revealing new biotechnological potentialities of the riboflavin producer fungus *Ashbya gossypii* by means of the construction and analysis of its metabolic network

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IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho

Systems biology has recently arisen as a promising and powerful tool for process development and optimization. The fast-growing number of sequenced genomes may have contributed to this phenomenon since sequenced genome is the starting point from where it is possible to associate by homology a specific function to the genes of a microorganism. From this entire set of functions we can disclose the main metabolic pathways of a specific microorganism. One of the genomes already sequenced is from the fungus *Ashbya gossypii*, an industrially relevant microorganism intensively used for industrial riboflavin production. Despite the high similarity with *Saccharomyces cerevisiae* genome *A. gossypii* presents a lower level of complexity containing only 4726 protein-coding genes distributed over seven chromosomes. The aim of this work is to construct a metabolic model from which we can retrieve valuable information concerning specific metabolic pathways and the optimum conditions for the production of other compounds besides riboflavin. The initial stage of this process, being performed at present, consists in collecting the all set of metabolic-relevant genes through a manual re-annotation of *A. gossypii* genome. Despite being a manual procedure, this step is made using the user-friendly software – MERLIN – that provides an automatic annotation for each gene, which speeds up the entire process. The user should then analyze the function automatically assigned by this application, accepting or suggesting another one. Each metabolic gene is assigned to an Enzyme Commission (EC) number that corresponds to a specific enzyme. For such procedure several databases are used such as UniProt, SGD, AGD, ExpASY and BRENDA. At the end of this phase we should obtain an extended set of EC numbers that represent the reactions possibly occurring on the cell. In a next stage some factors will be considered in order to elaborate a final mathematical model: reaction stoichiometry; distribution of reactions by different cell compartments; biomass formation; energy requirements; other constraints.

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# Cellular Microbiology and Pathogenesis

*Poster session from thematic symposium 9*

Coutinho | Pombinho | Santos | Amorim-Machado |  
Domingues | Fraga | Menino | de Brito | Rodrigues |  
Dias | Martins | Silva | Moreira | Milho | Paterson |  
Pinho | Choupina | Almeida | Pais | Vilanova | Simões  
| Leitão | Mil-Homens | Gomes-Alves | Mira | Esteves |  
Lopes | Nunes | Branquinho | Jorge | Santos | Silva |  
Valente | Henriques | Felix | da Silva | Vicente | de  
Brito | Ramos

PS9: 1

### ***Burkholderia cenocepacia* clonal phenotypic variation during long-term colonization of a cystic fibrosis patient lungs**

**Carla P. Coutinho<sup>1</sup>, Carla C.C.R. de Carvalho<sup>1,2</sup>, Andreia Madeira<sup>1,2</sup>, Ana Pinto-de-Oliveira<sup>1</sup>, Isabel Sá-Correia<sup>1,2</sup>**

<sup>1</sup>IBB- Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal, Portugal;

<sup>2</sup>Department of Bioengineering, Instituto Superior Técnico, Technical University of Lisbon, Lisbon, Portugal

Chronic lung infection is the major cause of morbidity and premature mortality in cystic fibrosis(CF) patients. The systematic and longitudinal study carried out by our laboratory in the major Portuguese CF Center at Santa Maria Hospital (HSM), in Lisbon, during the last 16 years showed that around 70% of the patients infected with *Burkholderia cepacia* complex species are persistently colonized. A better understanding of how *Burkholderia cenocepacia* bacteria adapt to the CF lung environment while resisting host defense mechanisms and therapeutically administered antibiotics is crucial to deal with these chronic infections. To get insights into the adaptive strategies adopted by these bacteria during long-term colonization of the CF lungs, we carried out the phenotypic assessment of eleven *B. cenocepacia* clonal variants, obtained in HSM from the same CF patient sputa during three and a half years, until the patient's death with the cepacia syndrome [1]. Phenotypic characterization included susceptibility assays against different classes of antimicrobials, cell motility, cell hydrophobicity and zeta potential, colony and cell morphology, fatty acid composition, growth under iron limitation/load conditions, exopolysaccharide production and size of the biofilms formed [2]. For a number of the characteristics tested, no isolation time-dependent consistent alteration pattern could be identified. However, the values of antimicrobial susceptibility and swarming motility for the first *B. cenocepacia* isolate, though to have initiated the infection, were consistently above those for the clonal variants obtained during the course of infection and the contrary was found for the zeta potential. An adaptive strategy to long-term colonization was identified and described for the first time [1], involves the alteration of membrane fatty acid composition, in particular the reduction of fatty acid saturation degree in *B. cenocepacia* variants retrieved along with the deterioration of the pulmonary function and severe oxygen-limitation [2]. Results of the phenotypic characterization suggests a genetic adaptation to the stress conditions to which they are exposed over time in the CF airways, promoting the successful colonizing and long-term survival in the lungs [2-3].

[1] Cunha, M. V., et al., 2003, *Journal of Clinical Microbiology* **41**(9): p. 4113-4120; [2] Coutinho, C. P., et al., 2011, *Infection and Immunity*. **79** (7): p. 2950-2960; [3] Madeira, A., et al., 2010, *Proteomics*. **11**: p. 1313–1328.

PS9: 2

**Characterization of the role of the multifunctional glyceraldehyde-3-phosphate dehydrogenase in *Listeria monocytogenes* virulence****Rita Pombinho, Pierre Ferreira, Ana Camejo, Filipe Carvalho, Sandra Sousa, Didier Cabanes**

IBMC, Portugal

*Listeria monocytogenes* is a Gram-positive human pathogen with the ability to cross the intestinal, blood-brain and materno-fetal barriers causing listeriosis, a disease mainly characterized by gastroenteritis, meningoencephalitis and abortions. It is a facultative intracellular bacterium capable to survive in macrophages and invade non-phagocytic cells. Among the complex arsenal of bacterial virulence factors interacting with host tissues and involved in virulence [1], secreted proteins are privileged candidates. We showed that the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an anchorless glycolytic protein essential to pathogen survival, is up-regulated *in vivo* [2]. In addition, the secreted form of *Listeria* GAPDH was previously shown to interact with Rab5, preventing phagosome maturation as a survival strategy [3]. In order to decipher the exact role of *L. monocytogenes* GAPDH in critical steps of the *in vitro* and *in vivo* infectious processes, we evaluated the role of GAPDH in *Listeria* virulence using the purified recombinant protein, as well as a *L. monocytogenes* GAPDH-overexpressing strain. *In vitro* assays showed that the overproduction of GAPDH compromises neither the mutant growth profile in standard conditions, nor the intracellular survival pattern after internalization in the murine macrophage-like cell line J774. However, the overexpression of GAPDH inhibited *Listeria* adhesion and entry into eukaryotic cell lines, as compared to entry levels of the wild-type strain. Competition tests seemed to indicate that defects observed on adhesion and entry in non-phagocytic cells are not due to the blockade of a cell surface receptor. Accordingly, *in vivo* assays demonstrated that the overproduction of GAPDH, as well as the co-infection of *Listeria* with purified GAPDH caused a striking decrease of virulence after intravenous infection of BALB/c mice. Contrariwise, the relative virulence was only slightly attenuated upon co-infection of *Listeria* with *Streptococcus agalactiae* purified GAPDH. Taken together, this study points out new specific roles for GAPDH in two major steps of the *L. monocytogenes* cellular infection cycle, as well as in the bacterial virulence profile.

(1.) Camejo, 2011. *Virulence* 2 (5): 1-16 (2). Camejo, 2009. *PLoS Pathog* 5 (5): e10004493. Alvarez-Dominguez, 2008. *Traffic* 9 (3): 325-337

PS9: 3

## Cell Entry to Nuclear Import: HIV cycle may be influenced by phosphatases and kinases

**Ana Godinho Santos<sup>1,2</sup>, Sylvie Rato<sup>1,2</sup>, João Gonçalves<sup>1,2</sup>**

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Human Immunodeficiency Virus (HIV) depends on the host cell machinery to complete its life cycle. Several host proteins may help viral replication and others have the ability to suppress it. These helper and restriction factors might be involved in many different pathways, where regulation of protein activity by protein phosphorylation is essential. Both kinases and phosphatases are required for this crucial process that regulates many cellular processes in eukaryotes. Moreover, they constitute the largest subset of the druggable genome, sustaining kinase/phosphatase modulation as approach for the development of novel therapeutic strategies. These features prompted this study, which was designed to characterize kinases and phosphatases identified as helper factors for HIV-1 replication in a previous shRNA screen. For this purpose, we attempted to assess: 1) the effect of the 13 of the 14 identified proteins in HIV-2 replication cycle; and 2) the mechanism by which two of the identified proteins, SGK and CIB2, affect the early phase of HIV-1 life cycle, more precisely in viral fusion and in post-reverse transcription pathways. To accomplish this, the constructed shRNA clones were infected with HIV-2 particles to evaluate the effect on HIV-2 replication. When we compared the amount of virus in all tested shRNA clones challenged with HIV-1 and HIV-2, all proteins exhibited a similar outcome from the one observed in HIV-1. This evidence could indicate that HIV-1 and HIV-2 share cellular pathways while hijacking these host factors to assure its survival. Since CIB2 potentially interacts with integrins, and both SGK and CIB2 are involved in regulation of several ion channels, we inquired if these proteins could affect virus-cell fusion and performed a virus-cell fusion assay. We assessed that both SGK and CIB2 are important in HIV-1 entry, since their knockdown reduces the number of fusion events. Furthermore, we conducted the quantification of 2-LTR circles by Real Time-PCR in SGK and CIB2 shRNA clones infected with HIV-1, in order to establish if SGK and CIB2 play a role in steps previous to integration. Preliminary data suggests that SGK and CIB2 could lead to 2-LTR circles formation, affecting HIV-1 replication. This study provides new insights for the complex host-HIV interactions and proposes several mechanisms by which some of these kinases and phosphatases affect HIV infection, instigating further studies and new possibilities for antiviral strategies.

PS9: 4

### **The Proportion of Regulatory CD4+ T cells in the immune system reconstitution of HIV-infected patients under antiretroviral therapy**

**Pedro Amorim-Machado<sup>1,2</sup>, Palmira Barreiro-Silva<sup>1,2</sup>, Vítor Teixeira<sup>1,2</sup>, Ana Aboim Horta<sup>1,2,3</sup>, Cláudia Nóbrega<sup>1,2</sup>, Rui Sarmento e Castro<sup>1,2,3</sup>, António Gil Castro<sup>1,2</sup>, Margarida Correia-Neves<sup>1,2</sup>**

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The antiretroviral therapy (ART) has reduced the morbidity and mortality associated with HIV infection by decreasing the viral load and increasing CD4+ T cell counts. Even though most of HIV-infected individuals are able to recover to normal CD4+ T cell counts while on ART, the degree of immune recovery that is achieved is highly variable. Nearly 8% to 17% of the patients show increases in the CD4+ T cell counts reaching a “plateau” below the standard values of CD4+ T cells. This arrested CD4+ T cell recovery can have detrimental clinical consequences, such as sustained susceptibility to opportunistic diseases. To re-establish the homeostasis of the immune system, a complex equilibrium between distinct T cell populations has to be achieved and it is now firmly validated that regulatory T cells (Treg) play a major role in this equilibrium. Treg cells prevent the excess activation during certain immune responses to infection and “control” the activation of auto-reactive T cells. Since both conventional T cells and Treg cells express the HIV co-receptor CCR5, they are both susceptible to HIV infection and replication, which leads to the depletion of these populations. Therefore, the correct recovery of these cells during immune reconstitution due to ART treatment may be crucial to the maintenance of the immune system homeostasis. We performed a cross-sectional study to evaluate the progression of Treg cells in HIV-infected patients under ART. Our results show that (i) higher values of Treg cells associates with a poor recovery of CD4+ effector T cells and (ii) the levels of Treg cells correlate with the Nadir value (lowest CD4+ value before starting therapy).

