BOOK OF ABSTRACTS OF

CENTRE OF
BIOLOGICAL
ENGINEERING

ANNUAL MEETING 2017

6th July, Campus de Gualtar

University of Minho
School of Engineering

BOOK OF ABSTRACTS OF CEB ANNUAL MEETING 2017

6 JULY 2017, BRAGA, PORTUGAL

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Publisher:
Universidade do Minho, Centro de Engenharia Biológica
Campus de Gualtar, 4710-057 Braga, Portugal

ISBN:
978-989-97478-8-3

DOI:
10.21814/CEBsam2017

This publication contains research works sponsored by Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684), and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte.

Foreword

The Centre of Biological Engineering (CEB, from the Portuguese title 'Centro de Engenharia Biológica') is a research unit of the School of Engineering of the University of Minho, which is recognized as a strategic infrastructure for the development of the Portuguese Biotechnology and Bioengineering fields. The mission of CEB is to generate, disseminate, and apply knowledge with relevance for society in the economic, social and cultural dimensions, and to contribute to the expansion of the scientific and technological fields under its scope of activity. The research carried out at CEB covers the molecular, cellular and process scales, combining knowledge from the exact, natural, health, environmental and engineering sciences, in order to develop new products and processes as well as a wide range of bioengineering and biotechnological applications in the agro-food, environmental, energy, industrial fine chemistry, biomedical and health fields. CEB combines R&D activities with advanced training, technology transfer, consulting and services, with the aim of fostering the industrial and agro-food, health, and environmental sectors.

The CEB's activities developed within three main thematic lines (industrial/Food; Environment; Health) follow closely the guidelines defined by the European Commission concerning the establishment of the Knowledge-Based Bio-Economy concept and are in agreement with the targets defined by the EU HORIZON 2020 program and the research and innovation strategies for smart specialisation (RIS3) defined by the Portugal North region. CEB has a strategic and ambitious research plan to respond to the new the challenges, which have resulted from the extraordinary developments and breakthroughs in subjects like Molecular Biotechnology, Systems and Synthetic Biology, and Nanotechnology. All these cutting-edge and fast growing/moving topics clearly demand for a mid/long term investment in both research infrastructures and human resources. CEB's contribution to these efforts relies on the integration of different scientific and technological subjects and competences of excellence, through the complementarities and synergies of its seven research groups. All this is confirmed by the excellent records of accomplishment of its members, namely international scientific articles and respective citations, publication of books, PhD theses, and the volume of funding obtained in competitive national and European programs, editorial memberships of international journals and technical committees, the organization of international conferences, industry-led projects, patents, the creation of spin-offs, etc. Working as a research network in Portugal with strong international connections, CEB also plays a key role in the deployment of advanced doctoral programmes with close links to highly reputed institutions, such as MIT, aiming at contributing to the establishment of new start-up companies in the area of Biotechnology and Bioengineering.

This one-day meeting is intended to bring together all CEB researchers from across the different groups and thematic lines. We have lined up 21 oral presentations of research highlights and 59 poster communications. This meeting is also characterized by providing significant amount of time dedicated to discussion. We expect that such discussion, with the help of the external advisory board, will identify strategic issues to foster CEB competitiveness at both the national and international levels.

Eugénio Campos Ferreira

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Research Highlights in Industrial and Food Biotechnology and Bioengineering

Unravelling the behavior of nanostructures during digestion and absorption

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The food industry is increasingly focused on preventing nutrition-related diseases and improving consumers' wellbeing. As a result, there is a growing trend towards healthy foods, enriched with bioactive compounds (such as vitamins, probiotics, bioactive peptides and antioxidants) produced through the application of innovative and safe technologies. In this context, the development of novel delivery systems for food applications through the use of nanotechnology has been extensively explored [1]. In fact, the encapsulation of bioactive compounds in bio-based nanostructures have been reported as promising mean of protecting the valuable bioactive compounds and providing new functionalities (e.g. increase of bioavailability). However, the use of very small particle sizes may alter the biological fate of the ingested materials and bioactive compounds, which could potentially have adverse effects on human health [2].

Therefore, the emerging field of nanotechnology offers new challenges to food industry not only by offering novel tools to improve food quality and human health, but also by introducing questions about nanostructures' behaviour within the human body. The challenges that must be overcome before nanotechnology can be entirely embraced by food industry, includes the optimisation of nanostructures' formulations to increase stability and bioactive compounds' bioavailability and the risk assessment of their use in food. The understanding of the behaviour of different nano-based delivery systems (e.g. nanoemulsions, nanoparticles) under digestion conditions, assessing their efficiency and safety is therefore of utmost importance to enable its widespread application in the food industry.

This evaluation can be challenging, however, there are opportunities to take advantage from the lessons learned from pharmaceutical industry and of the considerable progress in the development of more realistic *in vitro* models to more accurately predict the behaviour of bio-based nanostructures once ingested.

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Greener technologies in by-products and wastes processing - the case of electric fields in extraction and proteins functional modification

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Several technologies utilizing electrical fields directly into food processing, such as pulsed electric fields and ohmic heating, are currently being used on a commercial scale for of an extensive range of food products. They have shown to be environmentally clean technologies (at least locally), that can bring added-value to the products, improving the overall energy efficiency of the process and reducing the use of non-renewable resources [1]. During the last decade, much research on ohmic heating and the effects of its moderate electric fields has been addressed with a view to combating pathogens and improving the nutritional and sensorial properties of thermal processed food. Recently it has been demonstrated that electro-heating appears also as an interesting processing tool to be used in extraction of bioactive compounds from food by-products, as well as a way to modulate functional and technological aspects of important food ingredients, such as whey proteins. Results shows that the presence of electric fields during heating contributes to a change thermodynamic and kinetic behaviour of protein denaturation, as well in the shape of produced aggregates, highlighting the influence of non-thermal effects. Transmission electron microscopy unveils that the morphology of the protein aggregates is different under the influence of electric effects, which seems to increase the appearance of dispersed short fibrillar structures. Electro-heating treatment can be designed together with gelation techniques for the development of biodegradable protein-based gels as potential devices for the incorporation of food nutraceuticals, thus creating novel applications not only for food industries, but also in the pharmaceutical area; results have shown that 33 mmol.L^{-1} of Fe^{2+} can be associated to a whey protein gel network providing an opportunity for the development of innovative functional foods that can be used as an oral dietary supplement. Electrical and thermal effects can be optimized into a single step treatment enhancing thermal stabilization (i.e. inactivation of microorganism and enzymes) and extraction of anthocyanins and phenolic compounds from vegetable and fruit tissues. Electro-heating at high electric field (200 V/cm) and high temperature (~100 ºC) enhanced considerably the extraction of chlorogenic acid from purple potato wastes, but also allowed to extract ellagic and ferulic acids, catechin and rutin in a very comparable way to a freeze/thawing treatments Electro-heating capability of applying high heating rates with a precise temperature control together with putative electroporation effects in cell tissues presents an interesting solution for several biotechnological processes.

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Genetic engineering approaches for enhanced lignocellulosic-based bioprocesses

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Lignocellulosic biomass is the most abundant, low-cost, bio-renewable resource. It has a recognised potential as a sustainable platform for the production of biofuels and other bio-chemicals. To improve the accessibility of the cellulose component from complex lignocellulosic structures to the enzymes, a pretreatment step is necessary. Enzymatic saccharification of resulting whole slurry is highly desirable as it avoids the solid-liquid separation step, the need for detoxification and related waste disposal problem, and increases final sugar concentration. However, lignin residues and other inhibitory compounds resulting from pretreatment negatively affect the digestibility of the whole slurry and compromise fermentation efficiency. To tackle these pitfalls, genetic engineering strategies have been developed and integrated in the process to improve both stages.
For improving the fermentation efficiency, our approach has been to intensify the process by using high solid loadings and both pentose and hexoses fractions, enriching sugar concentration available for fermentation. To work under such demanding conditions robust yeast strains are crucial. We have selected natural robust yeast isolates and identified key genes necessary for yeast growth and maximal fermentation rate in hydrolysates. Selected robust yeast chassis have been metabolic engineered for co-fermentation of glucose and xylose from hemicellulose fraction using a novel metabolic assembly tool and key tolerance genes expression has been simultaneously evaluated for the valorization of biomass of different origins. Results obtained pointed to the importance of designing from the very beginning a tailor-made yeast considering the specific raw material and process [1]. The flexibility of the metabolic assembly tool developed and the selected robust yeast backgrounds envisioned the developing of effective yeast platforms for biomass processing into different products. For improving the saccharification of whole slurry, our strategy has been to use the efficient recombinant protein production system from *Escherichia coli* to produce hydrolysis enhancers, namely a family 3 carbohydrate-binding module (CBM3). The purified CBM3 was used as an additive in the enzymatic hydrolysis of the whole slurry from hydrothermally-pretreated *Eucalyptus globulus* wood among other biotechnological applications [2]. The results obtained show an increase in glucose yield when CBM3 was added, compensating the negative effect of inhibitors on the enzymatic efficiency of whole slurry saccharification. Thus, CBM3 is a valid additive for enhanced lignocellulosics saccharification and a valuable alternative to costly additives (e.g. BSA) as it can be affordably obtained from heterologous bacterium or integrated in the developed yeast platforms, thus contributing to more cost-efficient and environmental-friendly biomass conversion bioprocesses.

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Contributes for the development of *Ashbya gossypii* as a cell factory

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Ashbya gossypii is a filamentous Saccharomycete long known by the scientific and industrial communities, first as a cotton pathogen and subsequently as a riboflavin overproducer. Its industrial relevance combined with its high genetic similarity with *Saccharomyces cerevisiae* promoted the development of a significant molecular and *in silico* toolbox for its genetic engineering. This, together with the increasing knowledge of its genome, transcriptome and metabolism has helped designing effective metabolic engineering strategies for optimizing riboflavin production, and also for developing new *A. gossypii* strains for novel biotechnological applications [1]. Here, we will address our main contributes for the development of *A. gossypii* as a cell factory organism, by presenting an overview of the most representative outputs from our research [referenced in 1, 2].

Envisioning its exploration as a recombinant protein producer, our main efforts focused on the characterization of the *A. gossypii* protein secretory pathway at the genomic, transcriptomic and proteomic levels [2]. Based on experimental observations and on data from omic analyses, a hydrolytic enzyme, invertase, was deduced to be natively secreted by *A. gossypii* and molecularly characterized. The N-glycosylation pattern of the proteins natively secreted by *A. gossypii* was also characterized, as well as the recombinant production by this fungus of secreted proteins from different fungal origins. Among these, the β-galactosidase from *Aspergillus niger* was expressed in *A. gossypii* under the regulation of several native and heterologous promoters, presenting the highest extracellular production levels.

A new molecular tool based on the Cre-*lox*P recombination system was also developed for generating *A. gossypii* strains free of exogenous selection markers for industrial applications. With this tool, we were able to block the *A. gossypii*'s *de novo* pyrimidine biosynthetic pathway and thus generate a uridine/uracil (*Agura3Δ*) auxotrophic strain, which presents improved riboflavin production under limited uracil/uridine supplementation.

While characterizing the different *A. gossypii* strains (wild and engineered) we have worked with, we have also gathered important information on the physiology of this fungus (e.g., in defined media and in raw substrates such as crude glycerol), which have helped us design strategies to improve its performance, namely improved tolerance to osmotic stress.

These results allowed a considerable advance in the understanding of the biotechnological potential of *A. gossypii* and the production of other metabolites of interest is now being rationally evaluated. To support the rapid development of new strains, more flexible genome editing tools are also being constructed.

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Mycotoxins in Food and Mitigation of its effects

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Mycotoxins are toxic compounds mainly produced by fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium*. These toxins are frequently detected in many food commodities including cereals, fruit and vegetables, even after processing [1]. Since the same fungus may produce more than one toxin and the same toxin may be produced by different fungi, mycotoxins often occur as a mixture. Their ubiquitous presence represents a challenge to the health of humans, animals and the environment. Hundreds of compounds are listed as possible mycotoxins; however, only a few occur at levels that may are really challenging.

The main focus of AMG is to study and develop integrated and innovative methods, supported in fundamental and applied research, for the mitigation of the occurrence of mycotoxins in food and feed, promoting complex system understanding and knowledge gaps identification.

Currently available approaches to control mycotoxins cannot assure their complete elimination from food and feeds chains. Most of them are based on prevention, either pre or post-harvest, or on the segregation of contaminated kernels after harvest. Other strategies will partially remove mycotoxins from commodities and are applied on a case by case approach. These will include: (i) biological ones, inactivation of patulin by *Saccharomyces* strains or the degradation of ochratoxin A by enzymes; (ii) chemical ones, the use of ozone in food processing; (iii) or physical ones, food irradiation to inactive mycotoxin producing fungi and degrade mycotoxin. However, these methods have not a broad application. The lack of practical solutions to control mycotoxin contamination in the field, at harvest and of processed products leads to the demand of methods for their partial or total elimination.

Over the last years, AMG studies the application of some of these strategies, including the use of ozone, the application of irradiation, and the use of enzymes and of lactic acid bacteria to inactivate or inhibit mycotoxigenic fungi, and to degrade mycotoxins. The main outcomes of this work will be reviewed.

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Bioprocesses development based on low-cost feedstocks by fermentation technology for added-value compounds production

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Bio-based industries are focused on the use of renewable biological resources for the production of bio-based products and biofuels. Under this scope, bioprocesses development based in low-cost substrates has been the major goal of the team. The main objective is to give a competitive solution for the biotechnological industry to re-use sub-products or wastes as feedstock, improving the sustainability of biotechnological processes, through the use of greener and more competitive technologies. Thus, is of most importance to demonstrate that these technologies enable the production of new chemical building blocks and new products from feedstocks that replace the need for fossil based inputs.

In this context, the team has been focused on the study of the potential of different low-cost and renewable material to develop and optimize fermentation processes. Submerged fermentation technology has been applied for several applications using the yeast *Yarrowia lipolytica* such as: crude glycerol from the biodiesel industry to produce organic acids (ex. citric acid), the renewable substrate castor oil for aroma production and oily wastes for microbial lipids and lipase production. This yeast species has been considered as cellular model for dimorphism studies. Its ability to change from oval typical yeast shape to a pseudo hyphae morphotype has been studied by the team and correlated with operational factors in bioreactors [1]. This morphological characteristic of *Y. lipolytica* makes it one of the few yeast species able to grow under solid-state fermentation. With this knowledge, new opportunities will be explored by the team under the scope of the recent financed project Waste4Lip, such as the transformation of wastes into feedstock for biorefineries by solid-state fermentation with *Y. lipolytica*.

Solid-state fermentation (SSF) has many advantages in comparison to traditional submerged fermentation, such as: higher products titers, better yields, easier recovery of products, smaller reactor volumes and low energy requirements. Over the last years, this technology has been applied by the team to up-grade solid wastes from olive oil and wine industries by SSF with *Aspergillus* spp, producing added-value compounds like enzymes and phenolic compounds, and at the same time, obtaining a fermented solid with improved nutritional value to be used as animal feed. Strategies of wastes mixtures and pre-treatments have been developed as well as scale-up of the process to prove its feasibility [2].

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Synthetic biology: Heterologous production of bioactive agents

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Synthetic biology provides powerful tools to design innovative products and technologies. In the medical field, examples include the elucidation of disease mechanisms, identification of potential targets, discovery of new chemotherapeutics or design of novel drugs [1]. Additionally, it enables the development of economically attractive microbial production processes for complex natural products.

Plants secondary metabolites are considered high-value chemicals exhibiting interesting biological activities. However, they are present in plants in low amounts and accumulate during long growth periods. Their extraction is often problematic since their purification is difficult originating low yields, which are also consequence of environmental and regional factors. In addition, chemical synthesis is complex and environmentally unfriendly. Therefore, the biosynthesis of these high-value chemicals in engineered organisms has emerged as a competitive alternative compared to chemistry-based methods.

Curcuminoids and coumarins are polyphenolic compounds produced in plants that exhibit very interesting pharmacological properties. Under this scope, we designed and constructed an artificial pathway using codon-optimized enzymes for the production of these compounds in *Escherichia coli* [2]. Both types of polyphenolic compounds can be produced from tyrosine or hydroxycinnamic acids as precursors. To produce curcumin, the most studied curcuminoid for therapeutic purposes, 4-coumaroyl-CoA ligase (4CL) from *Arabidopsis thaliana*, curcuminoid synthase from *Oryza sativa* or diketide-CoA synthase and curcumin synthase from *Curcuma longa* were used. Using this pathway 354 mg/L of curcumin was produced, which corresponds to the highest concentration obtained so far using a heterologous host. Curcumin was also produced for the first time using tyrosine as precursor and caffeic acid as an intermediate. Other curcuminoids, such as bisdemethoxycurcumin and demethoxycurcumin were also produced using as precursors tyrosine or hydroxycinnamic acids (p-coumaric acid or a mixture of p-coumaric and ferulic acids, respectively). Based in this pathway, a similar pathway was designed and constructed to produce coumarins. The enzymes 4CL and p-coumaroyl-CoA 2′-hydroxylase from *Ipomoea batatas* were used to produce the coumarins umbelliferone, scopoletin and esculetin from p-coumaric acid, ferulic acid and caffeic acid, respectively. Approximately 20-50 mg/L of each coumarin was produced. The optimization of coumarins production from tyrosine is being conducted.

Saccharomyces cerevisae, which is also an interesting host, has only been used to produce other polyketides (e.g. resveratrol) [2]. However, it presents some unique advantages over *E. coli* for the design and construction of biosynthetic pathways to produce curcuminoids or coumarins. Hence, we are also exploring *S. cerevisae* as a potential chassis for the production of these valuable chemicals.