PS9: 5

**The *Salmonella* type III secretion effector SteA subverts host cell membrane trafficking, possibly by manipulation of molecular motors****Lia Dora Domingues, Luís Jaime Mota**

Instituto de Tecnologia Química e Biológica, Portugal

*Salmonella enterica* serovar typhimurium (*S. typhimurium*) is a facultative intracellular bacterium, which causes gastroenteritis in humans and replicates intracellularly in a membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). *S. typhimurium* injects several virulence proteins (effectors) into host cells using type III secretion systems (T3SSs) encoded in *Salmonella* Pathogenesis Island-1 (SPI-1) and SPI-2 to promote host cell invasion and intracellular replication, respectively. SteA is an effector injected into cells by both the SPI-1-encoded T3SS (T3SS-1) and T3SS-2. A *S. typhimurium steA* deletion mutant shows defects in mice models of salmonellosis. Here, to study cellular mechanisms underlying SteA-mediated virulence, we analyzed macrophage-like RAW267.4 murine cells or human epithelial HeLa cells after infection with wild-type *S. typhimurium*, *steA* deletion mutant, or *steA* deletion mutant expressing SteA *in trans*. We did not observe a defect of the *steA* mutant in intracellular bacterial replication within macrophages or in invasion of epithelial cells. However, infection of HeLa cells revealed that SteA is important for the appearance of *Salmonella*-induced membrane tubules enriched in late endocytic markers (known as SIFs) and of tubules (known as SISTs) enriched in SCAMP3, a host cell protein that normally concentrates at the *trans*-Golgi network. SIFs and SISTs are a hallmark of the infection of epithelial cells by *S. typhimurium* and represent bacterial subversion of host cell membrane trafficking. Because SteA was not required for the appearance of *Salmonella*-induced tubules enriched in T3SS-2 effectors, this indicates that SteA subverts trafficking pathways by which late endocytic or TGN-derived membrane are transported into *Salmonella*-induced tubules, but does not play a prominent role in the biogenesis of the tubules *per se*. Infection of HeLa cells also showed that SteA controls clustering of bacterial vacuoles and might participate in normal partitioning of the SCV membrane during bacterial intra-vacuolar replication, processes that like appearance of SIFs or SISTs are known to depend on host cell molecular motors. Thus, SteA subverts host cell membrane trafficking possibly through manipulation of molecular motors. We are currently investigating which motors (dynein, kinesins, or myosins) are affected by SteA and the results will be presented and discussed.

PS9: 6

### **Cellular immunity confers transient protection in experimental Buruli ulcer following BCG or mycolactone-negative *Mycobacterium ulcerans* vaccination**

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Buruli ulcer (BU) is an emerging infectious disease caused by *Mycobacterium ulcerans* that can result in extensive necrotizing cutaneous lesions due to the cytotoxic properties of the exotoxin mycolactone. There is no specific vaccine against BU but reports show some degree of cross-reactive protection conferred by *M. bovis* BCG immunization. Alternatively, it has been suggested that a species-specific immunization could be a better preventive strategy. In this study, we used the footpad mouse model to characterize histological and cytokine profiles triggered by vaccination with either BCG or mycolactone-negative *M. ulcerans* followed by infection with a virulent strain of *M. ulcerans*. We observed that BCG vaccination significantly delayed the onset of *M. ulcerans* growth and footpad swelling through the induction of an earlier and sustained IFN- $\gamma$  T cell response in the DLN. BCG vaccination also resulted in cell-mediated immunity in *M. ulcerans*-infected footpads, given the predominance of a mononuclear infiltrate, as well as increased and sustained levels of IFN- $\gamma$  and TNF. No relevant IL-4, IL-17 or IL-10 responses were detected in either the footpad or DLN. Despite this protective Th1 response, BCG vaccination did not completely avoid the progression of *M. ulcerans* infection, regardless of challenge dose. Immunization with mycolactone-negative *M. ulcerans* also significantly delayed progression of infection but ultimately pathogenic mechanisms prevailed with the consequent emergence of footpad lesion. Our findings emphasize the relevance of protective Th1 responses against *M. ulcerans* infections in the context of cell-mediated immunity triggered by vaccination. In future studies it will be important to determine how the partial protective immune mechanisms induced by these vaccination protocols are compromised.

PS9: 7

**Differential response to *Candida albicans* and *C. non-albicans* of human cells carrying the Asp299Gly polymorphism in TLR4**

**João Filipe Menino<sup>1</sup>, Agostinho Carvalho<sup>2</sup>, Pedro Costa<sup>1</sup>, Cristina Cunha<sup>2</sup>, Nuno Osorio<sup>1</sup>, Agostinho Almeida<sup>1</sup>, Margarida Saraiva<sup>1</sup>, Jorge Pedrosa<sup>1</sup>, Luigina Romani<sup>2</sup>, Antonio Gil Castro<sup>1</sup>, Fernando Rodrigues<sup>1</sup>**

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The recognition of pathogenic microorganisms by the innate immune system relies on pattern recognition receptors (PRRs) that detect preserved structures of microorganisms; so-called pathogen associated molecular patterns (PAMPs). A major group of PRRs are the Toll-like receptors that after recognizing PAMPs, activate cellular signalling pathways to induce immune-response genes. Toll-like receptor 4 (TLR4) is an important PAMP that recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, as well as structures from fungal and mycobacterial pathogens. Polymorphisms in this receptor have been associated to functional alterations, mainly in what concerns the release of cytokines. Recent findings show an increased genetic susceptibility of individuals carrying the Asp299Gly polymorphism to *Candida non-albicans* species comparatively to *Candida albicans* species. Our data revealed that the mRNA levels of several genes involved in the two distinct signalling TLR4 pathways (MyD88/TIRAP and TRIF/TRAM) shows a biased cytokine expression profile for Type 1-IFNs in PBMCs from heterozygous individuals stimulated with non-*albicans* species, but not with *C. albicans*. A higher expression of intermediary molecules involved in TRIF/TRAM pathway (TRAM, TBK1, IRF3, CD80) was also found, indicating a preferential activation of this pathway in these individuals. These data suggest that the presence of the Asp299Gly polymorphism may impact the outcome of infections with different pathogenic fungi, by modulating the differentiation of the adequate immunological response. The elucidation of TLR-4 downstream key players involved in the cellular signalling pathways will be of major interest.



PS9: 8

**DNA-PK, a cofactor in HIV-1 LTR-driven transcription****Paula de Brito<sup>1,2</sup>, Sylvie Rato<sup>1,2</sup>, Ana Catarina Santos<sup>1,2</sup>, Leonor Resende<sup>1,2</sup>, André Ramos<sup>1,2</sup>, Luis Moita<sup>2</sup>, Joao Ferreira<sup>2</sup>, Joao Goncalves<sup>1,2</sup>**<sup>1</sup>URIA-Centro de Patogénese Molecular, Faculdade de Farmácia, Universidade de Lisboa, Lisboa, Portugal; <sup>2</sup>Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal

DNA-dependent protein kinase (DNA-PK), a multimeric nuclear kinase, is one of the key enzymes involved in the nonhomologous end-joining DNA double-strand break repair pathway and has been implicated in HIV-1 integration. However, its role in HIV-1 life cycle is still very controversial. The AIM of this work was to clarify DNA-PK contribution in HIV-1 life cycle and the mechanisms whereby it exerts its activity. To study the effects of DNA-PK in HIV-1 replication we first constructed a stable cell line that does not express DNA-PK, as indicated by mRNA levels and western blot analysis, by transducing Jurkat T cells with shRNA DNA-PK lentiviral particles. When this shRNA clone was challenged with pNL43<sub>HIV-1</sub> for 7 days, we observed a significant decrease in p24<sup>CA</sup> levels, determined in the supernatant by ELISA and intracellularly by western blot, suggesting that HIV-1 replication is significantly impaired by DNA-PK gene silencing. Subsequently, we investigated which HIV-1 life cycle phase is disrupted when DNA-PK is not expressed. Flow cytometry evaluation performed 48 h after shRNA DNA-PK clone transduction with lentiviruses expressing eGFP cassette under control of ubiquitin-C promoter pseudotyped with a VSV-G envelope or with HIV-1 gp120 shows that DNA-PK is not crucial for HIV entry and integration. In contrast, our results shows that 48 h after transient transfection of HeLa-P4 cells, containing the  $\beta$ -galactosidase gene under the control of HIV-LTR, with pNL43<sub>HIV-1</sub> and pLKO.1shDNA-PK DNA-PK is important for HIV-1 LTR-driven transcription. Concomitantly, a decrease in Tat mRNA levels is observed pointing out that DNA-PK is important for HIV-1 LTR-driven transcription of the viral elongated transcripts. In conclusion, our results suggest that DNA-PK is crucial for HIV-1 replication acting as a cofactor in HIV-1 LTR-driven transcription.

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PS9: 9

**Fighting mycobacterial infections within the thymus****Bruno Rodrigues<sup>1,2</sup>, Cláudio Nunes-Alves<sup>1,2,3</sup>, Margarida Correia-Neves<sup>1,2</sup>, Cláudia Nóbrega<sup>1,2</sup>**

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We have recently shown that, in the mouse model, mycobacteria are able to infect the thymus, the organ responsible for T lymphocyte differentiation. The bacterial load in the thymus slowly progresses, stabilizing at more advanced periods of infection [16weeks post-infection (wpi)] in comparison to the spleen which occurs at 4wpi. In the spleen, this stabilization is clearly associated to the activity of IFN- $\gamma$  secreting T cells, and so we hypothesise the involvement of these cells in the thymus as well. However, newly generated T cells are not fully differentiated when they leave the thymus. In addition T cells that differentiate within a mycobacterial infected thymus are tolerant to the invading pathogen. So being, we conjecture the involvement of mature T cells, re-circulating from peripheral organs back to the thymus, in the control of thymic infection. Evaluate the immune response against mycobacteria within the thymus and assess if T cell re-circulation from the periphery back to the thymus is involved in this process. Wild type and RAG-GFP mice were infected intravenously with  $10^6$  CFU *Mycobacterium avium* strain 2447 (intermediate virulence). Uninfected mice were used as controls. At specific time-points mice were sacrificed and gene expression was assessed in spleen and thymus by qRT-PCR. The numbers, specificity (assessed using MHC class II tetramer loaded with the mycobacterial epitope Ag85, kindly provided by the NIH tetramer facility) and IFN- $\gamma$  secreting ability of newly generated T cells (GFP<sup>+</sup>) and of re-circulating T cells (GFP<sup>-</sup>) in the thymus was determined. Coinciding with the stabilization of the bacterial load in the thymus, is an increased expression of IFN- $\gamma$  at 16wpi in infected mice in comparison to uninfected, followed by an increase in iNOS expression, a marker of macrophagic activation, at 24wpi. Increased levels of the Th1 recruiting chemokines IP-10, MIG and MIP-1 $\beta$  were detected in the thymus of infected mice from 16wpi on, in comparison to uninfected mice. Although the number of re-circulating T cells was not increased during infection, this pool was enriched with specific mycobacterial T cells from 16wpi on. This work presents evidence of an ongoing immune response in the thymus, occurring at later time-points and with a distinct profile from that occurring in the spleen. The thymus recruits mycobacterial-specific T cells from the periphery, which appear to be the major producers of IFN- $\gamma$  in response to mycobacterial antigens.

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PS9: 10

***In vitro* studies to characterize epidemiologically relevant *M. bovis* strains: resistance to oxidative and nitrosative stress and interaction with macrophages****Ana F. Dias<sup>1</sup>, Mónica V. Cunha<sup>1</sup>, Margarida Correia-Neves<sup>2</sup>, Margarida Saraiva<sup>2</sup>, Ana Botelho<sup>1</sup>**<sup>1</sup>Laboratório Nacional de Investigação Veterinária (INRB, I.P./LNIV), Lisboa, Portugal;<sup>2</sup>Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Braga, Portugal.

*Mycobacterium bovis* is the main etiological agent of bovine tuberculosis, a zoonotic disease with high relevance in the world. Once in the host, *M. bovis* has the ability to survive and replicate within macrophages, being the interaction of mycobacteria with host cell decisive in the establishment of infection. In this work, two epidemiologically relevant *M. bovis* strains were studied: LNIV 11428/003/05 (with spoligotype SB0121) and LNIV 7407/000/04 (SB1175), in order to evaluate if the differences observed among these two genotypes regarding colonization capability and host range could be explained by differences in phenotypic traits related to pathogenicity. Growth parameters and resistance to reactive oxygen and nitrogen intermediates were compared in broth medium. In addition, intracellular growth within macrophages and effect of mycobacterial infection on cytokine production were studied. In broth medium, growth was similar in both strains when evaluated by viable colony counting (CFU). However, by measuring the optical density (OD<sub>600</sub>), strain LNIV 7407/000/04 demonstrated a growth rate of 0.22 while strain LNIV 11428/003/05 exhibited a slightly higher growth rate (0.28, 1.3 times higher). This strain also showed greater resistance to hydrogen peroxide but, in contrast, exhibited greater susceptibility to 4 and 8 mM sodium nitrite, in comparison with the SB1175 strain. The multiplication in macrophages was significantly more efficient for strain LNIV 11428/003/05. The production of TNF- $\alpha$  by macrophages in response to LNIV 11428/003/05 infection increased between 6 and 24 hours after infection, but remained unchanged with LNIV 7407/000/04 infection. These results suggest that high prevalence, host range and wide geographical spread of predominant SB0121 spoligotype strains in Portugal, may be related with a greater ability of these strains to survive and grow within macrophages. This work initiates *in vitro* research concerning the behavior of *M. bovis* in relation to host cells, studies that are scarce until now.

PS9: 11

**Local and regional re-establishment of cellular immunity during curative antibiotherapy of murine *Mycobacterium ulcerans* infection****Teresa G. Martins<sup>1</sup>, Jose B. Gama<sup>1</sup>, Alexandra G. Fraga<sup>1</sup>, Margarida Saraiva<sup>1</sup>, Manuel T. Silva<sup>2</sup>, António G. Castro<sup>1</sup>, Jorge Pedrosa<sup>1</sup>**<sup>1</sup>ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal;<sup>2</sup>Institute for Molecular and Cell Biology (IBMC), University of Porto, Porto, Portugal

Buruli ulcer (BU), caused by the environmental pathogen *Mycobacterium ulcerans*, is a neglected necrotizing disease of the skin, subcutaneous tissue and bone. BU pathogenesis is associated with the production of mycolactone, a lipidic toxin with cytotoxic and immunosuppressive properties. Since 2004, the World Health Organization recommends the treatment of BU with a combination of the antibiotics rifampicin and streptomycin. Histology analysis of human tissue samples from successfully treated patients suggest that such antibiotic treatment reverses the mycolactone-induced local immunosuppression, leading to increased inflammatory infiltrations and phagocytosis of bacilli. To better understand the immunological alterations that occur during antibiotic treatment and their relationship with bacterial killing, we used a mouse model of *M. ulcerans* infection in the footpad. Analysis of macroscopic lesion progression in the infected footpads, bacterial burdens, histology and immunohistochemistry were performed. In popliteal draining lymph nodes, CFU counts, histology and immunohistochemistry were also performed. We observed a shift in the cellular infiltrates from a predominantly polymorphonuclear/macrophagic to a lymphocytic/macrophagic profile in the infected footpads of antibiotic-treated mice. This shift occurred before the complete elimination of viable *M. ulcerans*, and this was accompanied by an increased production of inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of nitric oxide by the phagocytes to kill mycobacteria. We also observed that the antibiotherapy prevented the draining lymph node destruction associated with lymphocyte depletion, which occurs during infections with virulent *M. ulcerans* in non-treated mice. Predominantly mononuclear infiltrates persisted in the footpads during and after treatment, as well as continuous production of iNOS, coincident with the long persistence of non-viable acid-fast bacilli. In summary, early rifampicin/streptomycin treatment of mouse footpad infections by highly virulent *M. ulcerans* results in the rapid elimination of viable bacilli and prevents destruction of the draining lymph nodes, allowing the rapid re-establishment of local and regional cell mediated immune responses and lesion healing.

PS9: 12

### Mucoid morphotype variation of *Burkholderia multivorans* during chronic persistence in the airways of cystic fibrosis patients

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*Burkholderia cepacia* complex (*Bcc*) bacteria are opportunistic pathogens infecting hosts such as cystic fibrosis (CF) patients. *Bcc* long-term infection of CF patient airways has been associated with emergence of phenotypic variation (Zlosnik et al. 2008). Here we studied two *Burkholderia multivorans* clonal isolates displaying different morphotypes from a chronically infected CF patient to evaluate traits development during lung infection. Expression profiling of mucoid D2095 and nonmucoid D2214 isolates using custom *Burkholderia* microarrays revealed decreased expression of genes encoding products related to virulence-associated traits and metabolism in D2214 (Silva et al. 2011). Furthermore, D2214 showed no exopolysaccharide production, lower motility and chemotaxis, and more biofilm formation, particularly under microaerophilic conditions, than the clonal mucoid isolate D2095. When *Galleria mellonella* was used as acute infection model, D2214 at a cell number of approximately  $7 \times 10^6$  c.f.u. caused higher survival rate than D2095, although 6 days post-infection most of the larvae were also dead. Infection with the same number of cells by mucoid D2095 caused larvae death by day 4. The decreased expression of genes involved in carbon and nitrogen metabolism may reflect lower metabolic needs of D2214 caused by lack of exopolysaccharide, but also by the attenuation of pathways not required for survival. As a result, D2214 showed higher survival than D2095 in minimal medium for 28-days under aerobic conditions. Mucoids vs. nonmucoid morphotype triggering cues include high antibiotic concentration, osmotic and oxidative stresses and nutrient limitation. Overall, adaptation during *Bcc* chronic lung infections give rise to genotypic and phenotypic variation among isolates, contributing to their fitness while maintaining their capacity for survival in this opportunistic human niche.

Zlosnik et al (2008). *Journal of Clinical Microbiology* 46:1470-1473. Silva IN et al (2011). *Microbiology*. DOI 10.1099/mic.0.050989-0.

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PS9: 13

**New molecular determinants of the symbiosis between *Sinorhizobium meliloti* and *Medicago sativa***Mário R. Santos<sup>1</sup>, Andreia T. Marques<sup>1</sup>, Leonilde M. Moreira<sup>1,2</sup><sup>1</sup>Instituto de Biotecnologia e Bioengenharia, centro de Engenharia Biológica e Química, IST, Lisboa, Portugal; <sup>2</sup>Departamento de Bioengenharia, IST, Lisboa Portugal

Legume plants have developed an intimate association with nitrogen-fixing rhizobial bacteria that provide plants with a reliable nitrogen source. Central to this plant-bacteria interaction is the formation of root nodules. Several symbiosis genetic determinants have been characterized, one of them being the outer membrane protein TolC from *Sinorhizobium meliloti*. TolC is required for establishing symbiosis with the plant *Medicago sativa* as well as for protein and exopolysaccharide secretion and protection against osmotic and oxidative stresses (Cosme *et al.* 2008). As a step toward understanding the physiology of the *S. meliloti* 1021 *tolC* mutant, its transcriptional profile was determined (Santos *et al.* 2010). The significantly induced genes suggest the activation of cytoplasmic and extracytoplasmic stress responses. These stress conditions are most probably caused by protein accumulation both in the cytoplasm and periplasm and by oxidative stress. The activation of an oxidative stress response in the *tolC* mutant was confirmed by the increased levels of the enzymatic activities of catalase, superoxide dismutase and glutathione reductase. The absence of a functional TolC caused a decreased in the expression of genes encoding products mainly involved in nitrogen metabolism, transport and cell division. Two genes strongly induced in the *tolC* mutant were homologues to CpxAR two-component regulatory system. Although Cpx proteins were initially known by responding to adaptation to cell envelope damage, recent studies link Cpx proteins to adhesion processes. *S. meliloti cpxA* mutant adherence to *M. sativa* roots was strongly decreased and the number of nodules after 5 weeks reduced by 50%. Further studies to assess the role of this two-component regulatory system in *Sinorhizobium* root-adhesion and symbiosis are being carried out.

Cosme *et al.* (2008). *Mol. Plant Microbe Interact.* 21:947-957. Santos *et al.* (2010). *BMC Microbiol.* 10:180. Funding is acknowledged to FCT (contract PTDC/BIA-MIC/113733/2009 and a Doctoral grant to MRS).

PS9: 14

**Screen for novel type III secretion effectors of *Chlamydia trachomatis*****Maria Cunha, Catarina Milho, Filipe Almeida, Rui Mauricio, Luís Jaime Mota**

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*Chlamydia trachomatis* are obligate intracellular and genetically intractable bacteria that cause ocular and genital infections in humans. Manipulation of host cells by *C. trachomatis* depends on the delivery of bacterial proteins (effectors) into the host cell cytosol and membranes. We aimed to screen for novel effectors of *C. trachomatis* injected into host cells by a type III secretion (T3S) mechanism. We used *Yersinia enterocolitica* as a genetically tractable surrogate bacterium assembling a T3S apparatus, which has been shown to recognize heterologous T3S substrates. We selected 48 genes that may encode uncharacterized effectors (e.g. no detectable amino acid identity to other proteins or with a particular putative biochemical activity) from the genome of *C. trachomatis* strain L2/434/Bu. Because T3S substrates normally possess a non-conserved and non-cleavable secretion signal within their first 15-30 amino acids, we constructed translational fusions comprising the first 20 amino acids of the candidate effectors and a reporter protein (the mature form of TEM-1  $\beta$ -lactamase), expressed under the control of the *Y. enterocolitica* *yopE* effector gene promoter ( $P_{yopE}$ ). As negative control, we used a fusion between the first 20 amino acids of a *C. trachomatis* ribosomal protein and TEM-1 (RplJ<sub>20</sub>-TEM-1). T3S assays performed with T3S-competent *Y. enterocolitica* or with an isogenic T3S-deficient strain individually expressing the TEM-1 fusions revealed a statistically significant higher level of secretion of 22 fusion proteins relative to RplJ<sub>20</sub>-TEM-1. Because T3S substrates have evolved to be transported within a narrow secretion tube (inner diameter of  $\approx 25$  Å), we tested if these 22 *C. trachomatis* proteins with an N-terminal T3S signal were also secreted as full-length proteins after  $P_{yopE}$ -dependent expression in *Y. enterocolitica*. Thirteen proteins showed a statistically significant higher level of secretion than full-length *C. trachomatis* RplJ. We also tested secretion of 11 out of these 13 proteins when expressed from an arabinose-inducible promoter in *Y. enterocolitica*, which revealed T3S-dependent secretion of 4 proteins. In summary, we have identified 22 novel putative *C. trachomatis* T3S effectors, four of which (CT082, CT105, CT143, and CT161) were also secreted as full-length proteins by *Y. enterocolitica* in different experimental conditions. Therefore, CT082, CT105, CT143, and CT161 are very likely novel effectors of *C. trachomatis*.

PS9: 15

### **Aflatoxins in Pakistani chilies - empirical data for improving quality**

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Background: Chilies are a major crop susceptible to aflatoxins (AF) contamination from fungi. AF are the most carcinogenic natural compound known and are implicated in causing liver cancers in thousands of humans. AF in Pakistani chilies is a serious constraint to exports and the health of the population of general significance to developing countries. Objectives: To establish empirical data for reducing AF in chilies. To introduce an "in house" analytical laboratory in Pakistan of use to developing countries and avoiding expensive extraction equipment, especially immuno-affinity columns. Methods: Samples were collected in Pakistan and analysed by chromatography using standardized methods, including a Pakistani-operated HPLC with fluorescence detection. In Pakistan, economical MycoSep columns were employed for separating AF. Results: All samples of Pakistani chilies contained AFB1 and high levels were obtained from ground samples in analysis carried out in the UK. There was no relation between AF and *Aspergillus flavus* quantification. Subsequently, the analysis was undertaken in Pakistan and 73.0% and 86.4% of different samples of whole and ground chilies, respectively were contaminated. A comparison was made of total AF in chilies collected in different seasons and winter chilies may provide a better quality product. AF in chilies from rural, semi-rural and urban areas of the Punjab region of Pakistan was determined. The data indicate that rural localities have particular problems. Finally, AF were analysed in chili varieties Longi, Wonder Hot and Skyline. The variety with lowest AF was Wonder Hot. The ability to undertake this analysis in Pakistan will enhance greatly the ability to improve chili production in that country. Lowest concentrations were from (a) urban and winter chilies and (b) the Wonder Hot variety. Hence, Wonder Hot chilies grown in winter and in urban locations are likely to have low AF relevant to improving exports and the health of the country.