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In silico metabolic engineering: from research to the market

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The emergence of industrial biotechnology in the last years has created the need to accelerate the tasks of strain development, as most strains have naturally evolved for growth and not for the production of desired compounds. Moreover, in many cases, microbial strains are being used to produce compounds that are not native to their metabolism, requiring the addition of heterologous genes.
Thus, concurrently with fast and novel developments in molecular biology, there has been a significant investment in modelling and computational tools to aid rational strain design efforts. In our research group, we have been involved in several projects where relevant tools have been developed such as the user-friendly, widely used software tool OptFlux [1]. More recently, we have also launched the merlin tool [2] for aiding in genome-scale model reconstruction processes.
Based on the knowledge accumulated in the *in silico* metabolic engineering field, the spinoff company SilicoLife was launched in 2010 to answer some market needs that could not have been addressed through the University. SilicoLife is now a fully independent company specialized in designing *in silico* metabolic engineering solutions for industrial biotechnology, having projects with some of the major players in the field worldwide, both from industry and academia. SilicoLife has several proprietary technologies, from a pipeline for microbial model reconstruction to tools that aid the identification of non-trivial interventions such as gene knockouts and over/underexpressions for re-directing the metabolic fluxes to the desired target.

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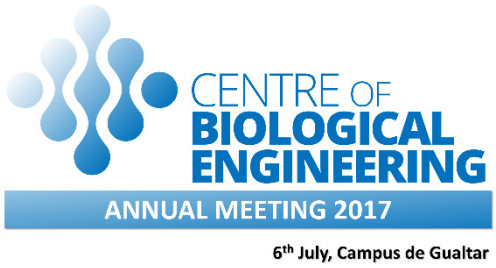

Research Highlights in Environmental Biotechnology and Bioengineering

Facts and challenges on hydrocarbons bioremediation

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The intense activity of the oil industry generates substantial amounts of contaminated wastes and wastewaters. Moreover, accidental oil spills occur frequently, causing severe damages in the marine environment and in the soil. Subsurface soil contamination is generally caused by oil leakages from underground storage tanks and transport pipelines that can further lead to groundwater contamination. To date, common techniques for remediation of petroleum-contaminated environments include physical removal, washing by cosolvents or surfactants, thermal desorption, electrokinetic movement of contaminants and oxidation/reduction via chemical agents. Biological technologies can be an alternative to the more aggressive physicochemical methods, as bioremediation exploits the metabolic diversity of microorganisms and their ability to degrade organic contaminants.
Aerobic bioremediation is frequently preferred over anaerobic processes, due to faster rates of hydrocarbons activation and biodegradation [1]. However, in subsurface environments oxygen is generally scarce and anoxic conditions prevail. Anaerobic microorganisms can biodegrade hydrocarbons coupled to the reduction of nitrate, iron(III), sulfate or under methanogenic conditions [2]. *In situ* bioremediation of hydrocarbons at anoxic conditions has not been extensively studied, despite the broad occurrence of these contaminants in the subsurface. Reduced knowledge on the catabolic mechanisms and microbial communities involved in anaerobic hydrocarbons biodegradation has limited this approach, and needs further research.
Our work has been focused on the bioremediation of petroleum-contaminated environments in the absence of molecular oxygen. Culture-dependent and independent approaches have been applied for improving knowledge on the key microorganisms involved in anaerobic biodegradation of hydrocarbons. The effects of different redox conditions in hydrocarbons biodegradation, and in the structure and composition of the microbial communities involved in these metabolic pathways, were investigated. Isolation of a novel *Desulfomonile* bacterium is ongoing, and its potential for bioremediation of halogenated compounds is being studied. By adding different co-substrates, methane production from olefins was accelerated. The work developed was awarded by NASA in the 12th International Workshop on Environment and Alternative Energy (Cape Canaveral, USA, 2014).

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Acceleration of methane production by carbon nanotubes

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Carbon nanotubes and other conductive materials have been found to influence the rates of several anaerobic reactions.

A range of different conductive carbon materials (CM) were reported to enhance methane production by anaerobic microbial communities. In most studies, the improvement of the overall process is attributed to the ability of these compounds to promote direct interspecies electron transfer (DIET) between bacteria, degrading more complex substrates, and methanogens, producing methane. The occurrence of DIET in the majority of these systems is not, however, proved and the effect of such conductive compounds on the activity of individual members, inside complex microbial communities, was never investigated. Thus, we herein present the results obtained when incubating pure cultures of methanogens, without any other microbial partner, in the presence of increasing concentrations of carbon nanotubes (CNT). Methane production from acetate, by the acetoclastic methanogens *Methanosaeta concilii* and *Methanosarcina mazei*, and from hydrogen plus carbon dioxide, by the hydrogenotrophic methanogens *Methanospirillum hungatei* and *Methanobacterium formicicum*, was accelerated, up to 17 times, in the presence of CNT [1]. Physical/chemical properties of the growth media changed in the presence of CNT, with redox potential decreasing with increasing CNT concentrations, and thus favouring methanogenesis. These findings show that CNT influences the microbial activity of methanogens in pure cultures and most likely this effect is extended to methanogens in complex communities as well, occurring in anaerobic bioreactors and in the environment.

Conductive materials also participate in biodegradation of recalcitrant compounds by acting as electron shuttles (ES), accelerating the process. Little amounts of different CM, namely activated carbon, carbon xerogels and CNT, act as ES in biotic and abiotic anaerobic degradation of azo dyes and aromatic amines, hasten considerably the reduction rates. CM associated with magnetic nanoparticles combine catalytic and magnetic properties. For example, CNT impregnated with 2% of iron (CNT@2%Fe), improved the rates of azo dyes reduction up to 79-fold and could be recycled as catalysts in successive decolourisation cycles [2].

In conclusion, addition of conductive materials is beneficial for accelerating biological and methane yielding biotransformations, improving the efficiency of environmental clean-up bioprocesses and bioenergy production.

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Perspectives on syngas fermentation

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The replacement of fossil fuels by renewable energy sources is, nowadays, a worldwide priority. Gasification processes and further bioconversion of syngas appears to be a promising alternative compared to the existing chemical techniques, since this process convert renewable sources into alternative fuels and commodity chemicals, such as CH_4, fatty acids, alcohols, etc., additionally contributing to the reduction of greenhouse gases [1]. Nearly any form of organic matter can be transformed through gasification, into syngas, mainly composed of CO, H_2 and CO_2. The biological conversion of syngas offers several advantages over catalytic processes, specifically the greater resistance to catalyst poisoning and the higher specificity for the substrates [2]. Syngas- and CO-fermenting microorganisms use the Wood-Ljungdahl pathway to produce several multi-carbon compounds such as short- and medium-fatty acids and alcohols. Even though many studies were performed in the last few years, fermentation of syngas still involves practical challenges due to limitations of the process. The major bottleneck of syngas fermentation that blocks the commercialization of this technology is gas-to-liquid mass transfer limitations, since it reduces the microorganisms' access to the substrate and consequently reduces the productivity rates. It is of utmost importance the development of alternatives that promote the enhancement of mass transfer, the improvement on the productivity rates from syngas fermentation and the deep study of the biocatalysts involved in syngas bioconversion pathways. Biological syngas conversion has been a research topic at the BRIDGE group since 2009, by studying both technological and microbiological aspects of the process. Previous work developed in our group focused on the use of anaerobic complex microbial communities to obtain enriched cultures and/or pure cultures that could convert syngas or CO into mainly acetate, CH_4 and H_2. Regarding to the technological aspects of syngas bioconversion process, a multi-orifice baffled bioreactor was used to study the effect of using different reactors designs to improve the gas-liquid mass transfer. Moreover, recent studies conducted at BRIDGE group with collaboration of BIOSYSTEMS group showed that the use of increased pressure (up to 5 bar) to increase gas-liquid mass transfer, leads to different metabolic routes on microorganisms. These results represent a step forward to direct the biochemical pathways of microbial community towards the specific products from syngas. As future perspectives, we aimed to continue a research line on syngas fermentation, by studying different operational approaches for this process and focusing on the production of butanol, 2,3-butanediol and propionate.

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Quantitative Image Analysis: a monitoring tool in wastewater treatment

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Computers are key equipment for the analysis of large amounts of data, for tasks requiring complex computation, and for the extraction of quantitative information, opposite to the qualitative evaluation of human analysis. Today, the automatic analysis of numerical images captured by digital cameras enables to rapidly extract quantitative information. Thus, quantitative image analysis (QIA) can be defined in general terms as the extraction of significant information from images, by means of digital image processing and analysis techniques.

In the last twenty years, QIA have gained an unquestionable role in several fields of research worldwide and our lab is considered a pioneer research unit on the development of QIA procedures for biological wastewater treatment processes monitoring. Over the years, the number of QIA studies [1, 2] for aggregated (granules and flocs) biomass and filamentous bacteria characterization has been increasing. It should be noticed, though, that some difficulties may be encountered in QIA procedures related to the suitability of the employed microscopy technique, regarding the intended biological process characterization.

So far, it has been demonstrated the applicability of QIA monitoring in pinpoint flocs formation, and filamentous and zoogleal bulking events identification in activated sludge (AS) systems, as well as in the prediction of sludge settling ability properties. QIA has proved to be adequate in terms of the main AS protozoa and metazoa recognition, as well as for operating conditions assessment. In enhanced biological phosphorus removal (EBPR) processes the ability to predict intracellular storage compounds, e.g. glycogen, polyhydroxyalkanoate, and polyphosphate concentrations, by QIA methodologies coupled to staining procedures, has been successfully proven. This novel approach, considered a faster technique to promptly monitor EBPR processes has the potential to surpass the off-line analysis, which is labor intensive and difficult to implement in full-scale plants [1]. Furthermore, it has also been found that the use of morphological and physiological data allowed predicting, at some extent, a number of effluent quality parameters. Other applications of QIA, in high-rate anaerobic processes, based on granular sludge, allowed detecting aggregation times and fragmentation phenomena during critical events, such as toxic and organic overloads [2]. Either way, the main goal is to improve the biological process efficiency through the combination of QIA information with operational parameters data.

Nowadays, great efforts are being made regarding the inclusion of staining procedures, with particular interest in the use of fluorescent dyes, due to the high amount of information provided by these techniques. In this way, it will be possible to obtain relevant data on the biomass characterization, viability and composition. However, further research is still needed to validate the obtained results with standard analytical analysis.

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Biobarriers for the Rehabilitation of Contaminated Systems

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The research activity of the Chemical Engineering Lab is defined within the mission and focus of BRIDGE group and aims to provide knowledge for environmental restoration, rehabilitation and sustainability by integrated recycling. As so, it aims the definition and development of innovative processes able to treat water/soils/sediments contaminated with metals, solvents and/or pharmaceuticals through the sorption concept, associated with co-adjuvant biological/chemical/electrochemical processes as biodegradation or oxidation. At present, different microorganisms as bacteria and fungi are under study, metabolically active or not, associated and/or supported by distinct sorbents that ranges from low-cost agro-forestry wastes (fern, eucalyptus leaves, oak leaves, grapefruit, cane pruning wine grapes, pine bark, cedar bark, rice husk, waste coffee grounds, eggshells, waste cork), natural materials like cork, clays, zeolites to designed sorbent materials, with chemically enhanced sorbing surface.
The general methodological approach used for the purpose includes: biosupports/biosorbents design and manufacture (equilibria, kinetics and mechanistic characterization), molecular and microscopy techniques and materials characterization by XPS, XRD, TGA, FTIR and RAMAN spectroscopy. The entrapment of metal ions with industrial interest has been under scrutiny in Chemical Engineering Lab for a long time and the effort led to catalysts design and manufacture, starting with waste metals that proved to have catalytic applications in liquid and gas phase mild oxidation [1]. On the other hand, several different industries use solvents on their productive processes making the problem of the deposition of these solvents on aquifer systems or on the soil very serious and the policy of the European Union in terms of solvent depositions is very restrictive. The same approach will be applied in this solvents issue (3-pentanone, DEK, MEK, toluene, 1,2-dichlorobenzene) adding a biodegradation step to the role played by the supported biofilm. The biodegradation of complex molecules as the active principles of pharmaceuticals (fluoxetine, ibuprofen) is also under consideration with very promising results [2]. Besides the kinetics, equilibria and mechanistic studies on the retention/degradation processes established at the possible combinations between sorbates/biosorbents, mathematical modeling and simulation is being performed in order to generalize the applicability of this approach to distinct rehabilitation pathways. These models will allow knowledge integration and upscale to reactors development and operation, defining a strategy for wastes valorization and sustainability by recycling or by downstream applications as it is the case of biobarriers made of recycled wastes, working as a support for eco-compatible biofilms that retain and degrade emerging contaminants.

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Bioprospecting fungi for biodegradation of textile dyes

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Reactive dyes are widely used in the textile industry. Coloured effluents from dyestuff and textile industries, the major producers and users of *azo* dyes, not only produce visual pollution but can also be detrimental to life, as they are usually resistant to biological treatment. In addition, fungi, mainly white rot fungi (wrf), have shown the ability to degrade numerous aromatic organopollutants, including textile dyes, via oxidative mechanisms till their complete mineralisation, avoiding the formation of anilines as intermediates. In our work, textile *azo* dyes were synthesized using aminobenzoic and aminosulphonic acids as di*azo* components and bioaccessible groups such as 2-methoxyphenol (guaiacol) and 2,6-dimethoxyphenol (syringol) as coupling components. The bioaccessible groups are present in the lignin structure and seem to be access points to the ligninolytic enzymes produced by wrf. The fungal biodegradation of the *azo* dyes were studied in order to establish the relationship between the chemical structure of the dye and the extent of biodegradation. The rule of the non-specific fungal ligninolytic enzymatic system, lignin peroxidases, manganese peroxidases and laccases, as well as the enzyme glyoxal oxidase wich produce H_2O_2 for the activities of both peroxidases were studied. Reactive Black 5 and the anthraquinone-based polymeric dye Poly R-478 have been currently used to screen the fungal biodegradation under alkaline conditions (pH ≥ 8.0). In order to adapt the fungi to this alkaline condition a chemostat was used [1,2].

To perform this work the wrf used were supplied by the fungal culture collection Micoteca da Universidade do Minho (MUM). To overcome current limitations in fungal biodegradation performance is desired that new strains well adapted to high osmotic pressure and alkaline conditions can be bioprospected preferentially from extreme environmental conditions worldwide.

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CENTRE OF BIOLOGICAL ENGINEERING

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Research Highlights in Health Biotechnology and Bioengineering

Liposomal formulations for rheumatoid arthritis

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Rheumatoid arthritis (RA) is the most common inflammatory rheumatic disease, affecting almost 1% of the world population. Although the cause of RA remains unknown, the complex interaction between immune mediators (cytokines and effector cells) is responsible for the joint damage that begins at the synovial membrane. Activated macrophages are critical in the pathogenesis of RA and showed specifically express a receptor for the vitamin folic acid (FA), folate receptor β (FRβ). This particular receptor allows internalization of FA-coupled cargo.
In this work we will address the potential of nanoparticles as an effective drug delivery system for therapies that will directly target activated macrophages. Special attention will be given to stealth degree of the nanoparticles as a strategy to avoid clearance by macrophages of the mononuclear phagocytic system (MPS).
This work summarizes the application of FA-target nanoparticles as drug delivery systems for RA and proposes prospective future directions [1].

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Bioproduction of polyesters

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Polyesters are polymers comprising of repeating ester groups as chain structure backbone, being the most popular biodegradable polymers. Many studies related with the synthesis of aliphatic and aromatic polyesters using chemical processes have been carried out [1]. Poly (ethylene glutarate) (PEG), Poly(ethylene malonate) (PEM), Poly(ethylene phthalate) (PEP) are attractive polyesters by virtue of their easiness in synthesis and widely diversity of applications such as textile manufacturing, microelectronics, bioprocessing, food packaging as well as in bio medical like surgical threads, contact lenses, supporting material in bone repairing, treat air leaks in lung injury. The chemical synthesis of these polyesters requires harsh conditions like high temperature and pressure, long reaction times and costly downstream processing. Many strategies such as the designing of recyclable catalysts, alteration of reaction conditions and the use of biocatalysts have been implemented to overcome these problems and make them green and environmentally friendly processes [2]. Recently, a new approach for synthesizing these type of polyesters has been developed by enzyme-catalysed polymerization. Potentially a solvent-free enzymatic system can offer better processing conditions without further complex purification process. The use of solid immobilized enzymes in solvent free systems where the reactants are the solvents itself might yield slow reaction rates due to the limiting diffusion between reactants and mass transfer limitations. The use of ultrasound might also contribute to push the reaction forward due to the cavitation effects, such as increased local temperature and pressure as well as the generation of micro level mixing and turbulence conditions. Template-assisted synthesis is also considered to study the effect of PEG addition, free or linked to the catalyst.
Herein, Poly(ethylene glutarate) was synthesized by reaction of equimolar amount of diethyl glutarate and ethylene glycol with 2 or 20% (w/v) of native and PEGylated enzymes (cutinase from *Fusarium solani pisi*, immobilized lipase B from *Candida Antarctica* and lipase from *Thermomyces Lanuginosus*). The mixture was placed in an ultrasonic bath for 1 or 2h and then under vacuum until 7h. The reaction products were characterized by NMR spectroscopy, Maldi-TOF mass spectrometry, FT-IR, differential scanning calorimetry (DSC) and thermogravimetric analysis (Tga).

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The *in vitro* and the *in silico* power couple: facilitating the discovery of novel anti–infective strategies based on antimicrobial peptides and quorum sensing inhibitors

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The persistent growth of antibiotic-resistance and the resilience of biofilm-related infections is pressing researchers to develop novel strategies to control infectious diseases. New antimicrobial strategies, namely concerning the use of i) antimicrobial peptides (AMP) (natural compounds with alternative mechanisms of action), ii) quorum-sensing inhibitors (QSI) (destabilisers of key communication mechanisms that regulate virulence and biofilm formation); and iii) antimicrobial combinations (can lower effective concentrations and achieve synergy), can lead to more effective therapeutics for this ever-growing, world-wide problem.

This work presents a two-fold approach regarding these strategies, namely i) comprehensive *in silico* characterisation of existing experimental results on AMP combinations and QSI through bioinformatics, and ii) *in vitro* (laboratorial) study of novel AMP and QSI combinations.

The *in silico* approach outputted a semi-automated curation workflow [1] that supported the mining of scientific literature on AMP combinations and QSI, enabling the reconstruction of antimicrobial networks that allowed the creation of two public databases. The first (http://sing.ei.uvigo.es/antimicrobialCombination/) contains information on AMP combinations against major pathogenic bacteria/fungi. Records describe species, strains, combination effects, methodologies, mode of growth, and expert observations. The second (http://pcquorum.org) contains QSI information on *Pseudomonas aeruginosa*, capturing the effects over QS genes, QS signals and virulence factors/mechanisms. These drug-QS interactions are contextualised by details on the experimental methods, drugs, QS entities and strains.

In the *in vitro* approach, colistin was combined with the AMP temporin-A, citropin-1.1 and tachyplesin-I (linear analogue) and tested to prevent (prophylaxis) or treat (therapeutics) planktonic and biofilm cultures of *P. aeruginosa* and *Staphylococcus aureus*. These tests included single- and double-species biofilms, encompassing six strains (two MDR). Results showed synergy and additiveness for both bacteria, even for MDR double-species biofilms. The most effective combinations, however, were toxic, but future work will tackle this issue [2]. Current work is testing AMP combinations with QSI, such as Azithromycin, which is the top QSI in the PCQuorum database, against biofilms.

The outcomes derived from both approaches were complementary: the databases aided in the AMP combinations and species selection to be tested *in vitro*, which in turn outputted valuable information to be added to the databases, thus bridging the gap between the two approaches. Globally, the use of the two-fold approach (*in silico+in vitro*) allowed not only the creation of important resources for fellow researchers, but also pointed out AMP combinations that were deemed promising in the treatment of double-species biofilms of relevant pathogens.

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Dextrin-based hydrogel for the development of injectable bone substitute

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The development of injectable bone substitutes (IBS) have garnered great importance in the bone regeneration field, as a strategy to reach areas of the body using minimally invasive procedures, and showing the ability of perfect fitting according to irregularities of bone tissue defects. In this context, the combination of injectable hydrogels and ceramic granules is emerging as a well-established trend. Particularly, *in situ* gelation hydrogels have arisen as a new IBS generation.

An injectable and *in situ* gelation hydrogel (HG) based on dextrin was developed aiming act as a carrier for bone graft granules and biomolecules [1] [2]. Dextrin is a low weight carbohydrate obtained by partial hydrolysis of starch that is already widely used in the cosmetic, textile and food industries. Furthermore, it is biocompatible, non-immunogenic and biodegradable, which renders dextrin a promising polymer for biomedical applications such as scaffolds and/or drug delivery systems.

To prepare the HG, dextrin was firstly oxidized with sodium periodate ($NaIO_4$) and then cross-linked with adipic acid dihydrazide, a non-toxic cross-linking molecule. Since HG will be a vehicle for medical application, a sterilization protocol for oxidized dextrin (ODEX) by gamma radiation was investigated, as well as, the effect of partial periodate oxidation effect on dextrin structure (oxidized derivatives), using mass spectrometry-based techniques. The results showed that gamma irradiation did not promote changes on the chemical structure of ODEX, and, so that, can be used as suitable terminal sterilization method for ODEX. *In vitro* cito- and genotoxicity assays performed in human lymphoblastoid TK6 cells revealed that HG displayed cytotoxicity depending on concentration, due to ODEX, but did not promote DNA damage. *In vivo* skin sentitization test showed that HG is not allergic.

The HG combined with commercial ceramic granules (macroporous Bonelike®, 250-500 μm) was tested in pre-clinical trials using a goat model, in two different bone defects: critical-sized calvarial defect (14 mm) and segmental tibial fracture (4 mm). The HG allowed the proper cohesion between the granules on the formulation and, also guaranteed mixture injectability. One the other hand, HG was able to stabilize *in situ* granules into the bone defects during tested periods (3-12 weeks) without affecting Bonelike® granules' osteoconductive properties neither impairing the bone repair/regeneration process.

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Application of antisense oligomers based approaches to control candidiasis

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Candidiasis is the primary fungal disease, with a mortality rate of about 40% and costs associated with hospitalized patients that range from €5700 to €85000 per episode. This important clinical, social and economic problem is due to the recognized phenomenon of *Candida* species multi-antifungal resistance, associated with the indiscriminate use of traditional antifungal agents. In clinical practice, *Candida albicans* continues to be the most commonly encountered member of the genus *Candida* with an incidence of approximately 47% in all infections caused by *Candida* species. However, in recent decades the number of candidiadis due to non-*Candida albicans Candida* species, particularly *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*, has increased significantly.
Candida species pathogenicity is facilitated by a number of virulence factors, most importantly adherence to medical and/or host cells, biofilm formation, filamentous forms development and secretion of hydrolytic enzymes [1].
Thus, new alternatives strategies/therapies, with novel mechanisms of action, improved pharmacokinetics, and less toxicity, are urgently needed to reach the marketplace to control *Candida* species infections.
Antisense therapy holds great promise for the treatment of many human diseases. The concept underlying antisense therapy is relatively straightforward: the use of a complementary sequence to a specific mRNA that can inhibit expression of the latter and induce a 'blockage' in the transfer of genetic information from DNA to protein. The antisense oligomers are a short strand of nucleic acids, which is complementary to the target mRNA, and generally composed by short sequences with 13-25 nucleotides of unmodified or chemically modified molecules [2].
Our key hypothesis is that, if in a pathogenic microorganism, the genetic sequence of a particular gene is known as a determinant agent of pathogenicity, synthesizing a strand of nucleic acid that will bind to the mRNA produced and inactivating it, in its translation into protein, we will be able to control its virulence.
In this sense, it is intended to develop cocktail(s) of therapeutic antisense oligomers to be used in drug nano-carrier formulations against the most pathogenic *Candida* species in order to control its virulence. Coating of medical surfaces, as well as, the antisense oligomers exploitation as new nano-drugs for *in vivo* administration, are exciting examples of future medical applications that will contribute for decreasing of candidiasis in the worldwide.

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Bacteriophage-derived enzymes: from exploration to exploitation

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Bacteriophages are viruses infecting bacterial cells. During their replication cycle, these viruses cross the bacterial cell wall twice. Firstly, bacteriophages have to inject their genome across the cell wall to initiate infection. Secondly, they must exit the host cell at the end of the replication cycle to infect new host cells. The principal barrier to overcome is the polysaccharides that can either be extracellular (slime or capsules) or structural (peptidoglycan). For this, bacteriophages encode two specific enzymes, called depolymerases and endolysins, to degrade these polymers to enable either infection or lysis.
Depolymerases, are specialized enzymes that degrade polymers (e.g. slime or capsular polysaccharides) to facilitate bacteriophage access to its hosts. They can be used to strip cells from their capsules, which is the main virulence factor of most pathogens. These enzymes have also the potential to digest the slime (i.e. matrix) and control infectious biofilms, which are problematic in food and clinical settings [1].
Endolysins are enzymes that specifically cleave the bacterial rigid peptidoglycan to release progeny bacteriophages at the end of its lytic cycle. This properties make them promising antibacterial agents, able to eliminate pathogens, including antibiotic resistant bacteria, in a quick and specific manner [2].
This talk will give an overview of the identification and characterization of bacteriophage-derived depolymerases and endolysins, and how they can serve as alternatives for existing antibiotics when applied against highly virulent and multidrug resistance pathogens, like *Acinetobacter* and *Pseudomonas*, with several *in vitro* and *in vivo* examples.

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Bacterial cellulose – from lab to market

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Bacterial nanocellulose (BNC) is a nanofibrilar exopolysaccharide synthesized by certain Gram-negative, obligate aerobic, acetic acid bacteria, the *Komagataeibacter* genus being the most important due to the high cellulose yield obtained. The unique properties of this biopolymer have supported a wide range of potential applications, in human and veterinary medicine, odonthology, pharmaceutical industry, acoustic and filter membranes, biotechnological devices and in the food and paper industry. The large-scale production of BNC, through advanced biotechnology has eluded many researches. Historical attempts but on low volume and high-value (mostly for biomedical applications) production can be traced back to the 90s.

This presentation will show the main work with BNC by the Funcarb group. Examples of these studies will include the use of BNC in biomedical and food applications. Finally, an overview on the main efforts towards the production of BNC at large scale and potential markets will also be presented.

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Poster Communications

Climate change affects fungal diseases of the important oil palm crop

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Palm oil is a very important commodity used in 40% of supermarket products, cooking, cosmetics, pharmaceuticals and increasingly, biodiesel. Eighty five percent of palm oil is produced in Malaysia and Indonesia. Also, palm oil production impacts the environment negatively and increases greenhouse gases (GHG). The crop is subjected to serious diseases, including those caused by fungi, which increase when oil palm (OP) are grown under sub-optimal conditions. Climate change is predicted to decrease the ability to produce disease-free OP after 30 and especially 80 years in Malaysia and Indonesia [1]. This affects negatively the sustainability of the palm oil industry, and increases pressure on destroying forests to further grow the cash crop. A limited number of areas gained more suitable climates for growing disease-free OP.

The situation was assessed in other OP producing countries in Latin America (*e.g.* Brazil) and Africa (*e.g.* Nigeria) in which OP are increasingly grown [2]. The decrease in suitability was even more severe in many cases, making fungal diseases more likely. These other countries are not a viable alternative to growing in Malaysia and Indonesia. A small number of countries were more suitable (*e.g*. Paraguay) over 30 to 80 years in a predicted movement towards the poles of suitable climate. Interestingly, the decreased ability to grow OP may have an ameliorating effect on climate change, as OP production increases GHG.

In summary, the effects of climate will have profound effects on OP and fungal diseases in the future.

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The Role of Filamentous Fungi in the Inter-Kingdom Complex Association of Water Biofilms

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The provision of safe drinking water (DW) is a top priority issue in any civilized society. Biofilms in drinking water distribution systems (DWDS) are responsible for several undesirable effects in the water quality. One the main drawbacks is their potential to protect pathogenic microorganisms from stress conditions. In such microcosms, microbial diversity leads to a variety of complex relationships involving interspecies and intraspecies interactions. However, most of the available information was obtained from studies with bacteria [1]. Very few reports on filamentous fungal biofilms can be found in the literature, probably because these microorganisms cannot fit completely within restrictive biofilm definitions based on bacteria [2]. In fact, the term "water biofilm" is rarely applied to filamentous fungi. However, fungi are especially adapted to growth on surfaces, as evidenced by their absorptive nutrition mode, their secretion of extracellular enzymes to digest complex molecules, and apical hyphal growth. Fungi are therefore excellent candidates for biofilm formation but this aspect is still poorly understood. Therefore, the understanding on the mechanisms underlying DW multispecies biofilm formation and behaviour is of utmost importance in order to develop more efficient control strategies. This includes the knowledge on the role of cell-surface properties, inter-kingdom interactions and chlorine effects on initial adhesion and biofilm control.

Members of the AMG group found in a recent study that *Penicillium expansum*, a filamentous fungi isolated from Braga DWDS, grows as a complex, multicellular biofilm in 48 h. Similarly to bacterial biofilms, it was possible to identify the different phases of filamentous fungi biofilm formation (induction, exponential, stationary, and sloughing off). Microscopic analysis allowed identifying several steps: the involvement of conidia on initial adhesion (4 h), germling (8 h), initial monolayer (12 h), a monolayer of intertwined hyphae (24 h), mycelial development, hyphal layering and bundling, and development of the mature biofilms (≥ 48 h). It was possible to apply several quantitative methods, previously used with bacterial biofilms, to study filamentous fungal biofilms. The metabolic activity and biomass of the fungal biofilms were shown to increase over time and a correlation between metabolism, biofilm mass and hyphal development was found. Additionally, the association of *P. expansum* with *Acinetobacter calcoaceticus*, a DW-isolated bacterium, increased resistance of the inter-kingdom biofilm to chlorine.

The overall results clear proposes that it seems fundamental to gain deeper insights into the mechanisms promoting multi-species biofilm ontogenesis in DWDS, including inter-kingdom (prokaryotes and eukaryotes), and the role of fungal-bacteria association on resistance to disinfection.

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Preserved Freeze-Dried and Aged Filamentous Fungi of Biotechnological Importance

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The bioindustrial production strongly depends on the reliable microbial preservation in microBiological Resource Centres. The implementation of consistent microbial preservation techniques and appropriate quality assurance are key issues for an effective and efficient preservation and consequently industrial production. Though, the cost and convenience of each method are important aspects to be taken into consideration such as the knowledge of all parameters capable of affecting the procedures [1]. Preservation methods currently used are highly empirical and in many instances do not provide reliable genetic and phenotypic stability. There is an increasing in the adaptation of the existent methodologies to the specific strain to preserve. But there is also the need to understand the alterations after preservation. Freeze-drying is commonly used to preserve biological materials for long-term storage at room temperature and protectants are generally added to these materials to reduce damage during the freezing and drying processes.

Therefore, the main goal of the present experimental study is to evaluate the freeze-drying preservation method for the effective long-term preservation of strains belonging to *Aspergillus* section *Nigri*. To perform this, twenty one strains representative of *Aspergillus* section *Nigri* were selected and preserved by freeze-drying. The strains were subjected to accelerated storage, equivalent to ageing, with two different time points (2 and 4 weeks of maintenance of the freeze-dried strains, in ampoules of borosilicate glass, at 37 ºC). These samples were morphological and physiological analysed. In addition, the genetic stability is under study applying molecular biology techniques.

For all the methodologies used to evaluate freeze-drying of fungi along time the major conclusions so far are: 1) Minor differences were found but there were no significant changes observed in the macroscopic and microscopic analysis; 2) In the screening for aflatoxins, all strains maintained their pattern before and after ageing; 3) In the detection of ochratoxin-A (OTA), most of the OTA producing strains did not present significant changes after preservation and ageing; 4) In the fumonisins detection, it was possible to observe that some strains changed their profile along the procedures, but for most samples no production was detected after ageing.

In conclusion, and so far, freeze-drying can be considered a technique of excellence to be used on the maintenance of biodiversity within the filamentous fungi, and more accurately for *Aspergillus* section *Nigri*.

Acknowledgments:

C. Santos and R. Rodriguez acknowledge Universidad de La Frontera (Temuco, Chile) for the partial funding from the Project DIUFRO DI16-0135.

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Effects of organic acids on mycotoxigenic fungi that produces aflatoxins and Ochratoxin A

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Mycotoxins are toxic fungal secondary metabolites that occur frequently in a great diversity of agricultural commodities and processed foods. Two of the main mycotoxins that can be found in foods are aflatoxins (AFs) and ochratoxin A (OTA). Some important producers of these mycotoxins are respectively *Aspergillus flavus* and *Penicillium nordicum*. *Aspergillus flavus* occurs mostly on corn, peanuts, spices, cottonseed and tree nuts; *P. nordicum* occurs on dry-cured meat and cheese products. In this study several organic acids were tested for the inhibition of *A. flavus* and *P. nordicum* growth and for the inhibition of the production of AFs and OTA. Experiments were done using the poisoned food technique and mycotoxins production was assessed by HPLC with fluorescence detection. Lactic acid (LA), phenyllactic acid (PLA), hydroxyphenyllactic acid (OH-PLA), indole lactic acid (ILA), propionic acid (PA), acetic acid (AA) and butyric acid (BA) were added to MEA or YES culture medium to achieve concentrations of 0.1 to 8.0 mg/mL. Fungi were inoculated on the centre of petri dishes with a spore suspension (10^6 spores/mL) and incubated at 25 ºC in the dark, in triplicate. Fungal colony diameters were measured daily and mycotoxins contents were analysed after 7 days of growth. The concentration of each organic acid, which inhibited by 90% the fungal growth and the production of mycotoxins (IC90) was derived from non-linear fit of Log doses versus normalized data using the Hill equation. The stronger effects on fungal growth were obtained with butyric, propionic and acetic acid. IC90 were between 1.1 and 1.9 mg/mL for both fungi. PLA and ILA also inhibited the growth of fungi but IC90 were considerably higher (8.2-23.9 mg/mL). For the inhibition of AFs, BA, PA and PLA were the most active organic acids with IC90 of 0.25, 0.38 and 0.87 mg/mL, respectively. Concerning the inhibition of OTA, BA, PA, AA, ILA and PLA had the strongest effect. Their IC90 were of 0.95, 1.53, 1.66, 3.15 and 4.50 mg/mL, respectively. These organic acids may be of interest to complement the action of commercial fungicides already available in the market to suppress the production of mycotoxins in crop fields.

Environmentally sustainable processes for biomass conversion into biofuels and value-added compounds: integrated and intensified approach within a biorefinery concept

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Lignocellulosic biomass conversion into biofuels is considered a promising alternative to replace fossil fuels, being one of investment priorities of European Union to attain a sustainable growth within Horizon 2020. Nevertheless, lignocellulosic biofuels are not widely implemented on large-scale due to the high initial investment and operational costs. The scientific research carried out has been focused in the development of biomass processing technology for bioethanol production making use of environmentally-friendly pre-treatments and molecular biotechnology tools (metabolic, genetic and physiological engineering) for yeast development.