PS9: 16

### Assessment of antimicrobial activity of textiles for wound dressing: methodology optimization

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Normally, the skin is capable of restore the tissue integrity, after wound injury. However, the deposition of bacteria on the wound site results on infection causing pain and healing delay. To control bacteria proliferation, antimicrobial textiles have been developed, and the assessment of their activity is a required step. Although, several standard methods were published to assess textiles antimicrobial activity, they are time and material consuming and have some shortcomings with regard to the real conditions of use. Therefore, the aim of this work was to optimize the method described on JIS L 1902:2008-Testing for antibacterial activity and efficacy on textile products, the most commonly used standard. Two textile samples were used: A-cotton without treatment (control) and B-cotton with 10% of the recommend concentration of Ruco-bac AGP. The microorganism used was *Staphylococcus aureus*-ATCC 6538. The first improvement was sample size. On the qualitative method, square samples with  $1 \times 1 \text{ cm}^2$  were used instead  $2.5 \times 2.5 \text{ cm}^2$  (suggested by the standard). For sample A no antimicrobial activity was observed and for sample B the halo size was similar for both sizes used. For the quantitative method, the samples used had 0.4g (standard suggestion) and 0.1g. Sample A had the same bacterial growth before and after contact with the fabric and sample B had no bacterial growth. With this improvement, the amount of sample and solutions need for the test was reduced four times. To reduce the use of disposable material, instead of 50mL falcons, 6 well plates were used. In this case, no bacteria were recovered from the sample A after incubation period on 6 well plates. These means, that the centrifugation is a crucial step to detach all bacteria from the fabric. The effect of the bacterial inoculum volume was also assessed. Three inoculum volumes (250, 100 and  $50 \mu\text{L}$ ) were added to 0,1g samples. No significant differences were observed for both samples. A healthy skin has  $10^5$  bacteria/ $\text{cm}^2$  and up to this value it is considered that the skin is infected. Therefore, 3 inoculum concentrations were tested:  $3 \times 10^5$ ,  $3 \times 10^6$ ,  $3 \times 10^7$  cell/mL. The results showed that the inoculum concentration had no significant changes for both samples after the incubation period. In conclusion, it is possible to use samples 4 times smaller than the standard suggestion, use higher inoculum volume to simulate wound exudate and higher concentration, to accurately predict the sample behaviour on an infected skin.

PS9: 17

### Isolation and characterization of necrosis-inducing phytophthora protein 1 (*npp1*) gene from plants pathogen *Phytophthora cinnamomi*

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Oomycetes from the genus *Phytophthora* are fungus-like plant pathogens that are devastating for agriculture and natural ecosystems. Due to their particular physiological characteristics, no efficient treatments against diseases caused by these microorganisms are presently available. To develop such treatments, it appears essential to dissect the molecular mechanisms that determine the interaction between *Phytophthora* species and host plants. One of the most widely distributed *Phytophthora* specie, with nearly 1000 host species is *Phytophthora cinnamomi*. Associated with this pathogen is the ink disease of *Castanea Sativa* Mill being one of the most destructive diseases in *C. Sativa* in the northeast of Portugal and the most common symptoms are root necrosis and reduction in root growth, which invariably lead to the trees death. *P. cinnamomi* is able to secrete a novel class of necrosis-inducing proteins, known as Nep1-like proteins (NLPs), more specifically necrosis-inducing *Phytophthora* protein 1 (*npp1*), that causes necrosis on leaf and roots of the plant, leading to the plant death. In order to better evaluate the mechanism of plant necrosis induced by *P. cinnamomi*, the study of factors that affect *npp1* gene expression is extremely important. The *npp1* gene ORF comprises 770 bp encoding a 256 aa protein with a molecular weight of approximately 25 kD. Gene expression *in vitro* in *P. pastoris* (heterologous expression), was studied during growth in different carbon sources, by RT-qPCR. Over expression of our gene in *P. pastoris* was also performed. *In vivo* expression technology has been used to study the expression of *npp1* gene from fungi during infection by RT-PCR. In our work chestnut roots were infected with *P. cinnamomi* and mRNA was extracted at different times of infection to analyze gene expression. These and other results will be presented and discussed.

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PS9: 18

**Phylogenetic and expression analyses of *Chlamydia trachomatis* inc genes****Filipe Almeida<sup>1</sup>, Vitor Borges<sup>2</sup>, Rita Ferreira<sup>2</sup>, Maria José Borrego<sup>2</sup>, João Paulo Gomes<sup>2</sup>, Luís Jaime Mota<sup>1</sup>**<sup>1</sup>Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Infection Biology Lab, Oeiras, Portugal; <sup>2</sup>Department of Infectious Diseases, National Institute of Health, Lisbon, Portugal

*Chlamydia trachomatis* serovars are genetically intractable obligate intracellular bacteria causing important ocular and genital infections in humans. Infections by serovars A-C are usually restricted to the conjunctival epithelium; infections by serovars D-K are restricted to genital epithelial cells; infections by serovars L1-L3 are sexually transmitted but with dissemination to lymph nodes, resulting in lymphogranuloma venereum (LGV). In spite of these differences, the genomes of ocular, genital, and LGV strains exhibit a high degree of synteny and more than 98% of identity at the DNA level. Inc proteins are an important group of potential chlamydial virulence factors. They share a unique hydrophobic motif thought to target them to the membrane of the large vacuole where *Chlamydia* resides intracellularly. Here, we analysed if differences in the nucleotide (nt) and amino acid (aa) sequence of 48 *C. trachomatis* inc genes and corresponding Inc proteins, or differences in the expression of incs, correlate to distinct tropism and disease outcomes. We identified 19 incs with a mean genetic distance among *C. trachomatis* reference strains from all serovars of at least 0.5% nt and 1.0% aa differences (ranging from 3.0-0.6% nt and 5.1-1.0% aa differences). This was largely due to differences in the sequences between distinct disease groups (ocular, genital, or LGV) and not to differences within groups. In phylogenetic reconstructions of nt or aa sequences of these 19 polymorphic incs, 6 of them showed separate clustering of the three disease groups, and the other 13 showed separate clustering of strains from at least one disease group. Moreover, 14 incs showed a ratio of nonsynonymous to synonymous substitutions of > 1 for all strains, which was also observed in analyses between disease groups, indicating an evolutionary trend towards protein modification. We used real-time quantitative PCR to determine the expression levels of the 48 incs during the infectious cycle of reference and clinical *C. trachomatis* strains representing the three disease groups. We identified 10 incs with notable differences in gene expression between serovars, which in most cases was due to higher expression levels in LGV strains. Overall, we identified nucleotide and expression differences between incs that may relate to distinct evolutionary pressures and could contribute to the specificity of host-pathogen interactions underlying infections with different *C. trachomatis* serovars.

PS9: 19

**Screen for type III secretion chaperones of *Chlamydia trachomatis*****Sara Vilela Pais, Luís Jaime Mota**

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*Chlamydiae* are a group of highly related Gram-negative bacteria, characterised by their obligate intracellular growth within eukaryotic cells and by their intractability to genetic manipulation. Among this group, *Chlamydia trachomatis* are important human pathogens, causing ocular and genital infections that have a significant clinical and public health impact. All *Chlamydiae* share a unique developmental cycle comprising an infectious but metabolically inactive form, which mediates cell invasion, and a non-infectious and metabolically active form, which replicates within a large membrane-bound compartment (inclusion). Both invasion and bacterial manipulation of host cell functions from within the inclusion are mediated by the injection of possibly more than 100 bacterial effector proteins through a type III secretion system (T3SS). Often, secretion of type III secretion (T3S) substrates involves characteristic chaperones (with a low molecular weight, an acidic pI, and which form dimers, and do not bind or hydrolyze ATP). Although the number of known chlamydial T3S effectors has been increasing, little is known about their possible cognate chaperones. Here, we aimed to identify new T3S chaperones of *C. trachomatis*. First, we analysed the genome of *C. trachomatis* strain L2/434/Bu by bioinformatics and selected 21 proteins with a predicted low molecular weight and pI. These proteins were subsequently analysed on their ability to oligomerise, by using a bacterial two-hybrid system (BACTH). The 7 proteins that self-associated were then systematically tested by BACTH for interactions with a collection of 38 known and candidate *C. trachomatis* T3S substrates. We identified two candidate T3S chaperones: CT043, which interacted with two known chlamydial T3S effectors (TARP and CT694) and with a candidate effector (CT696); and CT584, which interacted with a group of known chlamydial T3S effectors (CT619, CT620, CT621, CT711, and CT712), characterised by a domain of unknown function (DUF582), and with a candidate effector (CT082). T3S assays using *Yersinia enterocolitica* as heterologous bacteria revealed that the presence of CT043 seemed to help secretion of full-length TARP and CT694, which further strengthen the possible role of CT043 as chaperone. We are currently analysing the possible interactions involving CT043 or CT584 and chlamydial T3S substrates by biochemical methods and the results will be presented and discussed.

PS9: 20

***Staphylococcus epidermidis* biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages**

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*Staphylococcus epidermidis* an opportunistic pathogen due to its ability to establish biofilms on indwelling medical devices. The presence of high amounts of dormant bacteria is a hallmark of biofilms, making them more tolerant to antimicrobials and to the host immune response. We observed that *S. epidermidis* biofilms grown in excess glucose accumulated high amounts of viable but non-culturable (VBNC) bacteria, as assessed by their low ratio of culturable bacteria over the number of viable bacteria. This effect, which was a consequence of the accumulation of acidic compounds due to glucose metabolism, was counteracted by high extracellular levels of calcium and magnesium added to the culture medium allowing modulation of the proportions of VBNC bacteria within *S. epidermidis* biofilms. Using bacterial inocula obtained from biofilms with high and low proportions of VBNC bacteria, their stimulatory effect on murine macrophages was evaluated *in vitro* and *in vivo*. The inoculum enriched in VBNC bacteria induced *in vitro* a lower production of TNF- $\alpha$ , interleukin-1 and interleukin-6 by bone-marrow-derived murine macrophages and, *in vivo*, a lower stimulatory effect on peritoneal macrophages, assessed by increased surface expression of Gr1 and MHC class II molecules. Overall, these results show that environmental conditions, such as pH and extracellular levels of calcium and magnesium, can account to induce dormancy in *S. epidermidis* biofilms. Moreover, they show that bacterial suspensions enriched in dormant cells are less inflammatory suggesting that dormancy can contribute to the immune evasion of biofilms.

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PS9: 22

### **The DNA damage factors Ataxia-Telangiectasia –RAD3 related kinase and checkpoint kinase 2 are activated during African Swine Fever Virus replication**

**Margarida Simões, Carlos Martins, Fernando Ferreira**

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African Swine Fever Virus (ASFV) is responsible for a major threatening disease of swine integrating the Listed Diseases by the World Organization for Animal Health and for the past months, several new outbreaks outside endemic areas were registered in Georgia, Armenia, Azerbaijan, Iran and Russia. Given the worldwide economical context, an outbreak can have devastating consequences over production and commercialization; since there is neither a treatment nor vaccines available its control passes by severe restrain and sanitary measures. Until recently, ASFV was classified as a large nucleocytoplasmic virus but its nuclear replication phase is still to discover. The development of new molecules that selectively block nuclear proteins of DNA repair machinery and probably inhibit the viral replication and, the advances acquired on DNA damage mechanisms by studying viral survival mechanisms clearly justify a deeper analysis of ASFV demands on its host nucleus. Protein levels of sensors, mediators, transducers and effectors of the three pathways of DNA Damage Response (Ataxia Telangiectasia Mutated - ATM, Ataxia Telangiectasia-Rad 3 related – ATR and DNA dependent protein kinase catalytic subunit - DNA-pKcs) were evaluated during a 30 hour time course, after infecting Vero and HeLa cells with Ba71V ASFV strain, through Western Blotting. Regarding the viral recruitment/sequestration of specific nuclear DDR factors infected cells were analyzed by Indirect Immuno Fluorescence. Results show that ASFV activates the ATR pathway inducing a downstream phosphorylation of p53 “the guardian of the genome” and an increasing phosphorylation of the sentinel histone H2Ax (phosphorylation under the inhibitory drugs). Additionally, the protein levels of RPA<sub>34</sub> and p-Chk2 were also increased. This study shows that ASFV manipulates a DDR pathway (ATR) to insure that its DNA replication intermediates have short half-lives inside the host cell nucleus in order that viral genome is perceived as “self”. High levels of p53 and RPA<sub>34</sub> phosphorylation are related to homologous recombination and can be of importance during viral DNA concatemers formation. The G1/S cell cycle checkpoint arrest induced by p-Chk2 will allow the virus to use host DNA replication proteins in its favor.