The team has contributed to the development of environmentally-friendly processes for ethanol production following a biorefinery approach. Organosolv using glycerol as green solvent was optimized for fractionation of *Eucalyptus globulus* wood (EGW) in order to obtain a pretreated biomass susceptible to be used at high-solid loadings (>30%) on saccharification and fermentation process, as well as, a recovered lignin and hemicellulose [1]. As far as we know, the ethanol obtained (94 g/L) in this work was the highest ethanol concentration from lignocellulosic biomass reported in the literature [1].

Hydrothermal process as pretreatment (using water as only reaction medium) was also evaluated for the improving of enzymatic saccharification of cellulose to glucose, as well as the recovery of hemicellulose-derived compounds (as oligosaccharides) in liquid fraction in order to revalorize and optimize the process from integrated point of view [2]. By using a selected robust yeast strain, simultaneous saccharification and fermentation (SSF) of whole slurry from hydrothermally pretreated EWG was also optimized, achieving 23 kg of ethanol/100 kg of wood with 85.5% of ethanol yield [2]. Therefore, this approach has been also applied to other lignocellulosic biomasses (such as vine shoots, Paulownia wood, oat straw and Eucalyptus bark).

Together, these studies revealed the importance of integrating different approaches, from pre-treatment to yeast development, for cost-effective production of 2nd generation bioethanol.

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Encapsulation of omega-3 fatty acids in bio-based nanoemulsions: physical and chemical characterization

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The use of nanotechnology can offer several advantages, not only improving water solubility but also in the increase of bioavailability of lipophilic bioactive compounds. Omega-3 polyunsaturated fatty acids (ω-3 fatty acids) are known for their functional properties such as: improving cardiovascular health, decrease inflammation, increase cognitive function, and positively influence neurological and visual development. However, ω-3 fatty acids are highly susceptible to oxidation, have an intense odour and present low water solubility, which makes their direct application in foods extremely difficult. Nanoencapsulation (through nanoemulsions) may be used to reduce these problems.
In this work, lactoferrin (Lf), a protein derived from milk with a wide range of reported biological activities (e.g. antioxidant, antimicrobial and cancer prevention), was used as natural emulsifier for the development of oil-in-water nanoemulsions. Nanoemulsions were produced with a high-pressure homogenizer applied for 5 cycles at 20000 psi. Different Lf concentrations (0.2; 0.6; 1; 2; 3; 4 and 5% (w/w)) were tested. The nanoemulsions' physical properties were evaluated in terms of size and ζ-potential using dynamic light scattering (DLS) and by surface tension using the Ring method. The morphology of nanoemulsions was analysed by transmission electron microscopy (TEM). The physical and chemical stability of these nanoemulsions was assessed during 50 days, at storage temperatures of 4 ºC and 25 ºC, being the chemical stability of nanoemulsions evaluated by antioxidant activity measurements using the DPPH radical scavenging assay.
Results showed that according to the Lf concentration used, different properties were obtained. Nanoemulsions with Lf concentrations between 2 and 5 % (w/w) presented sizes around 160 nm and a ζ-potential higher than +30 mV. For concentrations below 2 % (w/w), nanoemulsions presented sizes around 200 nm and a ζ-potential below +30 mV. It was noticed that higher Lf concentrations lead to smaller sizes and higher ζ-potential values. Increasing Lf concentrations caused a decrease in the superficial tension of nanoemulsions. TEM measurements showed that nanoemulsions particles have a defined spherical shape. Results also showed that nanoemulsions with Lf concentration above 2 % (w/w) present better properties (smaller sizes and higher ζ-potential) regarding their storage stability. Nanoemulsions stored at 4 ºC did not exhibit significant variations in size and ζ-potential values, while at 25 ºC the nanoemulsions suffered a size increase (of around 35 nm compared to the initial value) and a reduction in ζ-potential (of around 20 mV compared to the initial value) during storage. Antioxidant activity did not demonstrate significant changes before and after storage at both temperatures (IC50 was around 14 mg/g of solution).

Strategies for Fructo-oligosaccharides production with high-content and purity

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The consumers' interest in healthy and high nutritional food has significantly increased in the recent years. This trend towards the adoption of healthier lifestyles has been the main driver for the great demand of functional ingredients, such as the prebiotics fructo-oligosaccharides (FOS). Industrially, FOS are produced from sucrose through purified enzymes, in two-step bioprocesses, with low theoretical yields (0.50-0.55 $g_{FOS}.g_{Sucrose}^{-1}$) and purities (50-55%). Downstream steps are therefore needed to remove the non-prebiotic sugars and enable the incorporation of these FOS mixtures in diabetic, dietetic and healthy foods.

In the last ten years, we have been investigating new strategies to produce FOS with higher contents, purities and differentiated functionalities. We have been exploring *Aureobasidium pullulans* and *Aspergillus ibericus* as FOS producers, in one-step fermentation processes, using the whole cells of the microorganisms instead of the isolated enzymes. This strategy proved to be efficient, fast and economic, yielding 0.64 $g_{FOS}.g_{Sucrose}^{-1}$. The FOS mixtures produced were able to stimulate the growth of probiotic strains and were simultaneously resistant to hydrolysis along the gastrointestinal system confirming their health claims as prebiotics. The probiotic strains preferentially metabolized the FOS mixture synthesized by *A. ibericus*, followed by the one from *A. pullulans* and lastly the commercial FOS.

The purification of FOS is not straightforward due to the physicochemical similarities between the different oligosaccharides and the smaller saccharides. To increase the FOS purity, we have been exploring different strategies including microbial treatments and downstream treatments as activated charcoal and ion-exchange chromatography.

As microbial treatments, we studied the use of a *Saccharomyces cerevisiae* strain, able to metabolize the small saccharides without FOS hydrolyse, in co-culture with the FOS microorganism producer or in a two-step fermentation, in which FOS are firstly synthesized and then purified by the *S. cerevisiae*. Fermentations in two-steps were found to be more efficient than the co-culture ones and purities of 82% (w/w) in FOS were obtained [1]. To avoid competition by the subtract in the co-culture, we are now evaluating the use of a *S. cerevisiae* strain with the SUC2 gene for invertase expression repressed. Using this strategy, FOS are being produced with yields of 0.64 $g_{FOS}.g_{Sucrose}^{-1}$ and purities up to 93% (w/w).

As downstream treatment we optimized an adsorption/desorption process of sugars using activated charcoal and ethanol as eluent. Mixtures containing 50.6% (w/w) of FOS were purified to 92.9% (w/w) with a FOS recovery of 74.5% (w/w) and some fractions were obtained with purities up to 97% (w/w) [2].

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Keratin peptides from chicken feathers for biomedical applications

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A chronic wound is a wound that does not heal in the expected amount of time and has a high risk of infection that can and will delay the healing process. Nowadays, the healing of chronic wounds continues to represent a significant challenge to physicians, as many wounds remain recalcitrant despite optimal standard care. Besides the acute pain that these patients endure, the economic burden of chronic wound care is "huge" on the health system. The design of wound dressings has suffered continuous and significant changes over the years. The development goes from natural materials used to just cover and conceal the wound to interactive materials that can facilitate the healing process. Keratins have been shown to play a key role in wound healing. Keratinocyte migration is one of the initial events of reepithelialization and is an important phase of wound healing [1,2]. It is know that water- soluble keratin peptides derived from an oxidative extraction from human hair, enhance the proliferation of human dermal fibroblasts. The use of keratin from human hair, can pose a problem due to limited supply, although another source can be used, chicken feathers. Chicken feather waste from the poultry industry is considered a critical problems in many countries, as it is responsible for a waste of 3600 million tons each year world-wild. The most common method to dispose this "waste" is by burning or burying, which is not an enviromment friendly procedure. Our research group in collaboration with the Universidade do Açores isolated and characterized microorganisms capable of degrading chicken feathers using a liquid-state fermentation. The project results clearly demonstrated the keratinolytic activity of the microorganism as well as the increase of protein on the culture medium. Most importantly the size of the peptides recovered range from 900 to 3000 Daltons, the most bioactive fraction. *In vitro* assays demonstrated that the recovered keratin peptides are not cytotoxic and that may have distinct effects on cell proliferation. If the chicken feathers were hydrolyzed by the S196D strain an increase on cell migration is observed, on the other hand is the peptides are a resulted of chicken feathers hydrolyzes by S188D there is a delay on the cell migration. The results obtained on this project are promising and will to the development of custom made wound dressings, depending on the migration rate required.

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Alternative strategies for the extraction of compounds from natural resources

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Exploitable compounds from natural resources include polysaccharides, proteins and peptides, gum exudates, lipids, polyphenols and other secondary metabolites.

Traditional-water extraction (TWE) of polysaccharides is a time-consuming process that requires high solvent and energy consumptions and generates large amounts of waste. Other bioactive compounds are traditionally extracted with organic solvents or mixtures of organic solvents and water, with or without heat.

Subcritical water extraction and hydrolysis have been used as an alternative to traditional solvent extraction for many compounds in different matrices (1). Advantages include absence of chemical solvents, lower solvent costs, lower extraction times at moderate operational conditions, resulting in higher quality extracts. Furthermore, water is a solvent with very interesting properties in subcritical conditions. As temperature increases hydrogen bonds break, significantly decreasing the dielectric constant and the polarity, thus increasing solubility of more hydrophobic compounds. On the other hand water ionization increases (thus increasing H+ and OH- ions concentration), meaning that reactions catalysed by acids or bases, like biomass hydrolysis, are accelerated.

Ohmic heating is defined as a process wherein an electric current is passed through materials with the primary purpose of heating them (2). Because heat is generated inside the material to be heated (Joule effect), the heating process does not depend on heat transfer between phases and interfaces, allowing uniform heating and an extremely rapid heating rate. Furthermore, it also allows heating of large particulates and fluids at comparable rates, as long as their conductivities remain similar. Many studies also suggest that EF has also a significant effect on the cell wall permeabilization, having EF been applied to vegetable cells with different purposes: enhancing diffusion of molecules into vegetable tissues, drying, pasteurization. The process has high energy conversion efficiencies resulting in lower operational costs and in an environmentally-friendly system (2).

Enzymatic hydrolysis has been used either as an extraction process (e.g. for bioactive peptides) or as a pre-treatment to facilitate extraction (e.g. for breaking the cell wall and hydrolysing the structural polysaccharides to be used in fermentation processes).

These processes (alone or in combination) are being applied to different matrices: seaweeds, olive oil residues, wine residues, pine bark, among others, mainly in the extraction of polysaccharides, proteins and phenolic compounds. Chemical, structural and functional characterization of different extracts allow exploiting different potential applications. On-going projects include "REDVALUE - Technological Alliance to complete the forest and agroindustrial production cycle".

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Biosurfactants: production, applications and future potential

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Surfactants are one of the most important classes of industrial chemicals in terms of production volume. These compounds exhibit a wide variety of applications in several industries and are present in nearly every product and aspect of our daily life. They can be found in detergents, laundry formulations, household cleaning products, cosmetics, herbicides or pesticides, and are also used in bioremediation, agriculture, food, pharmaceutical, textile, paper or the petroleum industries, among others. Most conventional surfactants available nowadays are derived from non-renewable resources and their use may lead to significant ecological problems due to their toxicity and low biodegradability. In the recent years, an increase in environmental awareness has led to much more interest in the use of renewable-based, biodegradable and more environmentally friendly surfactants. Among them biosurfactants, surface-active compounds synthesized by microorganisms, are attracting a pronounced interest due to their potential advantages over their synthetic counterparts, and to the fact that they could replace some of the synthetics in many environmental and industrial applications. They exhibit similar or better performances when compared with chemically synthesized surfactants, and due to their biological origin, they are less toxic and more easily biodegradable. Despite the clear advantages and the potential applications of biosurfactants, their overall use is hampered by their high production costs and their limited structural variation in contrast to chemically produced surfactants. In our group, several biosurfactant-producing microorganisms have been isolated from crude oil samples in the last years. In most of the cases, the biosurfactants produced by those isolates were similar to other previously reported (e.g., surfactin, rhamnolipids). However, a novel low molecular weight bioemulsifier, which structure is completely different to those previously reported, was also identified [1]. Biosurfactant production by these microorganisms was optimized through the development of alternative low-cost culture media containing exclusively agro-industrial wastes and by-products (i.e., sugarcane molasses, corn steep liquor, olive oil mill wastewater), which significantly contributed to reduce their production costs and, at the same time, allowed the valorization of those residues [2]. The biosurfactants produced exhibited a better performance when compared with several commonly used chemical surfactants, making them promising candidates for several applications, including microbial enhanced oil recovery and bioremediation.

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Bio-based nanocarriers incorporating curcumin – bioaccessibility and cell viability evaluation

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For decades, curcumin (Cur), a natural polyphenol product derived from turmeric (*Curcuma longa*) has been considered one of the most promising bioactive compounds due to its health benefits such as anti-inflammatory, antioxidant and anticarcinogenic properties. However, Cur application as functional compound in food products has been limited due to light, heat, and oxidation sensitive and mainly, to poor aqueous solubility which limit its bioavailability [1]. To increase Cur bioaccessibility and consequently, increase bioavailability, several carriers have been investigated, particularly nanocarriers. Among the various nanocarriers described in the literature, lipid-based nanocarriers may offer a promising tool to increase the stability, efficacy and safety of lipophilic compounds, namely Cur [2]. Moreover, the understanding of Cur-loaded nanocarriers' behaviour under gastrointestinal (GI) conditions is fundamental to produce safe and customized nanocarriers with optimized bioactivity for oral consumption.

The aim of this study was to comparatively analyze the impact of two different lipid nanocarriers incorporating Cur - solid lipid nanoparticles (SLN) and nanoemulsions (NE) – on bioaccessibility and Caco-2 cells viability.

The evaluation of the Cur-loaded lipid-based nanocarriers was performed based on their physicochemical properties and bioaccessibility under *in vitro* simulated GI conditions (using INFOGEST *in vitro* digestion method). During digestion process, samples were collected at each stage (i.e. mouth, stomach and intestine) and nanocarriers' size, ζ-potential, free fatty acids' release (FFA) and cur bioaccessibility were evaluated. Furthermore, *in vitro* cell viability of different nanocarriers was analyzed in Caco-2 cell line by MTT assay. Caco-2 cells were incubated with free Cur and different nanocarriers formulations at CU concentration of 0-25 $\mu g.mL^{-1}$ for 24 h.

The results from the *in vitro* digestion indicated Cur-loaded SNL and Cur-loaded NE stability under simulated mouth and stomach conditions. On the other hand, these nanocarriers were destabilized under simulated intestinal conditions; Cur-loaded NE showed less stability because particle size increased indicating droplet coalescence. Also, higher amount of FFA was released from NE compared to SNL. NE and SLN increased 3.4 and 2.5 times Cur bioaccessibility, respectively. *In vitro* cell viability assay revealed that the highest concentration of Curc on both NE and SLN formulations that one can use without interfere with cellular viability was 15 $\mu g.mL^{-1}$ after 24 h incubation.

This study showed the potential of fabricated NE and SLN for oral delivery of Cur, a hydrophobic nutraceutical molecule, by assembling essential information on digestion and safety of different nanocarriers.

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Optimizing CRISPR/Cas9 for high-expression genome *loci* in industrial yeast strains

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The yeast *Saccharomyces cerevisiae* is one of the key cell factories for the production of bio-based chemicals, from fuels and bulk chemicals to active pharmaceuticals. Generally recognized as safe (GRAS) by the U. S. Food and Drug Administration and with a broad array of tools available at the molecular level, *S. cerevisiae* has been successfully manipulated for a wide range of applications. For large-scale fermentations, particularly in biorefineries, yeast cells must perform under harsh conditions, such as fluctuating pH and temperature, high osmotic pressure and presence of inhibitors derived from biomass hydrolysis. In this context, robust and stress-tolerant yeast chassis are required to attain high titers and product yields [1]. Industrial environments have been identified as a bioresource of yeast strains with higher robustness and fermentation performance and distinct strains have been isolated. However, such strains are more difficult to genetically manipulate than the standard laboratory strains as they are typically prototrophic, diploid and often exhibit low transformation efficiencies and lower levels of homologous recombination. In this way, efficient genetic engineering tools are required to develop effective yeast platforms. Recently, the CRISPR/Cas9 system, based on RNA-guided nuclease activity, has been employed in industrial yeasts for efficient disruption of genes and DNA insertion. This technique allows for DNA integration in single and multiple *loci*, which might be an advantage for the fine tuning of gene expression. However, genome location must be investigated for gene integration suitability by testing whether a given region supports high gene expression without affecting the microorganism's fitness. Here, we designed guide RNA's targeting conserved *Ty* elements in the yeast genome, driving the Cas9 double-strand break for heterologous DNA insertion. The gene coding for *A. niger* extracellular β-galactosidase was used as reporter gene, as high level expression has been previously achieved in *S. cerevisiae* and its activity is easily detected by the hydrolysis of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) [2]. The *lac*A gene, under the control of ADH1 promoter and terminator (*lac*A cassette), and flanked by homologous *Ty* sequences was used as donor DNA for homologous recombination. Moreover, a multicopy yeast expression vector carrying the *lac*A cassette and the dominant G418-resistance marker was constructed, as a comparison to multi-copy genome integrations. Selected industrial strains were transformed and expression levels assessed.

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Integration of autohydrolysis and organosolv process for recovery of lignin from corncob

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Lignocelluloses, such as hardwood, softwood and agricultural residues, are low cost feedstocks mainly composed by cellulose, hemicellulose and lignin. Lignin is the third most abundant naturally synthesized polymer. It presents an amorphous polyphenolic structure, which can be used for the development of bio-based materials and chemicals. However, the bioconversion of renewable lignocelluloses to value-added products requires their fractionation through pretreatment technologies [1,2].

This work evaluated a combination of two environment-friendly pretreatments: liquid hot water (LHW) at 200 ºC for 30 min for removing hemicelluloses, followed by a delignification process using non-catalysed ethanol organosolv to obtain lignin with a high purity, and also cellulose for other purposes. For that, an experimental design was performed, as following: ethanol concentration 20–60%, temperatures: 140–180 ºC, and holding time 40–120 min. Lignins were recovered by precipitation after ethanol evaporation and air-dried. All fractions were characterized in terms of extraction yield, total phenolics, antioxidant capacity (DPPH), and thermal properties (differential scanning calorimetry – DSC). Corncob presented 35.9% cellulose, 30.5% hemicellulose and 21.1% lignin. During LHW, the hemicellulose was solubilized, and 62.7% cellulose and 30.4% lignin were recovered on solid fraction (HPLC analysis). Regarding to organosolv, only ethanol concentration influenced lignin extraction, being the maximum yield (65% on corncob dry matter) obtained with 60% ethanol at 180 ºC during 40 min. Glass transition temperature varied between fractions (60–90 ºC) and all fractions presented high antioxidant activity (comparable to BHT standard). Ethanol organosolv lignins are characterized by their high purity, low glass transition temperature, high solubility in organic solvents, a very low sulphur content, and their antioxidant capacity, showing a great potential to be used as radical scavenger (anti-oxidant), matrix material in bio-based composites, carbon fibres, phenolic resins and poly-urethane foams.

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Design of β-lactoglobulin nanostructures for encapsulation and controlled release of riboflavin in the gastrointestinal tract

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Bovine β-lactoglobulin (β-Lg) is a globular protein from milk and the major component of whey proteins (ca. 50 % of its protein content). It is a food-grade and Generally Recognized As Safe (GRAS) material, that have a high nutritional value and important biological and functional properties, particularly the capacity to form gels, which allows the formation of nanostructures that can be used to encapsulate nutraceuticals [1]. Besides, β-Lg is stable at low pH, and highly resistant to proteolytic degradation in the stomach. Riboflavin is an essential vitamin for the normal function of human brain and nervous system. However, this vitamin is poorly soluble in water and highly susceptible to light degradation, thus its encapsulation may represent a suitable solution to its protection, overcoming these issues [2]. This study aims at evaluating the ability of β-Lg food-grade nanostructures to encapsulate and control the release of riboflavin during the gastrointestinal (GI) passage.

In this study, aqueous dispersions of β-Lg (1%) were accordingly produced, and formation of stable β-Lg nanostructures was ascertained at pH 6.0, after heating at 80 ºC for 10 min. The nanostructures formed were characterized in terms of size, surface charge and stability, morphology and association efficiency (AE) of riboflavin. Riboflavin-loaded nanostructures were then submitted to an *in vitro* GI model system, simulating the conditions of human GI tract (i.e. stomach, duodenum, jejunum and ileum) and their condition (e.g. temperature, pH, mixing, transit time, enzymes and other constituents such as bile). The experiments were carried out for 5 h, and the nanostructures were structurally characterized after each stage of digestion.

Stable β-Lg nanostructures were obtained at pH 6, showing a spherical shape, particle sizes of 170.2±0.85 nm, low degree of polydispersity (i.e. PDI = 0.074±0.027), ζ-potential of -34.9±0.49 mV, and AE of 26%. β-Lg nanostructures showed to be stable in the stomach being mostly degraded in the small intestine (which was determined by electrophoresis assay), where most of their riboflavin content was released i.e. 46%, 84% and 89% in the duodenum, jejunum and ileum, respectively. Hence, β-Lg nanostructures showed to be suitable carriers for riboflavin until reaching the intestine, where destabilization eventually occurs.

This study represent a significant contribute in the food science field by providing knowledge related to the applicability of food-grade nanomaterials for incorporation and controlled release of functional compounds, thus improving their bioavailability through protection from harsh conditions during GI digestion.

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Screening of fungal sources of β-galactosidase with potential for the synthesis of prebiotics

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β-Galactosidases (EC 3.2.1.23), also known as lactases, are a family of enzymes able to catalyse two different types of reactions, namely hydrolysis and transgalactosylation. The hydrolytic activity is commonly applied in the food industries to reduce the lactose content of dairy products, preventing lactose crystallization problems and increasing sweetness, flavour and solubility. On the other hand, transgalactosylation reactions have been explored in the synthesis of lactose-based prebiotics, such as galacto-oligosaccharides (GOS), lactosucrose [1] and lactulose [2], with potential application in the pharmaceutical and food industry. These prebiotics are enzymatically produced through the hydrolysis of lactose and further transfer of a galactosyl residue to a suitable acceptor, i.e. fructose for the disaccharide lactulose; sucrose for the trisaccharide lactosucrose; and lactose for GOS. The sources of β-galactosidase are extensively distributed in nature, namely in microorganisms, plants and animal organs. Nevertheless, β-galactosidases from microbial sources exhibit higher industrial relevance mainly due to their easy handling, great catalytic activity and high production yields. In this study, fifty fungal strains obtained from MUM (Micoteca of University of Minho, Portugal) and from DIA-UAC (Food Research Department, Autonomous University of Coahuila, Mexico) were screened for β-galactosidase production. A chromogenic test performed in agar plates supplemented with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used in the screening study. Twelve promising fungal strains were identified and further validated as effective β-galactosidase producers under submerged fermentation (28 ºC, 150 rpm) using a culture medium composed of lactose, peptone, yeast extract and salts (KH_2PO_4, Na_2HPO_4 and $MgSO_4$). Under these conditions, only eight fungi (*Aspergillus brasiliensis*, *Aspergillus restrictus*, *Aspergillus uvarum*, *Penicillium brevicompactum*, *Penicillium italicum*, *Penicillium spinulosum, Mucor sp*. and *Trametes versicolor*) were able to consume lactose and produce β-galactosidase. The crude extract enzymes were characterized regarding their optimal pH and temperature. Additionally, their ability to synthesize lactose-based prebiotics was evaluated by incubating the crude enzymes with suitable mixtures of substrates (fructose + lactose or sucrose + lactose) at 37 ºC. Lactulose and GOS were produced by all the crude β-galactosidases when mixtures of fructose and lactose were used. However, the best results were obtained for β-galactosidases from *A. restrictus* and *A. uvarum*. When lactose and sucrose were used as substrates, GOS were the only lactose-based prebiotics obtained. Additionally, other type of prebiotic was synthesized in these conditions, namely fructo-oligosaccharides (FOS), suggesting also the presence of β-fructofuranosidase activity in the enzymatic extract. Overall, the eight fungi can be interesting biocatalysts for the prebiotic synthesis.

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Responses of the green alga *Pseudokirchneriella subcapitata* to short- and long-term exposure to heavy metals

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Algal cells can be exposed to toxicants for a short-term due to accidental discharges or, more commonly, for a long term. The green alga *Pseudokirchneriella subcapitata* has been widely used in ecological risk assessment, usually based on the impact of the toxicants in the alga growth. However, the physiological causes that lead algal growth inhibition are not completely understood.

This work pretends to elucidate the main targets of heavy metals toxicity in the alga *Pseudokirchneriella subcapitata* after a short (6 h) or a long (72 h) exposure time. For this purpose, the responses of *P. subcapitata* to three concentrations of Cd(II), Cr(VI), Cu(II) and Zn(II), corresponding approximately to 72h-EC_{10} and 72h-EC_{50} values and a high concentration (above 72h-EC_{90} values) on membrane integrity, esterase activity, mitochondrial function, photosynthetic activity, chlorophyll *a* (Chl *a*) content, intracellular accumulation of reactive oxygen species (ROS) and reduced glutathione (GSH) level were evaluated.

For a short-term exposure (6h), all metals studied (at all concentrations) induced a reduction of esterase activity. A loss of membrane integrity and a decrease of mitochondrial membrane potential in algal cells exposed to 72h-EC_{50} and >72h-EC_{90} concentrations of Cu(II) was also detected. Chl *a* autofluorescence was affected by the presence of Cr(VI) and Cu(II), which suggests the perturbation of photosynthesis.

A long-term (chronic) exposure of algal cells (72h) to Cd(II), Cr(VI) or Cu(II) at >72h-EC_{90} concentrations resulted in a loss of membrane integrity. For all metals tested, an inhibition of esterase activity, in a dose-dependent manner, was observed. Reduction of Chl *a* content, decrease of maximum quantum yield of photosystem II and modification of mitochondrial membrane potential was also verified. Cd(II), Cu(II) and Zn(II), at the highest concentrations tested, induced an increase of intracellular ROS and GSH content. The increase of GSH content might be a form of algal cells to redress the imbalance caused by the oxidative stress. However, the increase of GSH was not enough to protect the algal cells against the long-term exposure to oxidative stress.

In conclusion, the short- and long-term exposure of *P. subcapitata* cells to heavy metals had a negative impact on alga physiology and metabolism. Although a compromising of membrane integrity was observed for >72h-EC_{90} concentrations of Cu(II) and Cd(II), Cr(VI), after long exposure, cell membrane should not be the primary target of the metals action. The main targets of the heavy metals under study have an intracellular localization. The impairment of esterase activity combined with the reduction of Chl*a* content was related with the inhibition of growth caused by a prolonged exposure of Cr(VI) and Cu(II). In the case of Zn (II), in addition to these metabolic parameters, the damage of mitochondrial function was also associated with the growth inhibition. The identification of the targets of the heavy metals studied contributes to the elucidation of the mechanisms of action of these toxicants on the alga *P. subcapitata*.

Colour preservation of white wines using polyphenol compounds

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Gallic acid, caffeic acid and glutathione were used as additive for white wine preservation. Gallic acid or caffeic acid, in concentration of 60 mg/L and glutathione in concentration of 20 mg/L, were added in Vinho Verde white wine containing 35 mg/L of free SO_2 at bottling. For comparison white wine were bottled with 20 mg/L of free SO_2 and with 35 mg/L of free SO_2 (usual concentrations in wines), without gallic or caffeic acids or glutathione. Wine quality was evaluated in terms of sensory characteristic, color and aromatic compounds in the time of bottling and after 4 and 12 months of storage.
Sensory evaluation of the wines was made by a trained panel of 5 judges. The colour changes were assessed using CIELab method. Aromatic compounds in wine were quantified and identified, after liquid/liquid extraction using a gas chromatography coupled with mass spectrometry (GC-MS).
According to colour analysis, after 4 and 12 months, the wine with gallic acid was the one with better colour preservation and less oxidation, followed by the wine with caffeic acid. Moreover, the wine with gallic acid obtained the highest scores according the sensory evaluation. In terms of aromatic compounds all wines demonstrated a rich aromatic profile.
Present results indicate that gallic acid, caffeic acid and glutathione can improve sensory quality of white wine during storage and protect wine aroma volatiles.

Pathway for cyanotoxin valorization - Microscystin as case study

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The worldwide occurrence of hepatotoxic cyanobacterium *Microcystis aeruginosa* and the accumulation of its intracellular toxin microcystin, the most widespread cyanotoxin, have been commonly associated with water impairment causing several human deaths and various animal intoxication incidents. These findings led the World Health Organization and several national governments to establish guidelines and recommendation values for this toxin in water, which gave rise to an increasing demand for microcystin's analytical standards. These standards might be used either as laboratory standards for human and environmental risk assessment or as tools for molecular and cell biology studies. Also, recent research works highlighted the huge potential of cyanotoxins to be applied as anticancer/antitumor drugs or antimicrobial agents. However, the existing commercial microcystin solutions present prohibitive prices around 28000€/mg due to constraints found in up- and downstream processes.
Envisaging the need to decrease the production cost of such high added-value products the aim of our work was to i) evaluate the effect of environmental factors on *Microcystis aeruginosa* growth and toxin accumulation; ii) develop cultivation strategies to optimize cyanobacteria growth and maximize toxin productivity; iii) optimize downstream processing steps in order to obtain high yields of cyanotoxin.
Utilizing an innovative approach of combining and assessing the synergistic interactions of four different abiotic factors on growth kinetics and toxin production, it was possible to reach variations of approximately 2000-fold. Cultivation systems have shown to play a significant role on biomass productivity since the use of flat-panel photobioreactors resulted in similar maximum biomass concentrations in half of the time of the growth, when compared to bubble columns. The exposure of toxin-producer *M. aeruginosa* to extracts and filtrates of cultures of other microorganisms determined interesting effects on biomass growth. As example, extracts of non-toxic *M. aeruginosa* enhanced growth in 53% while extracts of *S. obliquus* had a negative impact decreasing growth in 19%. In biomass harvesting, despite all the four methods analysed presenting efficiencies above 90%, the addition of aluminium chloride has shown to be the fastest. Regarding cell disruption, amongst the methodologies studied, implementation of freeze-thaw cycles followed by sonication was found to be the best approach to promote intracellular organic matter release, resulting in approximately 100% of disruption efficiency. These results are a step forward in the path of implementing microcystin's industrial scale production in order to allow the development of innovative biotechnological products and approaches in distinct fields such as health and water quality.

Straighten curly hair with keratin peptides

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Chemical straightening curly human hair fibres involves the use of strong reducing agents at alkaline pHs. These treatments damage the hair fibre, reduce the cross-linking density and decrease hair's physico mechanical properties [1]. Human hair is made of keratin and the fixation of fibres shape involve the reduction and reformation of new disulphide bonds between keratin molecules. t is known that cysteine has been applied as a reducing agent for the substitution of environmentally harmful chemicals[2]. Here, we propose an alternative and green methodology using peptide sequences derived from human keratin genome. These peptide fragments have been designed by nature to interact with keratin and will penetrate on hair reducing and reforming the disulphide bonds at neutral pHs without the use of external harsh reducing agents. We tested 8 decapeptides which were selected from over 1235 decapeptides representing the all human genome of keratin and keratin associated proteins. All the peptides contain 2 or more cysteine residues in their composition and the 8 peptides were select based on their affinity for human hair keratin solutions. Here we found that 3 of the 8 selected peptides have high affinity towards hair keratin (measured as hair uptake) that it can re-shape disulphide bonds (as proven by MALDI-TOF/TOF) and change straighten hair. The proposed solutions presented here replace harsh reducing agents at alkaline pHs for peptide formulations acting at neutral pHs to change the shape of hair. These green solutions are therefore expected to have an high impact on haircare cosmetic industry with direct benefits for environment and humans (especially ethnic Africans).

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Size controlled protein nanoemulsions for cancer therapy

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Albumin nanoemulsions were produced by high pressure homogenization with a tri-block copolymer (Poloxamer 407), which presents a central hydrophobic chain of polyoxypropylene (PPO) and two identical lateral hydrophilic chains of polyethylene glycol (PEG). We observed a linear correlation between tri-block copolymer concentration and size – the use of 5 mg/mL of Poloxamer 407 yields nanoemulsions smaller than 100 nm. Molecular dynamics and fluorescent tagging of the tri-block copolymer highlight their mechanistic role on the size of emulsions. This novel method enables the fabrication of highly stable protein-based emulsions in the nano-size range, highly desirable for controlled drug delivery. Folic Acid (FA)-tagged protein nanoemulsions were shown to promote specific folate receptor (FR)-mediated targeting in FR positive cells. Carbon monoxide releasing molecule-2 (CORM-2) was incorporated in the oil phase of the initial formulation. FA-tagged nanoemulsions loaded with CORM-2 exhibited a considerable antitumor effect and an increased survival of BALB/c mice bearing subcutaneous A20 lymphoma tumors. The developed nanoemulsions also demonstrated to be well tolerated by these immunocompetent mice. The novel strategy presented here enables the construction of size controlled, functionalized protein-based nanoemulsions with excellent characteristics for active targeting in cancer therapy.

Multifunctional proteins for hair protection and coloring

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Hair is a complex protein-based fibre that plays a key role for the perception of human beauty. Human hair is composed mainly by proteins, lipids, water and pigments. The protein content is approximately 65 to 95%, and the major classes of hair proteins is composed by keratins. Hair's lightening is achieved by the degradation of the melanin pigment in a process designated by bleaching. This process causes a huge damage in the hair, proved by the decrease of it mechanical properties, shine and strength. [1]
There are already formulations composed by proteins which are capable to create suitable environments for healthy hair due to their water binding potential and amphoteric and buffering properties. Although these proteins can be used before or after the bleaching process to avoid hair damage, they do not have a role in the coloration of hair or in the properties of the hair colour. The main goal of the work was the bioproduction and application of multifunctional proteins for hair protection and coloration. These proteins simultaneously protect and colour the hair, without the need to use the traditional bleaching techniques that drastically damage hair. Crystallins, reflectins and chromogenic proteins were used in the design of the multifunctional proteins. Human γD-crystallin were used as restorative agents based on their ability to form thin coatings around hair fibres due to the presence of Greek key motif on its structure. [2] Reflectins and chromogenic proteins were used for the colouring of the hair fibres.
The multifunctional proteins will be used for the development of new hair cosmetic products with a high commercial potential.

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Chitosan-coated BSA nanoparticles for oral delivery

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Despite years of research, chronic pathologies, like cancer and chronic inflammatory diseases, are still in need of therapeutic approaches that allow easy administration, high compliance of the patient to the treatment and few or minor side effects. Engineered medicines, like surface-decorated nanoformulations, have the potential to accomplish all these important goals. However, oral administration of these formulations is a challenge due to the need to overcome the gastric harsh environment and be absorbed in the intestinal tract, reaching the blood flow as a whole functionalized particle.