PS9: 24

***Listeria monocytogenes* infection induces the activation of host cells DNA damage checkpoints****Elsa Leitão, Didier Cabanes, Sandra Sousa**

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*Listeria monocytogenes* is an intracellular pathogen that is known to subvert a number of host proteins and signaling pathways to modulate the host response favoring the establishment and maintenance of the infection. *Listeria* exploits and induces alterations in the host cytoskeleton, histone modifications and host gene expression modulation. In view of these facts, and considering what has been documented for other human pathogens such as *Escherichia coli*, *Helicobacter pylori*, *Cryptococcus neoformans* and *Porphyromonas gingivalis*, we hypothesized that *Listeria* might interfere with the host cell cycle in order to create a suitable niche for intracellular replication. The objective of this work is to explore the possibility that *Listeria* affects or manipulates the host cell cycle during cellular infection. Caco-2 (human epithelial colorectal adenocarcinoma) cells were infected for 1h with GFP-expressing *L. monocytogenes* EGDe or left uninfected and their flow cytometric DNA histograms were generated 20h after infection. Data revealed that there is a significant increase of infected cells at S-phase as compared to non-infected cells, concomitant with a decrease in the G0/G1 cells fraction. This observation suggests that *Listeria* infection could be inducing a delay and/or arrest in certain cell cycle phases through the activation of cell cycle checkpoints such as G1/S, intra-S-phase, G2/M, and replication checkpoints (DNA damage checkpoints). ATM and ATR kinases, two of the major DNA damage sensors, can be inactivated by caffeine inducing the override of the checkpoints even in the presence of damaged DNA. Interestingly, incubation of cells with caffeine reverted the effect of *Listeria* infection, with infected and non-infected cells showing similar DNA histograms 20h post-infection. These results indicate that *L. monocytogenes* infection could be activating DNA damage checkpoint(s) in an ATM/ATR-dependent manner. Moreover, infection of cells with a mutant strain defective in listeriolysin O allowed us to demonstrate that the consequence of *L. monocytogenes* infection on the host cell cycle is independent of the this toxin. Our data shows that *L. monocytogenes* infection has an effect on the progression of the host cell cycle possibly through the activation of DNA damage checkpoints.

PS9: 25

### Identification and characterization of novel multifunctional trimeric autotransporter adhesins in the cystic fibrosis pathogen *Burkholderia cenocepacia*

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*Burkholderia cepacia* complex (Bcc) bacteria have emerged as problematic opportunistic human pathogens in patients with Cystic Fibrosis (CF). Of the 17 species within the complex, *Burkholderia multivorans* and *Burkholderia cenocepacia* are the most dominant in CF patients. To initiate infection, *B. cenocepacia* must be able to colonize the respiratory epithelium. This step, although not fully characterized, is mediated by several adhesins, such as trimeric autotransporter adhesins (TAAs), a family of type V secretion system. These homotrimeric proteins are involved in various biological traits of pathogenic gram-negative bacteria including adherence, biofilm formation, invasion and survival within eukaryotic cells and serum resistance. We have conducted a computational analysis in the genome of the epidemic clinical isolate *B. cenocepacia* J2315 and we identified 3 clustered TAAs genes (*BCAM019*, *BCAM0223* and *BCAM0224*). The expression of these genes occurred preferentially for cells grown under high osmolarity, oxygen-limited conditions and oxidative stress. In order to investigate the contribution of these 3 TAAs for *Burkholderia* pathogenicity, we have constructed isogenic mutants, and tested their ability to adhere to extra-cellular matrix components, to form biofilm or to hemagglutinate red blood cells. We also used lung epithelial cell lines (16HBE14o- and CFBE41o-), which have a non-CF and a CF phenotype, respectively, to evaluate their involvement in adhesion and invasion. Furthermore, we analysed each *B. cenocepacia* mutant strain for resistance to complement killing. Finally, we used the killing of *Galleria mellonella* as a model host to study the role of the TAAs in virulence. Altogether our results demonstrate that these TAAs are important multifunctional virulence factors of *B. cenocepacia*, playing different roles in pathogenicity.



PS9: 26

### Identification and functional analysis of mating genes in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*

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The dimorphic fungus *Paracoccidioides brasiliensis* is the etiological agent of paracoccidioidomycosis, one of the most prevalent systemic mycoses in Latin America. *P. brasiliensis* exhibits thermodimorphic morphology, growing as a non-pathogenic mycelial/conidial form at environmental temperatures, and switching to a pathogenic multiple-budding and multinucleate yeast form at the host temperature. Sexual reproduction in *P. brasiliensis* has not been observed in nature or laboratory studies, though the genome sequences of three isolates (Pb01, Pb03 and Pb18) encode homologues for the essential mating genes described in other fungi. These include mating pheromones and their receptors, mating signaling pathway genes and heterothallic mating loci (MAT1-1 and MAT1-2). This work shows data regarding the presence of mating pathway components in *P. brasiliensis* through i) expression of mating pathway genes in *P. brasiliensis* isolates for both mating-types, ii) functional complementation by *P. brasiliensis* mating gene homologues upon heterologous expression in *Saccharomyces cerevisiae* mating-gene deletion mutants. Differential expression of alpha-pheromone (Pba) and alpha-pheromone receptor (PreB), as well as a-factor receptor (PreA) was observed in mycelial and yeast form of *P. brasiliensis* MAT1-1 and MAT1-2 isolates, indicating that an active mating system might be present in this fungus. The ability of *P. brasiliensis* mating gene homologues to complement mating-gene null mutants of *S. cerevisiae* was assessed using mating assays, pheromone-induced growth arrest and cell *shmoo* formation. Our data show that exposure of *S. cerevisiae* cells expressing the alpha-pheromone receptor (PreB) to either culture-purified or synthetic *P. brasiliensis* alpha-pheromone (Pba), leads to growth arrest (assessed by halo-assays and flow cytometric cell cycle analysis) and cell *shmoo* formation, indicating pheromone signaling activation. In addition, the heterologous expression of pheromone Pba and its receptor PreB in null-haploid yeast cells with opposite mating-types, functionally complements the mating defect of *S. cerevisiae* strains. Overall, our studies for the first time indicate the presence of a functional mating signaling system in the fungal pathogen *P. brasiliensis*. Further studies are underway to address the molecular details of mating signaling in this fungus, as well as to identify the conditions that promote sexual reproduction of *P. brasiliensis* in nature.

PS9: 27

**Transcriptomic and proteomic analyses reveal *Burkholderia cenocepacia* adaptive strategies to long-term colonization of the lungs of a cystic fibrosis patient under antimicrobial therapy**

**Nuno P Mira, Andreia Madeira, Pedro Santos, Carla P Coutinho, Ana S Moreira, Ana Pinto-de-Oliveira, Isabel Sá-Correia**

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Pulmonary colonization of patients with cystic fibrosis (CF) with *Burkholderia cenocepacia* and other bacteria of the *Burkholderia cepacia* complex (Bcc) is associated with worse prognosis and increased risk of death. During chronic colonization, the bacteria may evolve under stressing selection pressures, in particular, those resulting from host defenses, antimicrobial therapy, nutrient availability and oxygen limitation. Understanding the adaptive mechanisms that promote successful colonization and long-term survival in the lung is crucial. To get insights into the strategies adopted by *B. cenocepacia* to survive and resist host defense mechanisms and therapeutically administered antibiotics, the transcriptomes and proteomes of two clonal variants retrieved from a chronically infected CF patient were compared, based on DNA microarrays and two-dimensional difference gel electrophoresis (2D-DIGE) [2]. One of the isolates examined, IST439, is the first *B. cenocepacia* isolate thought to have started the infection and the other isolate, IST4113, was obtained three years later being more resistant to different classes of antimicrobials [2]. Results indicate the up-regulation of genes involved in translation, nucleotide biosynthesis, iron uptake (in particular genes of the ornibactin biosynthetic cluster) and in drug efflux in IST4113. Alterations related with adaptation to the nutritional environment of the CF lung and to an oxygen-limited environment are also suggested to be a key feature of transcriptional reprogramming occurring during long-term colonization and the progression of the disease.

[1] Coutinho C et al., *Infect Immun*, 79:2950-60, 2011 [2] Madeira A et al., *Proteomics* 11:1313-28, 2011

PS9: 28

### **A novel collagenase from *Aeromonas* sp.**

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*Aeromonas* sp. is an ubiquitous Gram-negative genus whose strains may cause septicaemia both in fish and humans. The main clinical symptoms associated with *Aeromonas* infection are gastroenteritis, wound infections, and systemic illness. Although during infection the expression of extracellular proteases capable of hydrolysing important host proteins, such as collagen, may contribute to the recognized multifactorial virulence of *Aeromonas* spp., the involvement of a true microbial collagenase in *Aeromonas* pathogenesis has not been established. Following a genomic analysis, we have identified a gene encoding a putative collagenase. This coding region shared high similarity values with sequences previously identified as coding members of the gluzincin collagenase family. The analysis of the predicted protein sequence allowed to identify the presence of the strictly conserved double-glycine motif, positioned 28 amino acids upstream of the HEXXH zinc binding site, critical for collagenolytic activity. The C-terminal region, including the catalytic domain, displays 47-51% amino acid identity and 65-67% sequence similarity to several microbial collagenases, but no similarity could be detected in the N-terminal regions. We have cloned and sequenced a collagenase gene from a strain of *Aeromonas*, expressed it using the *Escherichia coli* expression system and characterised the recombinant mature collagenase. This recombinant mature collagenase was cloned with an his-tag to allow a rapid purification on an affinity nickel column. Recombinant protein was purified and the purity of the collected sample was assessed by SDS-PAGE and zymographic analysis. We have also detected a cytotoxic effect of the recombinant collagenase on the growth and viability on Vero cell line. All taken together, the results suggest this gene in fact encodes a true collagenase whose expression may play an important role in *Aeromonas* virulence.

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PS9: 29

### Assessing *Diplodia corticola* infection mechanism

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*Diplodia corticola* is a fungal pathogen of *Quercus suber*, the cork oak, responsible, along with other pathogens, for the decline of the cork oak forest. In the Atlantic and West Mediterranean countries the cork transformation industry is of great economical importance. Recent data refer that in Portugal there are hundreds of companies related to cork with an estimated cork production of over than 550 million euros. This fungus is a pathogen of several oak species and recently was reported as causing cankers also on grapevines. Controlling the infection caused by *D. corticola* is a main concern. In order to achieve that, it is essential to understand how this organism infects its hosts. The infection mechanism of any pathogen relies on the molecules they produce and on the capacity of responding to environmental (host) alterations. Among these, proteases are one of the most important groups of molecules in the interaction with the host: they digest the intercellular matrix, a process that allows the spread of the pathogen through the host. This investigation aimed to unravel if tissues from different hosts induce the expression of different fungal proteases or alter the total proteolytic activity. In order to achieve this, *D. corticola* was grown in the presence of cork oak leaves, stem and cork, as well as in the presence of grapevine (*Vitis vinifera*) leaves and stems. The 'proteolytic secretome' of the fungus grown in these conditions was compared to the secretome of the fungus grown in the absence of plant tissues. Data shows that total extracellular proteolytic activity of *D. corticola* is dependent on the nature of the substrate. In general, both *Q. suber* and *V. vinifera* tissues induced an increase of proteolytic activity; nevertheless, different tissues induced the expression of different secretomes. Surprisingly, the presence of cork decreased the total proteolytic activity of *D. corticola*, which may be related to a protective effect of cork. At this point it is not possible to conclude on the physiologic relevance of these data, but this investigation is in agreement with the hypothesis that *D. corticola* entrance point is the stem.

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PS9: 30

**Bacterial resistance in commercial fish (*Solea* spp.) gastrointestinal microbiota in the public health context.****Nuno Miguel Mezia Lopes<sup>1</sup>, Pedro Rondon Werneck<sup>1</sup>, Karlos Ribeiro de Moraes<sup>1</sup>, Sónia Ferreira<sup>2,3</sup>, Fernando Morgado<sup>1</sup>**<sup>1</sup>CESAM - University of Aveiro, Portugal; <sup>2</sup>IEC - Institute for Education and Citizenship; <sup>3</sup>Hospital Infante D. Pedro

Great concerns are developing worldwide over the emerging threat of infectious diseases caused by antibiotic-resistant strains of bacteria, especially for the rise of nosocomial infections in healthcare units, placing a major peril over public health systems. Most of these strains have not fully known genetic material exchange paths and only very recently studies showed direct evidences of resistance genes being transferred from marine life to humans by several mechanisms such as Horizontal Gene Transfer (HGT), with potential reservoirs and vectors still to be determined. More still, the knowledge remains scarce about the effects caused by antibiotics and other antimicrobial compounds misuse in healthcare units or food industry on the gastrointestinal tract (GIT) microbiota of species with some degree of contact with human materials or wastes that could present themselves as vectors for a variety of antibiotic-resistant strains of bacteria. Therefore, this study was conceived to answer the questions: can the human consumed sole fish (*Solea* spp.) constitute a vector or natural reservoir for antibiotic-resistant strains of bacteria with some pathogenic role in public health? And if so, have this/ese strain/s acquired any resistance genes that might constitute a serious danger to public health safety? The research was developed by performing, in this first phase, the Kirby-Bauer bacterial growth inhibition testing using five different antibiotic pharmaceuticals, STX, Penicillin, Clavulanic acid, Amoxicillin and Ciprofloxacin on the GIT bacterial isolates of animals from two fishing harbors and two fishculture industries located both north and south of river Douro. Preliminary results revealed bacterial isolates to be highly resistant to Penicillin, Clavulanic acid and Amoxicillin per sampling site, except in the case of sampling site C, where bacterial isolates showed sensitivity to all antibiotics except to Penicillin. Contrary to this, site B showed bacterial resistance to Penicillin, Clavulanic acid and Amoxicillin. Bacterial isolates from site C also showed resistance to STX and intermediate sensitivity to Ciprofloxacin. Site A revealed a similar pattern presenting intermediate sensitivity also to Ciprofloxacin and STX.