This work aims to face this challenge using chitosan and/or poloxamer 407 as mucoadhesive and mucopenetrant polymers that will coat bovine serum albumin (BSA) nanospheres. Mucoadhesion increases the contact time of the particles with the intestinal epithelium, while mucopenetrance allows the progression of the particles through the intestinal mucosa, promoting their approach to the epithelial cells.

The nanospheres were produced by an emulsification method and then coated by incubation with polymers solution. The formulations were characterized by their size, zeta potential, morphology, and coating deposition in order to optimise the concentrations of the polymers.

Then, the nanoformulations were subjected to stability tests in simulated digestive fluids. The results obtained are promising since they showed diffusion of the coating polymers of the protein nanospheres to receptors fluids, liberating the protein nanospheres to be permeated throughout the intestinal mucosa. We detected differential profiles in terms of the size of the spheres coated with only one or with the two polymers and also in the amount of BSA that is released to the fluids, as measurement of spheres degradation. However, further improvements are being implemented in the analytic protocols.

Elemental biological tests were also performed. The formulations showed to be non-toxic to Caco-2 cells in the tested concentrations and preliminary results of *ex vivo* experiments with pig intestine showed the permeation of some material. However, it is necessary to develop new approaches to clarify these findings.

In conclusion, we believe that the use of chitosan and poloxamer 407 as coating polymers of nanospheres can mean a significant advance in the field of oral delivery and targeted-therapy and could have a high impact in the pharmaceutical industry.

Understanding the molecular basis behind hair morphology: development of new strategies to modulate hair from the follicle

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Understanding human hair biology and finding the genetic basis responsible for hair shape, colour and texture as well as for hair follicle (HF) aging, commonly perceived as hair graying will allow the development of new hair cosmetics able to modulate the levels of target genes and, ultimately, able to shape and colour hair according to our will. This is the main work goal of the BBRG people dedicated to hair research.

Scalp hair is an essential and defining element of our physical appearance with significant psychological and social impacts in our daily-life. Although HFs show a common morphology they give rise to shafts with an amazing natural variability of size, colour and shape that can be changed. However, common chemical hair styling processes are also known to induce changes in hair cuticle and cortex, damaging the fiber and in some extreme cases threatening human health [1]. The cosmetic industry has traditionally focused on the development of products or procedures to change hair fiber as it exits from the skin surface [1]. Due to the potential damage to the hair fiber, there is a huge interest in understanding the genetic basis associated with hair morphology, exploring whether hair appearance can be modified as the fiber is generated in the HF [2].

In the literature, some genes have already been associated with hair morphology and aging. Because an altered gene function is many times associated with a 'dose effect' on the protein product activity, and also due to the lack of public available information, we undertook two global high-throughput approaches to compare the levels of gene expression among Caucasian and African HFs, and among pigmented and grey HFs. Grasping complex mechanisms requires a global and parallel analysis of different cellular processes, often involving the interaction between different cell types; the microarray was the platform chosen to achieve a more integrated vision of the complex cellular events shape in the hair follicle. Based on the information available from the literature and on the team's unpublished results from the high-throughput gene transcript analysis, the work is now directed towards screening chemical libraries to find compounds able to alter the transcript levels and/or protein activities of highly interesting genes from the hair morphology point of view and to develop a targeted transfollicular delivery system able to specifically and efficiently deliver those compounds.

The promising results obtained so far will sustain new strategies for hair colour and shape modulation or even delaying rather than hiding the natural aging process by acting directly on the follicle. Innovation in haircare will be grounded in the most recent advances in HF biology and in follicle-targeted delivery for the development of new products that consumers can safely use at home.

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Hair Keratin Molecular Dynamics Studies

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The keratin is a key element of the hair, nails and skin in vertebrates. Understand the keratin features such its assembling in the mentioned structures, its interactions with some compounds or mechanical properties is of great interest in the fight against some diseases or in the development and optimization of cosmetic products.

Although molecular dynamics (MD) simulations provides unique information at molecular level in a dynamic way, there are only a few studies using this technique on the study of keratin. This is likely the result of the nonexistence of full length keratin crystallographic model. In the few published works the authors had to design and build the computational keratin model to perform the simulations of interest.

This work addresses some MD studies about hair keratin, from the physicochemical properties of the molecular models to the correlation of the simulations results with experimental data.

Our work on this field, with recently developed computational models of hair fibers, is also discussed. We built MD models able to reproduce in simulations some phenomena observed in experimental assays, providing important information at molecular level about the mechanisms that lead to the experimental results.

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Synergistic antimicrobial interaction between honey and phage against *Escherichia coli* biofilms

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Chronic wounds that take months, years or may even never heal present a major biological and financial problem on both individual patients and the broader health system. Chronic wounds afford a hostile environment of damaged tissues that allow bacterial proliferation and further wound colonization. Wound colonization by bacterial biofilms is one of the main obstacles of chronic wounds healing. Biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adhered to an inert or living surface. *Escherichia coli* is among the most common colonizers of infected wounds and it is a prolific biofilm former. Living in biofilm communities, cells are protected, become more difficult to control and eradicate, and less susceptible to antibiotic therapy. Due to the vast increase of antibiotic resistant bacteria, there is a renewed interest in pre-antibiotic therapies. Years before the discovery of modern antibiotics, bacteriophages (phages) that are bacterial viruses, and bee hive products such as honey were extensively used for their antimicrobial properties. Phages, are the natural bacterial enemies and have proven efficacy towards antibiotic-resistant bacteria, have self-replicating nature, do not interfere with the commensal flora and many studies acknowledge that phages can destroy, to varying extent, mono and mixed biofilm populations. Honey, on the other hand, has a broad spectrum antibacterial activity against bacteria and its high viscosity provides a protective barrier against infections being suitable for skin care, promoting the wound healing, tissue regeneration and anti-inflammatory process.

This work presents insights into the proceedings triggering *E. coli* biofilm control with phage, honey and their combination, achieved through standard antimicrobial activity assays, zeta potential and flow cytometry studies and further visual insights sought by SEM and TEM microscopy. Two Portuguese honeys (PF2 and U3) with different floral origin and an *E. coli* specific phage (EC3a), possessing depolymerase activity, were tested against 24 h and 48 h-old biofilms. Synergic and additive effects were perceived in some phage-honey experiments. Combined therapy prompted similar phenomena in biofilm cells, visualized by electron microscopy, as the individual treatments. Honey caused minor membrane perturbations to complete collapse and consequent discharge of cytoplasmic content, and phage completely destroyed cells leaving only vesicle-like structures and debris. Our experiments show that the addition of phage to low honey concentrations is advantageous, and that even 4-fold diluted honey combined with phage, presents no loss of antibacterial activity towards *E. coli*. Portuguese honeys possess excellent antibiofilm activity and may be potential alternative therapeutic agents in biofilm-related wound infection. Furthermore, to our knowledge this is the first study that assessed the impacts of phage-honey combinations in bacterial cells. The synergistic effect obtained was shown to be promising, since the antiviral effect of honey limits the emergence of phage resistant phenotypes and the use of a diluted honey solution is known to be advantageous, not only due to a potential lower cost of treatment, but also since a more liquid solution might be therapeutically more desirable as a topical rinsing solution maximizing the tolerability and practicality of the delivery technique. The pioneering combined delivery of phage and honey is thus a promising antimicrobial alternative towards *E. coli*.

Novel nanoparticle-based therapy to eradicate *Pseudomonas aeruginosa* infections in cystic fibrosis lungs: dual drug release by cubosomes

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Cystic fibrosis (CF) is a genetic disease that origins a defective chloride secretion resulting in the accumulation of thick and sticky sputum in lungs. The accumulated sputum is rich in nutrients being, thus, a good environment for microbial colonization. *Pseudomonas aeruginosa* have a significant prevalence among CF patients and its presence is highly associated with poor lung function and mortality of patients [1]. Numerous antibiotic strategies have been used varying in route of drug administration (systemic, oral, inhaled or route combination), classes of antibiotics and treatment duration in attempt to eradicate *P. aeruginosa* infections. Despite the long and aggressive antibiotic treatments using more than one antibiotic simultaneously, *P. aeruginosa* still persists causing chronic infections virtually impossible to eradicate [2]. Our recent data demonstrated that the failure of the current antibiotic treatments contributes to the emergence of multidrug resistant (MDR) *P. aeruginosa* compromising, thus, the second therapeutic line, which is quite alarming. According the same study, our results pointed out that antibiotic failure could be caused by the sub-optimal concentration that achieved bacteria. CF features such as extracellular DNA, mucin, pH, limited oxygen availability and biofilm formation can neutralize or diminish the concentration during antibiotic penetration into CF mesh.

The absence of new antibiotic molecules and the spread of MDR *P. aeruginosa* prompted us to search for innovative therapeutic strategies effective against *P. aeruginosa*. Nanoparticles (NP) exhibited exceptional properties for drug delivery with excellent pharmacokinetics profiles because it ensures deposition of the drug at the infection site with higher local drug availability using reduced dosages and avoiding systemic toxicity. NP such as liposomes, polymeric and lipid NPs have been explored for pulmonary delivery of antibiotics with promising results. The novelty of this study lays on the use of a NP poorly explored, the cubosomes, and novel synergic antimicrobial combinations, exploring anti-virulence agents and antimicrobial peptides with traditional antibiotics. Cubosomes can constitute a powerful and innovative delivery vehicle of antimicrobials for CF treatment due to the possibility of combining antimicrobials with different water solubilities that can display synergistic effects and combination of controllable release profiles.

To accomplish this purpose CEB and INL started working together. INL has been preparing the first set of cubosomes with selected antimicrobial agents, which will then be tested at CEB against *P. aeruginosa* bacteria grown in simulated CF environment.

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Disclosing the complexity involved in phage-biofilm interaction: the case study of a *Sep1virus* phage infecting *S. epidermidis*

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Staphylococcus epidermidis is a major causative agent of nosocomial infections, mainly associated with the use of indwelling devices, on which this bacterium forms structures known as biofilms. Due to biofilms high tolerance to antibiotics, virulent bacteriophages have been suggested as novel anti-biofilm therapeutic agents. In this study, we used the *S. epidermidis*-specific phage phiIBB-SEP1 (SEP1) [1] and evaluated its activity against biofilms. Despite its broad host spectrum and high activity against exponential phase cells, the same was not observed for cells encased in a biofilm structure. To understand the underlying factors impairing SEP1 inefficacy against biofilms, we tested this phage against distinct bacterial populations. Interestingly, SEP1 was able to infect late stationary-phase (dormant), persister and biofilm-released cells, suggesting that the inefficacy for biofilm control resulted from the biofilm structure. To demonstrate this hypothesis, SEP1 activity was tested against clusters of cells from scraped biofilms resulting in a 2 orders-of-magnitude reduction in the number of viable cells, after six hours of infection. Additionally, LIVE/DEAD staining allowed the observation that stationary-phase cells responded to phage addition, as determined by the increase in SYBR medium fluorescence intensity, which can be related with an increase on the cell metabolic activity.
These are promising results, since the rare feature presented by this phage of infecting cells with reduced metabolic activity allied with its ability to infect persister and biofilm-released cells, suggest its use as anti-biofilm agent when combined with enzymatic (dispersin B) or mechanic debridement (sharp).

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A bacteriophage-based platform for early diagnosis of Alzheimer's disease

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Alzheimer's disease (AD) is the most common neurodegenerative disease affecting a large proportion of the human population worldwide. One hallmark of AD is the increased deposition of plaques, which consist of amyloid-beta (AB) peptide, a key molecule to cause AD onset and progression. However, it is not AB immobilized in plaques, but in the still-soluble oligomeric/fibrillar form that impairs synaptic function and memory encoding. It is therefore important to develop tools that selectively target AB in oligomeric/fibrillar form, to diagnose and neutralize these detrimental AB-clusters during the early stages of the disease.

Homing peptides that selectively recognize AB-oligomers and fibrils have been described: AB30-39, reactive for AB fibrils and AB33-42, reactive to fibrils and oligomers [1]. However, these peptides are unable to cross the blood-brain barrier (BBB) by themselves. To overcome this limitation, viruses became a very interesting tool given their versatility to be modified through genetic or chemical manipulation. Bacteriophages (phages), are viruses that only infect bacteria (a major advantage in terms of safety when therapeutic use in humans is envisaged). M13KE is one of the most widely used phage which has been reported as capable to cross the BBB [2].

The present work describes the development of a phage-based system capable of diagnose AD at an early stage by shuttling amyloid-beta specific ligands across the BBB. M13 phages were genetically engineered with two peptide sequences to selectively recognize amyloid-beta oligomers in order to target and visualize amyloid-beta aggregates in the brain. Immunohistochemistry results successfully demonstrated that AB1-phages selectively target AB-protofibrils in brain slices from AD-model mice, but not in brain slices from age-matched wild-type littermate. In addition, control phage (carrying no AB-selective peptide) did not stain AD or WT tissue. Co-staining with both anti-M13 and 6E10 (an antibody reactive against all species of AB including plaques), confirm that AB1 phage target AB-protofibrils, but not plaques.

Future work will be devoted to test this system in AD-mouse and human models, first for diagnosis purposes at an early stage of the disease. Second, for therapeutic intervention, we will assess the inhibition of the oligomeric AB-mediated synaptic loss and memory impairment.

If successful, this approach will provide the neuroscience community with a faster, user-friendly and cheaper diagnostic/therapeutic tool for Alzheimer´s disease.

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Genomic analysis of *Acinetobacter baumannii* prophages reveals remarkable diversity and suggests profound impact on bacterial virulence and fitness

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Acinetobacter baumannii has been recently indicated by the World Health Organization (WHO) as the number one priority pathogen for research and development of new antibiotics. This is a direct consequence of the fast evolution of pathogenicity, and in particular of multidrug resistance, of this nosocomial pathogen.

While the development of new antibiotics is critical, understanding the mechanisms behind the crescent pathogenicity of this bacterium is equally relevant. Often, resistance and other virulence elements of pathogenic bacteria are contained on highly mobile pieces of DNA that can easily spread to other bacteria by a process of horizontal gene transfer (HGT). Among mediators of HGT we find bacteriophages (phages), viruses of bacteria thought to be the most abundant entities on Earth. When infecting a bacterial host, phages may follow a lytic path in which they replicate inside the bacteria and cause cell lysis for progeny release, or they may follow a lysogenic life cycle where they integrate the host genome and replicate in synchrony. Phages opting for the lysogenic life cycle are known as temperate phages, or prophages when integrated in the bacterial genome. Under certain stimuli prophages can excise from the host genome, entering the lytic cycle and resulting in cell death and release of phage progeny. During excision, a process of specialized transduction may occur, where parts of the bacterial genome adjacent to the prophages may be erroneously excised with the prophage genome and introduced with the virion into a new host. Often these pieces of DNA offer advantageous features to the bacterial host, as exemplified by the well-known prophage-encoded Shiga toxin of *Escherichia coli* O157:H7.

So here we question the contribution of prophages to the evolution of *A. baumannii* pathogenicity. We found prophages to be widely disseminated in 959 *A. baumannii* genomes, with a few also present in bacterial plasmids. Whole genome and proteome comparisons demonstrated a notable diversity of prophage sequences, with only a few small clusters of closer evolutionary relationships. Also remarkably, *A. baumannii* prophages encode for a multitude of putative virulence factors that may be implicated in the bacterium's capacity to colonize host niches, evade the host immune system, subsist in unfavorable environments, and tolerate antibiotics, including last resource agents as colistin.

Overall, our results point towards a significant contribution of prophages for the dissemination and evolution of pathogenicity in *A. baumannii*, and highlight the clinical relevance of these mediators of HGT.

The pathogenesis *of Staphylococcus epidermidis* biofilm-associated infections: the host and the pathogen perspective

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Staphylococcus epidermidis is a commensal bacterium that inhabits healthy human skin and mucosae. However, due to its capacity to attach to medical devices and form biofilms, *S. epidermidis* has emerged as one of the most common causes of healthcare-associated infections. Although biofilm formation on medical devices is classically associated with the development of chronic infections, the release of cells from the biofilm has been linked with the onset of acute infections, with bacteraemia as one of its major associated clinical manifestations. Despite the clinical relevance of biofilm disassembly, the interaction between biofilm-released cells and the host remains unclear. Hence, to better comprehend the pathogenesis of *S. epidermidis* biofilm-related infections, the interplay between *S. epidermidis* biofilm-released cells and the host immune system was investigated. The host immune response to the presence of biofilm-released cells was evaluated by analyzing the transcriptome of mouse splenocytes after 2 hours of the injection of the bacterium into the bloodstream. Data mining revealed that biofilm-released cells were particularly effective at activating inflammatory and antigen presenting cells and inducing cellular apoptosis. Moreover, these cells induced higher production of pro-inflammatory cytokines than biofilm or planktonic cells [1]. These results not only helped explaining the relapsing character of biofilm-originated infections but also raised important concerns regarding the use of compounds that cause biofilm disassembly as treatment strategy since biofilm-released cells can heighten the inflammatory response of the host, consequently augmenting disease severity. Finally, the response of biofilm-released cells to the presence of host factors was evaluated by analysing the transcriptome of the bacterium upon 2 hours of interaction with human blood. Data analysis showed that during interaction with human blood there was a dramatic alteration in the transcriptome of the bacterium, particularly in the transcription of genes encoding proteins involved in iron utilization, amino acids biosynthesis and biotin metabolism [2]. Iron and biotin are essential for prokaryotic central pathways being particularly important during infection constituting, therefore, potential targets for future studies aiming to develop strategies for the treatment of *S. epidermidis* biofilm-originated infections.