PS9: 31

### ***Borrelia burgdorferi* in patients with Multiple Sclerosis and others neurological disorders – Is there a connection?**

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Several studies have shown that the prevalence of Multiple Sclerosis (MS) follows the distribution of Lyme Borreliosis (LB). In North America and Europe, the large number of individuals who develop MS closely reflects the seasonal distribution of vectors (ticks) of the genus *Ixodes* responsible for the transmission of *Borrelia burgdorferi* sensu lato (s.l.) spirochaetes. LB is a disease whose epidemiology is associated with seasonal dynamics and geographic/epidemiological factors, suggesting that LB agents may be involved in clinical pictures diagnosed as MS. It is essential to understand the relationship between LB and neurological disorders, mainly MS, considering the increased number of patients affected by MS and the latest insights about LB aspects namely: the presence and diversity of vectors; the high-infection rates of the vectors with LB agents, and the diversity of genospecies of *B. burgdorferi* s.l., in particular, *B. garinii* a neurotropic species closely associated to neuroborreliosis patients. This study aims to check in Portugal, for the first time, *Borrelia* DNA and/or antibodies against LB agents in patients with probable or confirmed MS, or others neurological disorders, using highly sensitive molecular and immunologic techniques. Biological samples [15 blood, 101 cerebrospinal fluid (CSF) and 126 sera belonging to 151 patients with MS and/or other neurological disorders were analyzed by immunological and molecular methods, respectively ImmunoFluorescence Assay (IFA), Western Blot (WB) and traditional PCR targeting the *ospC* gene, and two nested-PCR, targeting, the 5S(*rrf*)-23S(*rrl*) intergenic spacer and flagellin gene. Presence of anti-*B. burgdorferi* s.l. antibodies were detected by IFA in 22 (18%) sera, 14 (14%) CSF and 4 (27%) blood samples. Concerning WB (confirmatory test) antibodies were found in 38 (30%) sera and 6 (40%) blood samples. *Borrelia* DNA was detected in 14 (13%) samples by the three PCR approaches. Therefore, 25 patients, with diagnosis or suspicion of MS and other neurological disorders, were positive for LB. These preliminary findings support the idea of a close relationship between MS and LB. The etiology of MS is still a matter of controversial discussion, and continues unknown causing considerable doubts in the medical community. However, if the etiology of MS is proven to be bacterial, the patients' future can be hopeful since LB often responds to antibiotic therapy, especially when treated early, whereas MS does not.

PS9: 32

### Experimental phage therapy against *Mycobacterium ulcerans* in mice model

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Buruli Ulcer (BU), caused by *Mycobacterium ulcerans*, is a difficult-to-treat, neglected necrotizing disease of the skin. Currently, an antibiotic regimen is recommended by WHO, but in advanced stages of the disease surgery remains the only effective recourse. In alternative or in association to antibiotherapy, the use of phages has been considered for the control of bacterial infections. In this study, we investigated the efficacy of lytic D29 phage in controlling *M. ulcerans* infection, using the mouse footpad model. A single dose of  $10^6$  D29 phage was administrated s.c., in *M. ulcerans* infected footpads, at a advanced stage of experimental BU. Control mice were injected with bacteria, phage or phage buffer only. In order to evaluate the efficacy of phage administration, *M. ulcerans* growth in footpad tissues was evaluated. As an index of lesion development, footpad swelling was also measured over time. Our results show that mice treated s.c. in infected footpads with D29 phage had a significant reduction in bacterial numbers as compared with untreated infected mice, at day 40 after treatment, and that phage administration prevented the ulceration of footpads induced by *M. ulcerans* infection. In order to monitor the bacteriophage persistence in footpads of mice, a 24 h assay was performed. Numbers of phages significantly decreased in footpads of treated mice from 2 to 24 h. More detailed studies will be needed to determine if the reduction of phage titres is a consequence of the phage property of replicating only when the bacterial density is above a threshold or due to recirculation to other organs like drain lymph nodes and spleen. Overall, our results suggest that a single dose of D29 phage seems to be an effective approach for reducing *M. ulcerans* colonization and the severity of BU disease.

PS9: 33

**EzrA is required for regulation of cell size in *Staphylococcus aureus***

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*Staphylococcus aureus* is a major human pathogen and the study of its cell division machinery may lead to the discovery of new targets for the development of more efficient antibiotics. EzrA is a negative regulator of FtsZ in *Bacillus subtilis*, involved in the coordination between cell growth-cell division and in the control of the cell elongation–division cycle. We have now studied the role of the *S. aureus* homologue protein and shown that it is not essential for cell viability. EzrA conditional and null mutants have an overall increase of the average cell size, compared to wild type strains. In the very large *ezrA* mutant *S. aureus* cells, with a diameter over 1.75  $\mu\text{m}$ , the main cell division protein FtsZ and the cell wall synthesizing Penicillin Binding Proteins (PBPs) are not properly localized. This suggests that there may be a maximum cell diameter that allows formation of an FtsZ-ring capable of recruiting the other components of the divisome and of driving cytokinesis and cell wall synthesis at midcell. We propose that the major role of EzrA in *S. aureus* is in maintaining cell size homeostasis.



PS9: 34

### New method of diagnosis of Pineapple fusariosis by MALDI-TOF MS technique

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Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) is a spectral technique that analyses the chemical cellular composition of microorganisms providing rapid and discriminatory fingerprints for identification. The remarkable reproducibility of this technique is based on the measurement of constantly expressed and highly abundant proteins (Santos *et al.* 2010). The usually observable molecular mass range is between 2000 and 20000 Da, where important ribosomal proteins appear, which is an advantage because these can be easily used as biomarkers. MALDI-TOF ICMS offers advantages over PCR. The method is now used in taxonomic assessments (e.g. bacteria, filamentous fungi, yeast, phages, virus, etc.) once it is capable to identify microorganisms up to level species and, in some cases, up to strain level. The procedure is rapid and in some cases the sample preparation does not need pre-treatment. Time required for the pathogen inactivation is an important determinant of infection-related mortality rates in contaminated crops. Costs associated with pathogen infections in crops could be significantly reduced by employing new rapid identification techniques such as MALDI-TOF ICMS (Santos 2011). In this work one susceptible and another one resistant to *Fusarium guttiforme* pineapple cultivars were infected with strains of this fungal species. Aqueous suspensions of *F. guttiforme* spores were inoculated into both resistant and susceptible pineapple stems and incubated at 25 °C during 5 days. For the case of the susceptible cultivar, after the MALDI-TOF ICMS analyses of the infected pineapple tissue it was possible identify the presence of the fungal mycelium proteins inside the pineapple stems. Additionally, based on the MALDI-TOF ICMS analyses of the pineapple health tissue, the proteins of the pineapple tissue were decreasing in concentration while the fungal proteins were increasing. For the case of the resistant pineapple cultivar, no fungal mycelium proteins were found inside the infected tissue.

Santos *et al.* (2010) *J. App. Microbiol.* 108, 375–385, Santos *et al.* (2011) *Tropical Plant Pathology* 36 (Supplement), August 2011

PS9: 35

### Presence of *Gardnerella vaginalis* in healthy portuguese women – a pilot study

**Débora Salgueiro Silva<sup>1</sup>, António Machado<sup>1</sup>, Patricia Alves<sup>1</sup>, José Martinez de Oliveira<sup>2</sup>, Ana Filipa Henriques<sup>1</sup>, Nuno Cerca<sup>1</sup>**

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Bacterial vaginosis (BV) has an important position worldwide, as the leading vaginal disorder in women, and affects 30-50% of African women and 10-20% of White women of reproductive age. This condition although not mortal causes great discomfort and may lead to other complications such as pre-term labour or increase susceptibility for HIV infection. During BV occur a decrease of *Lactobacillus* spp. present in the vaginal epithelium and an increase in the number of anaerobic microorganisms like *Gardnerella vaginalis*, *Prevotella* spp., *Mobilincus* spp.; *Mycoplasma hominis* and *Atopobium vaginae*. *Gardnerella vaginalis* is also responsible for the formation of a biofilm in the vaginal epithelium in sick women's. However, the direct correlation between the pathology and the causing agent (or agents) has not been clearly established. Currently there is only one article in PubMed (Guerreiro *et. al*, 1998) referring to the prevalence of bacterial vaginosis in Portugal and we aim to extend the research in this field specifically to the portuguese population. As part of this effort one of our aims it to characterise the bacterial population of portuguese women both healthy and diagnosed with BV. As such we collected swab samples of vaginal fluids from portuguese women with the help of health professionals and using self collection. The swabs were collected and treated within 24 hours at the University of Minho for the characterization of the bacterial population present, by using conventional microbiological growth techniques, PNA-FISH microscopy and 16S PCR. It was found that about 20% of the samples tested possessed *G. vaginalis* and all possessed *Lactobacillus* spp. using all 3 identification techniques described. This result is consistent with previous reports of *G. vaginalis* prevalence although slightly lower, and shows that traditional microbiological techniques, microscopy and molecular methods were consistent in terms of results.

Guerreiro *et al* (1998) *Int J Gynaecol Obstet. Dec;63 Suppl 1:S167-73*. This work was supported by Fundação para a Ciência e a Tecnologia (FCT) PTDC/BIA-MIC/098228/2008 (FCOMP-01-0124-FEDER-008991). AM was supported by FCT fellowship SFRH/BD/62375/2009.

PS9: 36

**Quorum sensing signals involved in regulating virulence in a plant pathogen****Rita S. Valente<sup>1,2</sup>, Karina B. Xavier<sup>1,2</sup>**<sup>1</sup>Instituto Gulbenkian de Ciência, Portugal; <sup>2</sup>Instituto de Tecnologia Química e Bioquímica, Portugal

Quorum sensing is a process in which bacteria sense their cell-population density by measuring the concentration of small secreted signal molecules and respond with changes in gene expression. *Pectobacterium carotovorum* is a Gram-negative bacterium and a plant pathogen that causes soft-rot diseases in various commercially important plants, including potatoes, by the production of an arsenal of plant cell wall-degrading enzymes (PCWDE) secreted by the bacterium. Previous studies identified three major pathways involved in the regulation of PCWDE production, specifically the acyl-homoserine lactone intra-species system, the autoinducer-2 inter-species system and the GacS/GacA two-component system, whose signal is still unknown. Here we want to determine how cell-cell signaling controls virulence in *P. carotovorum*. Towards the understanding of how virulence is controlled by cell-cell communication in *P. carotovorum*, we performed a genetic screen to identify new genes involved in signal production/regulation that contribute to virulence. We constructed a library of 15,000 mutants using the EZ-Tn5™ <R6Kgama-ori/KAN-2>Tnp Transposome™ kit and test the mutants for their ability to produce pectate lyase (Pectate Lyase Assay). All mutants with less than 40% of enzyme activity relatively to wild-type were selected. Approximately 500 mutants have impairment in pectate lyase activity and are currently being complemented with wild-type cells- and proteins-free fluids to test for restoration of pectate lyase activity. So far we have 26 mutants that have higher enzymatic activity in the presence of wt supernatant and are currently being analysed. These mutants are likely to have the transposon inserted in genes that control signal production/regulation and are being analysed. We found 500 candidate mutants involved in the regulation of virulence in *P. carotovorum*. Of these mutants, several recover pectin lyase activity when wild-type supernatant is added and we have the sequence of 8 candidate mutants. Three of them have the transposon inserted in different locations of *expl* gene, which is the synthase of one of the signals (AHL) controlling virulence in *P. carotovorum*. This shows that we have the appropriate set up to identify signals involved in the regulation of virulence in this plant pathogen.