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The fate of adhering bacteria on antimicrobial surfaces: transcriptomic analysis of resistance associated genes and macrophage-mediated phagocytosis

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The growing number of biomaterial-associated infections (BAI) has led to the need of developing novel antibacterial coatings. In our previous work [1], an antimicrobial lipopeptide and an enzyme were successfully co-immobilized onto polydimethylsiloxane (PDMS) imparting it with antimicrobial properties. Although results were quite promising, it could not be overlooked that some bacteria managed to adhere to these coatings. Thus, to know the fate of these bacteria on the pathogenesis of BAI became imperative. This study aimed: i) to inspect the susceptibility profiles of these remaining bacteria to lower doses of vancomycin and to the antimicrobials used to functionalize PDMS; ii) to investigate bacterial removal and digestion by macrophages. Results showed that the cells that managed to adhere to both unmodified and modified surfaces were able to grow into a biofilm with metabolic active cells. Vancomycin, used at a MIC, had no effect on the metabolic activity of biofilms formed on these surfaces. Conversely, biofilms formed on functionalized PDMS were more susceptible to vancomycin, suggesting a synergistic effect. To evaluate the development of bacterial resistance towards compounds immobilized onto PDMS, cells of a 72 h – 96 h-old biofilms formed on the functionalized surfaces were continuously recovered and allowed to adhere to new modified surfaces, for a total of 30 days, using PDMS surfaces as a control. Afterwards, the MIC and MBC of the lipopeptide were determined against the recovered cells and the transcript levels of several genes involved in antimicrobial resistance and virulence mechanisms were assessed using quantitative RT-PCR. Results showed that cells adhered to functionalized PDMS exhibited identical susceptibility patterns to those of cells recovered from unmodified surfaces, suggesting no development of resistance. The transcriptomic analysis also did not disclose any resistance development since cells in contact with modified surfaces exhibited some genes involved in microbial resistance equally or less expressed, as compared to the ones recovered from control surfaces. Regarding phagocytosis, it was found that macrophages adhesion to unmodified PDMS tend to cluster which may compromise their mobility and subsequently their phagocytic activity. After PDMS functionalization, a higher number of adhered macrophages were found and more evenly distributed along the functionalized surfaces, helping thus better clearance of bacteria. In conclusion, bacteria found on these surfaces did not develop resistance towards the compounds used to functionalize PDMS, being even more susceptible to vancomycin and macrophages action, which strengthens the great potential of our coating strategy to fight BAI.

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A genotypic analysis of five *P. aeruginosa* strains after biofilm infection by phages targeting different cell surface receptors

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Antibiotic resistance constitutes currently one of the most serious threats to the global public health and it urgently requires new and effective solutions. Bacteriophages are bacterial viruses increasingly recognized as being good alternatives to the traditional antibiotic therapies [1]. In the present study, the efficacy of phages against *Pseudomonas aeruginosa* PAO1 biofilm and planktonic cell cultures was evaluated over the course of 48 hours. Although significant reductions in the number of viable cells were achieved for both cases, the high level of adaptability of the bacteria in response to the selective pressure caused by phage treatment resulted in the emergence of phage-resistant variants. However, very few studies have explored this phenomenon. Here, the emergence of phage-resistant variants was tracked during the phage infection experiments. Resistant bacterial variants appeared as early as 6 hours post-phage biofilm treatment, depending on the phage used and the respective bacterial receptors. It was also found that phage-resistant variants appeared later in planktonic cultures than in biofilms, in most cases. Given the interest in further understanding the genetic makeup of these variants and possible mutations accumulated, some were selected for further phenotypic and genotypic characterization Whole genome sequencing was performed on five *P. aeruginosa* PAO1 phage-resistant variants and all revealed to carry mutations in the *galU* gene, which is involved in lipopolysaccharide core biosynthesis, as well as in one *pil* gene involved in the synthesis of type IV pilus. The sequencing analysis further revealed that three of the *P. aeruginosa* PAO1 variants carry large deletions (> 200 kbp) in their genomes. Overall, this work demonstrates that *P. aeruginosa* biofilms can survive phage attack and develop phage-resistant variants that are well adapted to the biofilm mode of growth.

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Antimicrobial activity of phenolic extracts of *Eucalyptus globulus* and *Juglans regia* against dairy industry pathogens

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Bovine mastitis (BM) is the most expensive pathology for dairy industry and *Staphylococcus aureus* is amongst the most prevalent causative agents of this disease. Nowadays, it is known that *S. aureus* contaminated milk can enter the dairy production chain and be the origin of food contamination. Due to the poor efficacy of antibiotics and to the ability to form biofilms evidenced by this pathogen, BM has become increasingly difficult to control and to eradicate. Phenolic plant extracts are nowadays being evaluated since they are a rich source of bioactive molecules. Thus, in this work the antimicrobial activity of *E. globulus* and *J. regia* alone and in combination against *S. aureus* BM isolates was evaluated and compared with penicillin G (Pen G), an antibiotic commonly used in the treatment of this pathology. The evaluation of the cytotoxic potential of both extracts was also performed.

E. globulus evidenced a bacteriostatic and a significant inhibitory effect against *S. aureus* planktonic cells and biofilms, respectively. *J. regia* only had inhibitory activity against biofilms. No synergy was observed when combining plant extracts, but on biofilms a slightly more effective activity was obtained, than when testing extracts individually. As expected, Pen G at MIC presented an inhibitory effect only against planktonic cells of some strains and at 16×MIC a slight antimicrobial activity against biofilms. Although *E. globulus* and *J. regia* were cytotoxic to animal cells at MIC concentration, non-toxic concentrations of *E. globulus* presented inhibitory effect on planktonic cells. On the other hand, higher concentrations can be used aiming their application as disinfectants in dairy industries.

In conclusion, as therapeutic strategy, plant extracts demonstrated to have effects higher than or similar to Pen G for penicillin resistant strains. As preventive approach such as equipment and surface disinfection, plant extracts seem to have promissory antibacterial potential.

Novel strategies to fight *Candida* infections: natural honey

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The incidence of *Candida* infections (Candidosis) has increased remarkably in the last years, being attributed to the rise in the elderly population, the increasing number of immunocompromised patients, and the widespread use of indwelling medical devices. *Candida albicans* remains as the most prevalent species of these infections, but a clear rise in the proportion of non-*Candida albicans Candida* (NCAC) species has been noted [1]. These species have an inherent level of resistance to certain antifungal agents higher than *C. albicans* though their virulence factors are much less understood. A major virulence factor is the ability to adhere and to form biofilms in medical devices and host tissues, because of a higher tolerance to antifungal therapy. Consequently, there is an urgent need to develop new strategies to fight these infections. Natural compounds are attracting increased interest in this field, among which honey. Honey has long been used in traditional and complementary medicine because of its antibacterial, antifungal, antimycobacterial and antiviral activities, due to the acidity (low pH), osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors, increase in cytokine release, as well as, to immune modulating and anti-inflammatory properties [2].

So, the main aim of this work is to evaluate honey as a novel strategy to fight infections caused by NCAC species. Honey was tested alone and in combination with antifungal agents (fluconazole, chlorhexidine, nystatin) against *Candida* single and mixed (with *Pseudomonas aeruginosa*) biofilms. A first screening of the treatment with four *Candida* species (*C. albicans*, *C. tropicalis*, *C. papapsilosis*, *C. glabrata*) indicated higher efficiency of honey against *C. tropicalis*. For biofilms of this species, honey is capable of 2-log reductions after 24h of treatment. Furthermore, the combination of honey with nystatin and chlorhexidine reduces the antifungal dosage typically required in clinical settings by 50%, with a total biofilm reduction of 3-log with nystatin and 5-logs with chlorhexidine. In mixed biofilms of *C. tropicalis* and *P. aeruginosa,* honey at 50% or above reduces yeast and bacterial biofilm by 2-log and 4-log, respectively.

Altogether, our results highlight the great potential of honey as an alternative or complimentary strategy for the control of *Candida* infections.

The authors acknowledge funding and support from the Portuguese Foundation for Science and Technology, COMPETE 2020 and BioTecNorte operation.

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Inconsistencies in conventional culture *vs* molecular approaches: unveiling distinct dynamics in cystic fibrosis polymicrobial communities

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The complex cystic fibrosis (CF) microbiome has been inferred from molecular approaches, since culture-based methodologies are unreliable to detect polymicrobial biofilm-mediated infections. Still, CF microbiome profiling is primarily focused on identifying disease-causative agents, disregarding how CF ecosystems are influenced by external factors, dismissing implications for the disease and providing potential basis for clinical intervention. This study aimed at examining changes in microbial composition in CF polymicrobial (dual-/three-species) communities involving the CF-traditional pathogen (*Pseudomonas aeruginosa*, PA) and two less common species (*Inquilinus limosus*, IL, and *Dolosigranulum pigrum,* DP) challenged by different oxygen environments and following antibiotic intervention. Changes were evaluated through molecular methodologies (quantitative real-time polymerase chain reaction- q-PCR- and peptide nucleic acid probe-fluorescence *in situ* hybridization - PNA-FISH) and conventional culture techniques.

Results showed no significant differences in total cells detected by conventional and molecular techniques in biofilms developed under aerobic, microaerophilic or anaerobic conditions. However, estimation of PA, IL and DP cells within dual- and three-species biofilms was notably inconsistent, with q-PCR and PNA-FISH leading to higher microbial quantification in comparison with counts obtained by specific culture media. This variability enriched for PA+IL+DP biofilms, where both molecular techniques could detect up to 4-log more than cells estimated by culture. Once exposed to tobramycin, ciprofloxacin or aztreonam (antibiotics currently used to treat CF infections), viable but non culturable bacteria within three-species biofilms was clearly detected, with cells losing ability to grow on solid media but still being detected in greater numbers by PNA-FISH and q-PCR. This was particularly observed for all species in biofilms challenged by tobramycin (>5 ΔLog cells/cm^2, culture *vs* PNA-FISH), and for IL in biofilms exposed to ciprofloxacin (>5 ΔLog cells/cm^2, culture *vs* PNA-FISH or q-PCR) and to aztreonam (up to 3 ΔLog cells/cm^2, culture *vs* PNA-FISH). Intriguingly, q-PCR presented some shortcomings by showing lower sensitivity in DP detection (values limited to below 5 Log CFU/cm^2 under all circumstances), in comparison with conventional culture and even with PNA-FISH.

This study highlights incongruities in CF polymicrobial communities` dynamics depending on the methodology used to inspect the consortia. Generally, molecular methods afforded improved sensitivity in microbial detection/quantification within polymicrobial biofilms, compared with culture-based techniques. Aware of the requirements demanded by each technique (time-consuming, cost, easy-handling), the choice for the suitable approach should also rely on the community-residing microorganisms, aiming to give accurate comprehension of how polymicrobial infections respond to external stresses and contribute for disease progression.

Towards *Staphylococcus epidermidis* biofilm dormancy characterization

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Despite being a common colonizer of human skin and mucosae, *Staphylococcus epidermidis* has a strong ability to adhere to medical devices surfaces. Therefore, *S. epidermidis* is among the most common causative agents of biofilm-associated infections. Dormant bacteria may be found among the metabolic heterogeneous cells within biofilms. These cells present a low metabolic activity and contribute to tolerance to the host immune response and antibiotics, and relapsing infections.

We performed an integrative analysis of dormancy within *S. epidermidis* biofilms, using an *in vitro* model previously described by our group [1]. We conducted a whole-transcriptome and proteome analysis of biofilms with higher number of dormant bacteria. Our data highlighted that: translation process was decreased in dormancy; transcripts involved in oxidation-reduction processes and proteins involved in catalytic activity and GTPase activity were up-regulated in dormancy; genes involved in the pyruvate metabolism were upregulated in dormancy. Additionally, in order to assess if dormant *S. epidermidis* biofilms influence the reactivity to host immune system, we carried on an immunoproteomic analysis by evaluating the immunoreactivity pattern to human sera. Interestingly, CodY protein was only reactive to sera in biofilms with higher number of dormant cells and ClpP protein only reactive when dormancy was prevented. Curiously, the ClpP deletion was previously associated with reduced ability to form *S. epidermidis* biofilms and with reduced virulence in a rat model of biofilm-associated infection [2]. These results may also suggest that magnesium is important to prevent nutrient limitation in *in vitro S. epidermidis* biofilms. Lastly, the clinical impact of dormancy among *S. epidermidis* isolates was studied. It was observed that both clinical and commensal isolates were able to develop a dormant state. In parallel, the effect of three different antibiotics against *S. epidermidis* biofilms with induced and prevented dormancy was assessed. Interestingly, our results point out to the development of a viable but-non culturable state within biofilms exposed to tetracycline and rifampicin, although rifampicin was also causing bacteria death.

Overall, using a multiple combined strategy, it was demonstrated that this dormancy model has a particular transcriptomic and proteomic profile, a distinct interplay with host and a relevant clinical impact.

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New Bacterial Biorecognition Elements from Phage Origin

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Bacteria are responsible for numerous infections and diseases with severe implications in animal's and human's health and also causing great economic and production loss in the community. The use and misuse of broad-spectrum antibiotics has created a new problem: antimicrobial resistance which is currently the second leading cause of death worldwide and has exacerbated the number and severity of bacterial infections.

Proteins able to bind problematic bacteria present high potential in the battle against bacterial infections. They enable the development of fast and accurate bacterial detection methods allowing a sooner application of a correct and efficient therapeutic. On the other hand they can be fused to unspecific drugs to target specific bacteria increasing this way antibacterial activity and decreasing the likelihood of antimicrobial resistance.

Bacteriophages (phages) are virus that specifically infect bacteria and are innocuous to eukaryotic cells. Their specificity, which can go up to the strain level, means that they naturally present the necessary proteins/structures to recognize their bacterial hosts. Consequently, phages are a powerful source of bacterial cell binding proteins.

In this work, we have used bioinformatics to identify different proteins from phage origin with potential binding ability to cells of problematic pathogenic bacteria. The genes encoding those proteins were cloned in a frame with a green fluorescent protein (GFP) gene creating fusion proteins that were heterologous expressed in *E. coli*, purified and incubated with the target bacteria to enable decoration of the cells. After washing the unbound fusion proteins, observations at the fluorescence microscope enable to visualize the target cells and assess binding and specificity.

This functional analysis enable to identify proteins able to bind specifically to four problematic bacteria: i) *Paenibacillus larvae*, the responsible for the American foulbrood (AFB) disease in larvae bees that causes enormous economic losses in honey production; ii); *Salmonella*, the main foodborne pathogen worldwide; iii) *Staphylococcus aureus*, a major cause of bacteremia with high morbidity and mortality and responsible for food poisoning, representing an important social and economic burden worldwide and; iv) *Citrobacter koseri*, an opportunistic pathogen in a variety of human infections with serious impact in neonates.

These new bacterial biorecognition elements from phage origin will now be used to design new, fast and accurate diagnostic methods as well to design new tailor-made antimicrobials enabling the efficient control of these problematic pathogenic bacteria.

Specmine: an R package for metabolomics and spectral data analysis and mining

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In the last years, interest in the field of metabolomics has been growing, materialized by the advances in experimental techniques, growth in available data and novel biological applications. Techniques as Nuclear Magnetic Resonance, Gas or Liquid Chromatography, Mass Spectrometry, and Infrared, UV-visible or Raman spectroscopies have provided extensive datasets that can help in tasks as biological and biomedical discovery, biotechnology and drug development. However, as it happens with other omics data, the analysis of metabolomics datasets provides methodological and computational challenges. Indeed, from the available software tools, none addresses the multiplicity of existing techniques and data analysis tasks.

At the Biosystems group, in collaboration with the UFSC in Brazil, we have developed a novel R package, named *specmine*, which provides a set of methods for metabolomics data analysis, including data loading in different formats, pre-processing, metabolite identification, univariate and multivariate data analysis, machine learning, and feature selection. Importantly, the implemented methods provide adequate support for the analysis of data from diverse experimental techniques, integrating a large set of functions from several R packages in a powerful, yet simple to use environment.

The *specmine* package is available in the CRAN R repository to be installed by any interested user. It has already been used to address data analysis tasks in different scenarios, considering natural products analysis, food and environmental research, tackling challenges as the characterization of bees propolis, cassava post-harvest deterioration or carotenoid contents or the exposure of algae to pollutants as diesel or gasoline.

Currently, we are finalizing the development of *webspecmine*, an online data analysis tool, which aims to provide the features of *specmine* through a web-based interface, making it easier to use by non-informaticians. This site will also prvide tools to enable researchers to deposit their datasets and make them available for the community, boosting data sharing and data analysis reproducibility.

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Bioprocessing of olive mill and winery wastes by solid-state fermentation for simultaneously enzymes production and to increase their nutritional value

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One of fundamental challenges of olive oil and wine industries is to develop the new model of circular economy, that should lead to improve their competitiveness. In Portugal, these sectors generate more than 600,000 t and 300,000 t of wastes each year, respectively. Solid-state fermentation (SSF) is an eco-innovative process that allows to use the agro-industrial wastes as substrate to obtain value-added products as enzymes. In addition, it can increase the protein and lipids content by fungus growth, turning a poor nutritional quality materials into a fortified feed.