PS9: 37

**Synthesis of capsular polysaccharide at the division septum of *Streptococcus pneumoniae* is dependent on a bacterial tyrosine kinase****Mafalda Xavier Henriques, Tatiana Rodrigues, Madalena Carido, Luís Ferreira, Sérgio R. Filipe**

ITQB - UNL, Portugal

One of the main virulence factors of *Streptococcus pneumoniae* is the capsular polysaccharide (CPS), present at the bacterial surface, surrounding the entire cell and forming the outermost layer of all fresh clinical isolates. Currently, more than 90 different serotypes of *S. pneumoniae*, which vary in the chemical composition of the CPS, are known. The 5' end of the *cps* operon, where the genes that encode proteins involved in the synthesis of the capsule are located, is highly conserved between serotypes and encodes for, among others, the Wzd and Wze proteins. These two proteins are proposed to regulate the rate of synthesis of the CPS and were the focus of our work. Wze is a bacterial tyrosine kinase that possesses the conserved motifs of this family of proteins: a Walker A and B ATP binding domain and a C-terminal tyrosine cluster. Wzd is a membrane protein that functions together with cytoplasmic Wze in the CPS synthesis, by promoting its phosphorylation. We have found that when functional fluorescent derivatives of Wzd and Wze were expressed together in pneumococcal cells, they localized at the bacterial division septum, where the new peptidoglycan is normally synthesized. This septal localization occurred without the help of additional proteins encoded in the capsule operon and it was dependent on a functional ATP binding domain of Wze, which was also required for the direct interaction between the two proteins, as demonstrated by a Bacterial Two-Hybrid assay. Interestingly, Wzd and Wze null mutants were able to produce capsule that, although tightly linked to the cell surface, was absent from the division septum of the bacteria. We therefore propose that Wzd and Wze are spatial regulators of capsular polysaccharide synthesis that, in the presence of ATP, localize at the division site and ensure that capsule is produced where the new cell wall is being assembled. The observation that full encapsulation of the pneumococcal cells requires functional Wzd and Wze raises the hypothesis that the capsular polysaccharide synthesis in *S. pneumoniae*, similarly to what has been proposed for peptidoglycan synthesis, takes place independently at two different locations, one where the new cell wall of septal origin is formed and another where the new cell wall of peripheral origin is assembled.

PS9: 38

### **Why phytopathogenic fungi infect humans? The case of *Lasiodiplodia theobromae***

**Carina Felix, Ana Sofia Duarte, Artur Alves, António C. M. Correia, Ana Cristina Esteves**

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*Lasiodiplodia theobromae* is a phytopathogenic fungus responsible for a countless number of diseases such as twig blight, tip dieback, gummosis and bark splitting in coconut-trees, mangoes, grapevines, amongst others. Although more typical of tropical and sub-tropical regions, this fungus also occurs in other climates, at a temperature range of 12-25°C. Besides causing infection in a large number of plants, which has been causing an enormous economic prejudice (mostly in agriculture), it has also occurred as an opportunist in humans, causing infections with different levels of gravity. *L.theobromae* thus presents a great adaptability to different environments, being able to use its virulence mechanisms at a very wide range of temperatures. The objective of this investigation was to understand how a phytopathogenic fungus is able to colonize and infect other hosts such as humans. At this point of the investigation, we focused mainly on the effect of temperature (25 and 37°C). Covering two very distinct and yet so important areas as human health and agriculture, this study is of extreme relevance to international economy, as well as considering the potential development of effective treatments to human infections. In order to define the optimal growth temperature and the best culture media, the fungus was cultivated in three different solid media :Potato Dextrose Agar, Corn Meal Agar e Oatmeal Agar , at eight different temperatures [5°C, 15°C, 20°C, 25°C, 30°C, 35°C, 37°C and 40°C], revealing a maximum growth at 30°C on Potato Dextrose Agar. Extracts from solid and liquid cultures growth at 25°C, 30°C and 37°C were tested for cytotoxicity against Vero cell line (cells from African green monkey's kidney) using the resazurin assay. Our preliminary study shows that this fungus is cytotoxic to Vero cells. Although it is not yet possible to identify all molecules involved in cytotoxicity, we were able to detect the presence of secreted proteases which have been described as virulence factors for other organisms.

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PS9: 39

### **Characterization of the oral fungal microbiota in smokers and non-smokers**

**Filipa Monteiro da Silva<sup>1</sup>, Ricardo Araújo<sup>2</sup>, Benedita Sampaio Maia<sup>1</sup>**

<sup>1</sup>Faculty of Dental Medicine of the Porto University, Portugal; <sup>2</sup>Institute of Molecular Pathology and Immunology of the Porto University, Portugal

Background: The dynamic oral microbiome is an essential component of the on changing balance between health and disease. Although overlooked in the majority of published studies, fungi are also important components of the oral microbiome. Smoking has several deleterious effects on human health, including a variety of changes in the oral cavity. Its effects on oral bacterial have been well characterized, but its impact on fungal population is still largely unknown. Aim: To characterize the oral fungal microbiota – yeasts and moulds - of healthy young subjects and assess the effect of smoking in this mycobiome. Methods: A group of 40 volunteer students from a Dental Medicine faculty, 20 smokers and 20 non-smokers, were selected. The final sample was composed by 26 females (65%) and 14 males (35%), with a mean age of  $24.0 \pm 2.8$  years old. Clinical examination was performed to obtain caries prevalence and plaque index. Oral rinse was collected and incubated in a fungi selective medium at 25°C and 37°C for 7 days. The final number of fungal colonies was obtained and the fungi identified, when possible, based on macro and microscopic morphology. Results: All subjects showed fungal growth, 38% yeasts and 62% moulds (at 25°C). The most prevalent mould genera identified were *Penicillium* and *Aspergillus*. Smokers' presented significantly higher levels of yeasts in samples incubated at 25°C and higher levels of moulds potentially pathogenic, in comparison to non-smokers. Conclusions: The characterization of oral microbiome is still not completely clarified. Fungi's role on the oral microbiota equilibrium, including the overlooked moulds, is apparently more relevant than previously perceived. Tobacco smoking appears to alter the oral mycobiome, facilitating the colonization of oral cavity

PS9: 40

### **Lyme Disease diagnostics: HILYSENS-Highly sensitive and specific low-cost lab-on-a-chip system**

**Leona Gilbert<sup>1</sup>, Leena Merilainen<sup>1</sup>, Nitipon Puttaraksa<sup>1</sup>, Armin Schwarzbach<sup>2</sup>, Mário Vicente<sup>3</sup>, Paula Garcia-Nogales<sup>4</sup>**

<sup>1</sup>Department of Biological and Environmental Sciences and Nanoscience Center, University of Jyväskylä, Jyväskylä, Finland; <sup>2</sup>BCA Borreliose Centrum Ausburg Betriebs GMBH & Co KG; <sup>3</sup>STAB Vida, Portugal; <sup>4</sup>Applied Research using Omic Sciences, S.L. (AROMICS), Barcelona, Spain

HILYSENS is a FP-7 European Union-funded project that intends to develop a specific and sensitive lab-on-a-chip detection kit for Lyme Disease. The end goal will allow improved clinical diagnostic, disease monitoring and treatment of *Borrelia burgdorferi* infections. In addition, the project will test for acute and chronic infections with the use of a disposable microfluidics chip integrating all the analysis functions and mass-produced using nanoimprint technology. Pleomorphic forms of *Borrelia* exhibit differential proteins expression profiles than the parent form. Current diagnostic tools for Lyme disease do not detect the pleomorphic bacterial forms, are laborious and tend to lack the required sensitivity leading to false negative results. As a result, this all leads to patients' misdiagnosis as well as mismanagement, and Lyme disease incidence are usually underestimated, as many cases are mis- or undiagnosed. This project Consortium is formed by a multidisciplinary and transnational group of Small / Medium Enterprises and Academic Research Centres brings together expertise in molecular biology, material physics, optical and electronic engineering and medicine. This sensitive and reliable patient diagnosis offered by the HILYSENS device will optimise resources available to medical practitioners, reduce the current costs of the disease, and most importantly, increase patients' quality of life.

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PS9: 41

### The fast identification by MALDI-TOF ICMS of *Fusarium guttiforme* infecting pineapple stem and being antagonised by *Trichoderma asperellum*

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<sup>1</sup>Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural – INCAPER – Vitória - ES, Brazil; <sup>2</sup>IBB/Centre of Biological Engineering, University of Minho, Braga, Portugal; <sup>3</sup>Núcleo de Biotecnologia, Universidade Federal do Espírito Santo, Vitória - ES, Brazil

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) is a spectral technique that analyses the chemical cellular composition of microorganisms providing rapid and discriminatory fingerprints for identification. The remarkable reproducibility of this technique is based on the measurement of constantly expressed and highly abundant proteins (Santos *et al.* 2010). The usually observable molecular mass range is between 2000 and 20000 Da, where important ribosomal proteins appear, which is an advantage because these can be easily used as biomarkers. MALDI-TOF ICMS offers advantages over PCR. The method is now used in taxonomic assessments (e.g. bacteria, filamentous fungi, yeast, phages, virus, etc.) once it is capable to identify microorganisms up to level species and, in some cases, up to strain level. The procedure is rapid and in some cases the sample preparation does not need pre-treatment. Time required for the pathogen inactivation is an important determinant of infection-related mortality rates in contaminated crops. Costs associated with pathogen infections in crops could be significantly reduced by employing new rapid identification techniques such as MALDI-TOF ICMS (Santos 2011). In this work pineapple stem was infected with an aqueous suspension of *Fusarium guttiforme* spores and incubated at 25 °C during 5 days. After direct *F. guttiforme* mycelium analysis by MALDI-TOF ICMS the infected pineapple stem was naturally contaminated by *Trichoderma* sp. *Trichoderma* sp. antagonising *F. guttiforme* was directly analysed by MALDI-TOF ICMS. In order to confirm its identity *Trichoderma* sp. was purified, grown in culture plate and analysed by MALDI-TOF ICMS and, macro- and micro-morphology. The results showed that the *F. guttiforme* isolate infecting pineapple stem presented a mass spectrum close to the same isolate grown in Potato Dextrose Agar (PDA) medium and those stored on the SARAMIS™ data base. Additionally, *Trichoderma* sp. spectra (before and after isolate purification) were compared with other spectra stored on the SARAMIS™ data base and identified as *Trichoderma asperellum*. The morphological analyses for the *Trichoderma* isolate corroborate with the MALDI-TOF ICMS results.

Santos *et al.* (2010) *J. App. Microbiol.* 108, 375–385, Santos *et al.* (2011) *Tropical Plant Pathology* 36 (Supplement), August 2011



PS9: 44

### Genome-wide screening to uncover new restriction cellular factors against HIV-1 with artificial transcription factors libraries.

**Paula de Brito<sup>1,2</sup>, Andreia Amaral<sup>2</sup>, Sylvie Rato<sup>1,2</sup>, Carlos Barbas<sup>3</sup>, Joao Goncalves<sup>1,2</sup>**

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Until now, and despite the development of several aggressive anti-retroviral drugs, there is no effective therapy against HIV. This is mainly due to cellular latent reservoirs and to the virus high mutation rate, which leads to differences in protein sequences and structures. One strategy to bypass the lack of effectiveness of anti-retroviral drugs is to pharmacologically target host cellular proteins, more stable and less prone to mutations, important for virus replication. Thus the main goal of this study was to identify new restriction cellular factors, and potential pharmacological targets, that hinder HIV-1 life cycle. Our approach for this challenge was to develop an innovative genome wide-screening where systematic overexpression of every gene in an HIV-1 permissive human cell is performed with engineered polydactyl zinc-finger transcription factors libraries. Each library, composed by  $\approx 1 \times 10^6$  members, differs by the DNA sequences subset, 5'-GNN-3', 5'-ANN-3' or 5'-G/ANN-3', recognized at each finger position. Artificial transcription factor (ATF) had five zinc-fingers domains, recognizing a 15-bp target site, fused to VP-64 transactivator domain and cloned in the lentiviral transfer vector pLVEF1a-IRES-EGFP. We set up a 2-phase selection strategy. In phase 1, Jurkat E6-1 T cells were transduced with 3 different ATFs libraries, selected and isolated by their resistance to infection with HIV-1 HSA and HIV-1 NL4-3. In phase 2, we identified 2 ATF (ATF1, ACA GGT ACA AAT GGT, and ATF2, GGT ACA AGC GAA GGC) responsible for HIV-1 non-replication after genomic DNA extraction, PCR amplification and sequencing. Subsequently, we identified differentially expressed genes that were under ATF transcriptional control, by three different analyses: microarray, bioinformatics and ChIP seq to determine theoretical and real ATF binding hits on the human genome. The overlap of the results from the 3 experiments generated a low number of genes with high probability of involvement in permissive to non-permissive cells transformation. In conclusion, through our novel genome-wide screening we have identified two ATF that inhibits HIV-1 replication and putative cellular genes responsible for this inhibition.

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PS9: 45

**The cis-encoded h2cR small non-coding RNA of *Burkholderia cenocepacia* J2315 is a post-transcriptional negative regulator of *hfq2***

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*Burkholderia cenocepacia* J2315, as well as all other members of the *Burkholderia cepacia* complex (Bcc), harbours two distinct *hfq*-like genes, *hfq* and *hfq2*. *In vitro* binding experiments of purified 6-His-tagged Hfq with total RNA extracted from *B. cenocepacia* J2315, led to the identification of the h2cR small non-coding regulatory RNA (sRNA). Results are presented showing that the h2cR sRNA is a cis-acting sRNA, encoded within the intergenic region upstream the *hfq2* start codon, in the opposite direction of transcription of *hfq2*. The h2cR transcripts are maximal in cells at the early exponential phase of growth, decreasing to undetectable levels in cells at the stationary growth phase. EMSA assays showed that h2cR can form complexes with the 5'UTR of *hfq2*, leading to structural changes and increased decay of the *hfq2* mRNA. Our results indicate that the distinct levels of *hfq2* mRNA in cells at different growth phases results from transcription from the  $\sigma^{70}$  or the Cep promoters, and the post-transcriptional negative regulation exerted by h2cR sRNA.

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# Nanotechnology

*Poster session thematic symposium 10*

Peiro | Fernandes | Figueiredo | dos Santos | Silva |  
Varastehmoradi

PS10: 1

**Development of a vaccine against shigellosis using poly (anhydride) nanoparticles as a non toxic adjuvant.****Ana Isabel Camacho Peiro<sup>1</sup>, J. De Souza<sup>1</sup>, S. Sánchez-Gómez<sup>1</sup>, J.M. Irache<sup>2</sup>, C. Gamazo<sup>1</sup>**<sup>1</sup>Microbiology and Parasitology Department, University of Navarra, Spain;<sup>2</sup>Pharmaceutical Technology Department, University of Navarra, Spain

Shigellosis represent a significant public health burden in developing countries, with about 160 million cases occurring annually, leading to possibly one million deaths per year worldwide. Vaccination appears to be the only rational prophylactic approach to control shigellosis. Unfortunately, none safe and efficacious vaccine is still available. We investigated the protection conferred by a new vaccine containing outer membrane vesicles from *Shigella flexneri* with an adjuvant based on nanoparticles in an experimental model of shigellosis in mice. Outer membrane vesicles (OMV) were obtained from supernatants of stationary phase cultures of *S. flexneri* 2a (OMV). Tangential filtration was used to enrich the antigenic complex. Poly (anhydride) nanoparticles (NPs) of the copolymer of methyl vinyl ether and maleic anhydride (PVM/MA) were prepared by a solvent evaporation method. NPs were further purified by centrifugation (3000g, 20 min) and lyophilized. NPs were characterized by size, zeta potential and antigenic content. The method of encapsulation was successful, with a yield of 23 ng/mg NP. Protection studies: BALB/c mice (females, nine-week-old, 20±1 g) were immunized by intradermal, nasal, oral or ocular route with 20 ng of free or encapsulated OMV (n=6). Thirty-five days after administration, mice were infected intranasally with a lethal dose of *S. flexneri* (1 x 10<sup>7</sup> CFU). This new nanovaccine was able to fully protect against infection when it was administered by nasal or oral routes. In contrast, when it was inoculated intradermally, only 40% of the animals resulted protected. Under the experimental conditions used, the adjuvant did not induce any adverse effect.

PS10: 2

**Model interface for pathogens detection systems**

**Elisabete Ramos Fernandes<sup>1</sup>, Deepa Bedi<sup>3</sup>, Suiqiong Li<sup>2</sup>, Shin Horikawa<sup>2</sup>, Andreas Ebner<sup>4</sup>, Michael Leitner<sup>4</sup>, Peter Hinterdorfer<sup>4</sup>, Leon Kluskens<sup>1</sup>, Joana Azeredo<sup>1</sup>, Bryan Chin<sup>2</sup>, Valery Petrenko<sup>3</sup>**

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The prevention of food-borne illness has become a very important factor in public health that stimulated development of new technologies for pathogen detection. Biosensing systems can circumvent the limitations of conventional techniques, such as time consuming, requirements of appropriate laboratory conditions and expensive techniques. Recently, there has been an extensive work undertaken towards the development of diagnostic biosensor devices for on-site detection of biological threats that explore a diversity of transduction mechanisms and bio-recognition elements. In particular, the environmentally robust filamentous phages have been successfully used as an alternative to fragile antibodies in wireless biosensor system for real-time pathogen detection. However, one of the challenges in using of these phages as biorecognition elements is creation a uniform coating to improve the detection system. Filamentous phage can aggregate forming bundles of fibers that cannot cover completely the sensor's interface leading to the decrease in sensor's performance. Moreover, in detection system they can give some false positive result due their availability to detect *Escherichia coli*. Therefore, in this work was developed a new interface for wireless magnetoelectric biosensors by a new form of phage, which we called "nano-phage". "Nano-phage" comprises nanoparticles composed of self-assembled fusion major coat protein of landscape phages selected against the target analyte. For proof-of-concept, we investigated interfaces formed by a model phage selected from landscape libraries as a streptavidin binder. The major goal of this effort was the evaluation of the nanoparticles in different conditions in order to improve the affinity to the target of interest. The method developed can be in future applied for the detection of food pathogens and help the developing of biosensors with increased performance.

PS10: 3

### **New approach on the development of a recombinant vaccine against *Helicobacter pylori*: from genetics to delivery system**

**Lara Figueiredo<sup>1</sup>, Teresa Carvalho<sup>2</sup>, Cecília C.R. Calado<sup>2</sup>, António J. Almeida<sup>1</sup>, Lídia M.D. Gonçalves<sup>1</sup>**

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*Helicobacter pylori* infection affects at least half the world's population and implies a high risk of peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. Although the antibiotic treatment is often successful, the increasing resistance to antibiotics, problems with patient compliance and the possibility of re-infection demands the urgency of the vaccine development. However, this strategy is impaired by the strain genetic microheterogeneity, being each patient colonized by a cloud of closely related organisms [1]. In order to overcome the strain genetic heterogeneity, a multiantigenic *H. pylori* vaccine was developed based on fragments of the following six antigens: CagA, VacA, HpaA, UreB, HomB and GroEL. The mouse immunization strategy, with these multi-epitope vaccine, followed a prime-boost regime based on a DNA-vaccine and protein-vaccine, respectively. DNA vaccines provide several important advantages over current vaccine strategies such as live or attenuated vaccines, as they mimic the effects of natural infection in their ability to endogenously express foreign proteins, also having the ability to induce humoral as well as cellular immune responses. A particle mediated delivery system, based on chitosan nanoparticles, was used, in order to protect the antigens degradation while acting as adjuvant, and since this polymer is non-reactivity, biocompatible and biodegradable [2]. The oral and the intra muscular routes of immunization were tested, as the bacteria primarily colonizes the gastric mucosa, the production of antibodies in the mucosa would constitute an important primary line of defense. Based on the analysis of the Th2 interleukines IL-4 and IL-6, the Th1 interleukines IFN- $\gamma$  and IL-2, the ratio of the antigen-specific IgG2a-IgG1 in the serum samples, and IgA in the gut, it was observed that the oral immunization presents promising results pointed out by the mucosa specific IgA produced in spite of leading to a less efficient production of specific serum antibodies when compared with i.m. administration. Hopefully, the present multi-functional vaccine associated to the immunization protocol developed will strongly contribute to a protective immunogenic response.

[1] Vitoriano I. et al. (2011). *Helicobacter* 16(2):153-68 [2] Cadete A. et al. (2011). submitted. This work was supported by Fundação para a Ciência e Tecnologia (Portugal) (PTDC/BIO/69242/2006).

PS10: 4

**Susceptibility assays in bacterial cellulose coated with silver nanoparticles****Carolina Alves dos Santos<sup>1</sup>, Angela Faustino Jozala<sup>2</sup>, Adalberto Pessoa Jr<sup>2</sup>, Marcelo Martins Seckler<sup>1</sup>**<sup>1</sup>School of Chemical Engineering, University of São Paulo, Brazil, Brazil; <sup>2</sup>School of Pharmaceutical Science, University of São Paulo

Silver nanoparticles have attracted much interest in biomedical engineering, since it has excellent antimicrobial properties by killing both gram-positive and gram-negative bacteria. Bacterial cellulose (BC) is an extracellular polysaccharide produced by strains of *Gluconacetobacter xylinus*. It has a promising application as a biomaterial, specially as temporary skin for burns, ulcers and others skin pathologies. The coating of BC with silver nanoparticles could be employed to develop medical products for skin pathologies without contamination. The aim of this work is to obtain BC coated with silver nanoparticles and to evaluate its ability to inhibit different microorganisms. For BC production, *G. xylinus* was grown in Hestrin and Schramm broth under 30 °C for 96 hours in static conditions. Silver nanoparticles were produced by chemical reduction of silver nitrate with sodium citrate at 90°C. Coated BC was obtained by its immersion in a silver nanoparticles aqueous dispersion and incubating for 4 hours at 30°C at 100 rpm. Susceptibility assays were performed with *Escherichia coli* ATCC 25922 in LB broth, *Pseudomonas aeruginosa* ATCC 9721 and *Staphylococcus aureus* ATCC 10390 in TSB broth, utilizing 24-wells microplates. The microplates were incubated at 37 °C for 24 h. After this period 10 µL of each well and the BC were inoculated in agar growth media. Results showed total inhibition of *P. aeruginosa* and *E. coli*. It was observed that *S. aureus* grew in BC, although silver nanoparticles dispersion inhibited the microorganism growth. Future assays are being delineated targeting possible therapeutic applications.

PS10: 6

**New sanitization method: synergism between silver nanoparticles and microwave radiations****Bardia Varastehmoradi<sup>1,2</sup>, Ahmadreza Shahverdi<sup>1</sup>**<sup>1</sup>Tehran University of Medical Sciences, Iran, Islamic Republic of; <sup>2</sup>Student Scientific Research Center, Tehran University of Medical Sciences, Islamic Republic of Iran

Different sanitization methods are important aspects in health, especially in pharmaceutical sciences to decline microbial population. Metal nanoparticles such as silver nanoparticles can be used to gain this goal as they have significant antimicrobial effects. Electromagnetic radiations such as microwave radiations have also power to destroy microorganisms. This investigation discusses about a new experimental method to kill bacteria by silver nanoparticles and low frequency of microwave radiations. We produced silver nanoparticles with biotechnological process that their sizes are less than 100 nm and average size is 22.5 nm. In this procedure the supernatant of *Klebsiella pneumoniae* was added to Silver nitrate at concentration of  $10^{-3}$  M and exposed to sunlight. We studied antibacterial effects against *Escherichia coli* with low power of electromagnetic radiation in a microwave oven (100 watt and 180 watt) in the absence and presence of sub-inhibitory concentration of silver nanoparticles (10 µg/ml). Colony-forming unit (CFU) was used for determination of viable cell at different incubation time. This investigation proved that utilizing of the low frequency of microwave radiations significantly increased the antimicrobial activity of silver nanoparticles in just about 10 seconds. The electromagnetic radiation can damage most of microorganisms. This study described a simple method to improve antibacterial effects of electromagnetic radiations with sub-inhibitory concentration of silver nanoparticles. This concentration of silver nanoparticles (10 µg/ml) was chosen to indicate that the effect observed was due to the combination and not to the effect of the silver nanoparticles itself or low frequency of microwave radiation itself. It shows that in just about 10 seconds of radiations, the silver nanoparticles are more toxic than non-radiated nanoparticles. This method can be used in the most fields of sciences as a sanitization method for fast killing of microorganisms inexpensively.



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