In this work, several enzymes have been produced using a filamentous fungus. A novel strain isolated from grapes, *A. ibericus*, was evaluated for the first time on SSF. This strain was able to produce lipases using olive pomace mixed with wheat bran (1:1), which achieved a maximum activity of 223 U/g of dry solid [1]. On the other hand, it has been proved the potential use of *A. ibericus* for lignocellulolytic enzymes as cellulases and xylanases. For that, olive mill wastes as olive pomace and winery wastes as exhausted grape marc and vineshoot trimmings were used as substrate. The mixtures of these wastes appeared to be positive for enzymes production compared to the separate use of wastes. Thus, this work proposes a novel strategy to create synergies between two industries that are often located in the same area. In addition, the SSF of these wastes were scale-up to tray type, packed-bed and pressured bioreactors, achieving similar results to those obtained in flask experiments.
After enzymes extraction, fermented wastes were characterized to evaluate their potential to be used as animal feed. The growth of fungus allowed to increase the protein content from low protein value (8%) to high value (17%) after 6 days of fermentation [2]. Because of this improvement in their nutritional value, the fermented waste was used as ingredient in fish diet. It was observed that fermented waste has potential as a feedstuff for European sea bass. In this way, all agro-industrial wastes were valued following the strategy of zero wastes, emulating sustainable natural cycle.
In line with strategy of simultaneously use of olive mill and winery wastes as low-costs substrates, in future works it will be evaluated the potential to obtain novel products by SSF as polyunsaturated fatty acids and antioxidant compounds. This will allow to develop the model of circular economy in these industries.

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Modelling interspecies interactions of syntrophic communities

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Microbial communities have gained special interest by reconstructing models for practical applications such as biorefineries, bioelectricity generation and bioremediation. However, studying these communities has proven to be difficult due to the absence of experimental protocols and computational tools like the ones available for single organism. Using the genome annotations of two mutualistic species, single species modelling protocols can be adapted to microbial communities and the resulting microbial community genome-scale model will allow identifying several types of possible metabolic interactions between the two microorganisms. Hence, the main objective of this project is to develop a microbial community genome-scale metabolic model of syntrophic communities of anaerobic bacteria and archaea, namely *Desulfovibrio vulgaris* Hildenborough and *Methanococcus maripaludis* S2. For this purpose, models for both species will be integrated integrated into a single consortium model.

Focusing on reconstructing *Desulfovibrio vulgaris* Hildenborough model, the *merlin* framework [1], developed in-house, will be used to perform all steps of the reconstruction process. Initially, this process starts by uploading the genome annotation from UniProt and into *merlin'*s database. *merlin* is a user-friendly Java application that performs the reconstruction of genome-scale metabolic models for any organism that has its genome sequenced. It performs the major steps of the reconstruction process, including the functional genomic annotation of the whole genome and subsequent collection of the portfolio of reactions. The reconstruction process involves identifying metabolic reactions, verifying the stoichiometry of the reactions, confirming the localization of the reactions, assembling the biomass equation and including it in the model together with other constrains. This process is constantly being refined by identifying and removing pathway dead ends, creating drains and enriching the composition of other metabolites, such as e-lipids, based on literature. Regarding the second organism, the *M. maripaludis* genome-scale metabolic model was previously published by another group [2].

Lastly, the *D. vulgaris* and *M. maripaludis* genome-scale metabolic models will be merged and *OptCom,* which is a comprehensive flux balance analysis framework for microbial communities, will be used to test and validate the above results from the organisms' metabolic interaction *in silico*. The availability of this framework besides *merlin* is a great addition to facilitate working process and obtain more accurate results.

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OptimModels: a framework for strain optimization using kinetic models

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Mathematical models have been applied to represent the complexity of cellular metabolism over the last decades. Two types of mathematical models are used for this purpose: kinetic and stoichiometric models. The reconstruction of kinetic metabolic models, however, is a complex task due to the difficulty in obtaining detailed information of enzyme kinetics.
Despite the early stage of their development, when compared with the stoichiometric metabolic models, kinetic models have already proven their capability to improve phenotype predictions and consequently more precise *in silico* strain design approaches [1].

One of the goals of Metabolic Engineering is the identification of genetic manipulations that will result in a microbial strain with a high yield/productivity of the desirable compound. This task can be reached using optimization algorithms based on metaheuristic approaches, such as Evolutionary Algorithms [2]. Although they do not guarantee the convergence to the best solution, these algorithms require relatively low computational time and provide a family of optimal or sub-optimal solutions that can be further inspected to select the most promising ones. Moreover, they allow the implementation of flexible objective functions and multi-objective design, and are easily parallelizable.

In this work, we developed a python package, named *optimModels*, which implements strain design methods based on Evolutionary Algorithms, using large-scale kinetic models as input.
Our case study uses two of the published kinetic metabolic models for *Escherichia coli*, proposed by Chassagnole and co-workers in 2002, and Jahan and co-workers in 2016.
We selected the maximization of serine and succinate production as objective functions and applied two different approaches, knockouts and under/over expression of enzymes, for strain design.

Preliminary results show that the framework presented here can be used for *in silico* strain design with kinetic metabolic models by finding the combination of genes to be knockout or/and their optimal levels of up/down-regulation.

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Yarrowia lipolytica as a cell factory to produce valuable compounds

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Yarrowia lipolytica, a strictly aerobic yeast, with GRAS status, has an intense secretory activity and can metabolize a wide range of substrates that guaranteed a spot as an interesting industrial organism. Recently, food supplements derived from this yeast were approved for commercialization, which is particularly important for broadening the range of possible applications of the compounds produced by *Y. lipolytica* [1]. Within "Bioprocess Development and Optimization" research team, *Y. lipolytica* has been used as a cell model and exploited applying the biorefinery concept for the production of enzymes, microbial lipids, aroma and organic acids, using low-cost renewable substrates.

A yeast-based integrated system was developed to valorize agro-industrial wastes, namely waste cooking oils and lard (used as a model of animal fat), by producing microbial lipids and lipase. The yeast oil content accumulated by *Y. lipolytica* from these inexpensive wastes was one of the higher ever reported for a non-genetically modified *Yarrowia* strain. Moreover, the simultaneous production of lipase may reduce the production cost of the microbial lipids and demonstrates that a biorefinery approach may be designed based on fat raw materials, allowing at the same time the reduction of fatty wastes surplus.

Lipids accumulation on *Y. lipolytica* may also occur from non-oily substrates such as glucose or glycerol that can also lead to citric acid (CA) production under nitrogen-limitation conditions. Crude glycerol, by-product from Biodiesel industry, has been used for CA production. Optimization of CA has been carried out by enhancing oxygen transfer rate at different types of bioreactors, such as STR, pressurized and airlift, as well as by mutagenesis strategies for strains improvement.

Yarrowia lipolytica is a model microorganism for lipids metabolism. It can produce several compounds from fatty acids catabolism, mainly aromatic compounds, such as lactones. This species is able to transform ricinoleic acid into γ-decalactone, a peach-like aroma compound of great importance for flavoring industry. The production of γ-decalactone has been intensively studied in order to better understand all process and optimize it. The role of lipases in substrate hydrolysis, the effect of substrate concentration, dissolved oxygen concentration and different fermentation strategies - batch and step-wise fed-batch – and bioreactor designs (STR and airlift) in the γ-decalactone production was investigated [2]. The characterization of γ-decalactone production by genetic modified strains at lab-scale bioreactor was also performed. *Yarrowia lipolytica* potential to produce other aromatic compounds, such as 2-phenylethanol, is been now explored.

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Eicosapentaenoic acid (EPA) production *in silico* by *Pythium irregulare*, as value-added product, using sugarcane vinasse as carbon source

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This study aims to assess *in silico* the production of Omega-3, mainly Eicosapentaenoic acid (EPA), as a value-added product, by *Pythium irregulare*, using sugarcane vinasse as carbon and nitrogen source. EPA is a 20-carbon polyunsaturated fatty acid with five cis double bonds, with its first double bond at the third carbon from the omega end, as an Omega-3 fatty acid. The Food and Agriculture Organization of the United Nations recommends ingestion up to 500 mg per day of EPA in the early years of life and for prevention of cardiovascular diseases, since it is not naturally synthesized in humans. EPA is an important dietary supplement, highly expensive ($600 – $4000 per kg of omega-3), with a promising market. The expected Omega-3 revenue is US$ 2.7 billion for 2020, with a Compound Annual Growth Rate (CAGR) of 17.5% (2014-2020), only in the pharmaceutical market.

Sugarcane vinasse is low cost carbon source produced in large amount. In 2019, it is estimated that 413 Brazilian sugarcane and bioethanol mills will produce more than 588 billion litters of vinasse, equivalent amount of the total of sewage produced by the world population (based on COD and Volume).

Pythium irregulare is an oleaginous Oomycete, a microscopic Stramenopile, able to accumulate large amounts of lipids, including Eicosapentaenoic acid (EPA). Previous studies have highlighted the promising production of EPA by *P. irregulare*, exploiting diverse low cost carbon sources, which include wastewaters as vinasse from corn-meal bioethanol production, glycerol and several sugars, but not from vinasse of sugarcane bioethanol plants. Moreover, there is still a lack of knowledge about its biosynthetic pathways.

For this propose, the genome-scale metabolic model will be constructed using Merlin, user-friendly software, in order to evaluate and validate *in silico* EPA and biomass production. Finally, OptFlux software will be applied in order to maximize *Pythium irregulare* Eicosapentaenoic acid and biomass production pathways using sugarcane vinasse as carbon and nitrogen source.

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Unmasking admixtures of extra virgin olive oils with olive oils containing sensory defects using a multi-sensor taste device

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Olive oils may be graded according to its overall physicochemical composition and sensorial attributes as extra-virgin (EVOOs), virgin (VOOs) or lampante olive oils (LOOs). Since olive oils are a food product quite prone to frauds, protection legal regulations have been implemented by the European Union Commission [1], which take into account the levels of chemical and physicochemical parameters (e.g., free acidity, peroxide value, UV extinction coefficients and alkyl esters content) as well as sensory evaluation (presence/absence of organoleptic defects and the positive fruity sensation) [2]. Unfortunately, the admixture of expensive olive oils with low quality oils aiming fraudulent economic revenue is still a common practice difficult to detect using the official methods.

In this work, it is evaluated, the capability of a lab-made potentiometric electronic tongue (Figure 1) for assessing blending levels of adulterated extra virgin olive oils with low quality olive oils for which an intense sensory defect could be perceived.

The preliminary results pointed out that the taste sensor device, together with chemometric tools (e.g., linear discriminant analysis coupled with simulated annealing variable selection algorithm) could be successfully applied to semi-quantitatively discriminate olive oils with a blend level lower or equal to 2.5% from those with higher adulteration percentages (correct classification rate greater than 82%±10% for cross-validation procedures). Thus, these results showed the practical potential of the E-tongue as a taste r device for the successful detection of EVOOs adulterated with LOOs containing organoleptic defects.

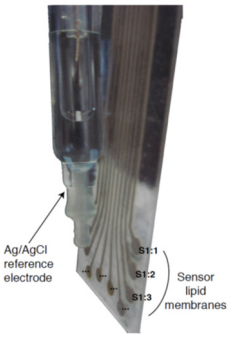

Figure 1: Lab-made E-tongue device.

Acknowledgments

This work was financially supported by Project POCI-01–0145-FEDER-006984 – Associate Laboratory LSRE-LCM, Project UID/QUI/00616/2013 – CQ-VR, Project UID/BIO/04469/2013 - CEB and Project UID/AGR/00690/2013 – CIMO all funded by FEDER - Fundo Europeu de Desenvolvimento Regional through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI) – and by national funds through FCT - Fundação para a Ciência e a Tecnologia, Portugal. Nuno Rodrigues thanks FCT, POPH-QREN and FSE for the Ph.D. Grant (SFRH/BD/104038/2014). Souheib Oueslati is grateful for the support of the Tunisian Ministry of Agriculture.

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Application of near infrared spectroscopy as a process analytical technology

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Food and pharmaceutical industries have a significant role related to global economy and hence such industries need to meet health and safety requirements, increasingly demanding environmental legislation, security, and sustainable production requirements. The determination of the chemical and physical properties and the detection and quantification of microbiological contaminations in these industries is a crucial step. The traditional methods used are laborious, time-consuming and/or requires expensive equipment and/or reagents. Near-infrared (NIR) spectroscopy has recently become increasingly important as a process analytical technique (PAT) due to its speed, low cost, and non-destructive characteristics. This spectroscopic technique is widely used for the analysis of different materials and its modes are transmittance, interactance, transflectance, diffuse transmittance and diffuse reflectance [1]. Many studies have been conducted to apply NIR to quality and safety measurements for food and agricultural materials, including fruits, vegetables, food and beverages as well as for pharmaceutical materials, namely for the detection, identification and quantification of bacteria in liquid suspensions and tablets.

It is difficult to analyze spectral data from complex functional groups of the materials directly because they contain many superposed overtones and combination bands. However, through the extraction of appropriate information from data sets using chemometric analysis - a multivariate statistical technique - it is possible to interpret spectroscopic data. This enables researchers to identify chemical and biological structures and determine the chemical/biological compound concentrations in the different materials. External environmental factors such as illumination and temperature play an important role when samples are measured. To eliminate undesirable effects from the external environment, pre-processing methods, such as averaging, centring, smoothing, standardization, normalization, transformation, multiplicative scatter correction (MSC), and standard vector normalization (SNV), must be conducted prior to chemometric analysis. The most frequently used multivariate statistical techniques are principal component analysis (PCA) and partial least-squares (PLS) [1].

At our lab several works are being carried out using NIR spectroscopy. The ability of this technique to detect and quantify bacterial contaminations in saline solutions (NaCl 0.9%) and pharmaceutical preparations was tested. Five different bacterial species usually responsible for microbial contamination of pharmaceutical products were used. The methodology was successfully tested in saline solutions (NaCl 0.9%) and validated in three different pharmaceutical preparations (contact lens solution, cough syrup and topic anti-inflammatory solution) [2]. The potential of NIR spectroscopy to determine the concentration of different compounds in white wines and cookies is also examined.

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Microbial evaluation of full-scale wastewater treatment plants by microscopy survey and chemometric analysis

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Activated sludge (AS) systems, are constituted by living organisms, mainly bacteria (floc-forming and filamentous), protozoa and metazoa. The later play an important role on grazing bacteria, and are known to be dependent on the working operational parameters (incoming effluent, dissolved oxygen, nitrification, hydraulic and sludge retention times, transient phenomena, etc.) and the system itself (conventional activated system – CAS, oxidation ditch – OD, trickling filter – TF, etc.). Floc-forming bacteria, such as aerobic heterotrophic, autotrophic (nitrifying and sulfur-oxidizing), denitrifying, sulfate-reducing and phosphate accumulating bacteria (PAO), are the main organisms responsible for pollution reduction in AS systems. On the other hand, the major role played by filamentous bacteria, rests on the establishment of the microbial aggregates structure, a key feature regarding sludge settling ability.

It is known that AS systems are prone to be affected by bulking, foaming, pin point flocs and dispersed growth occurrences, causing poor sludge settling abilities and affecting the wastewater treatment plant (WWTP) performance. In fact, an excess of filamentous bacteria, resulting in filamentous bulking or foaming events, or a shortage, resulting in dispersed growth or pinpoint flocs formation, leads to settling problems in the secondary clarifier. Furthermore, it is possible to establish a close correlation between the predominance of certain protozoa and metazoa taxa, several AS systems operational and settling problems occurrences.

In a previous study [1] the protozoa (crawling, free-swimming and sessile ciliates, testate amoeba and flagellates) and metazoa communities of three different WWTP types (one OD, four TF and three CAS reactors), were determined in terms of contents and relative abundance. The collected data was further processed by chemometric techniques, such as cross-correlation (CC), principal components (PCA) and decision trees (DT) analyses, allowing to successfully identify, and characterize, the different studied WWTP, and being able, thus, to help diagnosing and solving operational problems. In fact, the protozoa and metazoa based chemometric analyses allowed distinguishing the extended aeration systems (presenting high sessile and crawling ciliates contents), high sludge retention times systems (high metazoa contents), high nitrification abilities systems (highly predominant testate amoeba) and systems presenting transient phenomena (higher contents on flagellates and swimming ciliates).

On the other hand, another study [2] focused on the assessment of foaming events in full-scale WWTPs (one CAS and one OD) by surveying their biota and sludge characteristics. The biota community (protozoa, metazoa and filamentous bacteria) was monitored by microscopic observation and a new filamentous bacteria index was developed to quantify their occurrence. Furthermore, sludge structural characteristics (aggregated and filamentous biomass contents and aggregates size) were also determined (by quantitative image analysis) and further used. The obtained data was then processed by PCA, CC and DT to assess the foaming occurrences. It was found, in this study that such events were best assessed by the combined use of the relative abundance of testate amoeba and nocardioforms filamentous index, presenting an overall 92.9% success rate for foaming events.

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Bioelectrochemically-assisted recovery of valuable resources from urine

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Source separated urine is highly concentrated in nutrients and biodegradable compounds. This work explores the potential of combining nutrient recovery from urine with simultaneous energy production in bioelectrochemical systems (BES), under the FP7 project "ValueFromUrine".
Non-spontaneous phosphorus (P) recovery by struvite precipitation was analysed by adding three different magnesium (Mg) sources (magnesium chloride ($MgCl_2$), magnesium hydroxide ($Mg(OH)^2$) and magnesium oxide (MgO)). A statistical design of experiments was used to evaluate the effect of Mg:P molar ratio (1:1, 1.5:1 and 2:1) combined with stirring speed (30, 45 and 60 rpm) for each Mg source tested. MgO at 2:1 molar ratio and a stirring speed of 30 rpm allowed to achieve the highest P recovery efficiency (99 %) with struvite crystals size of 50 to 100 µm [1].
Urine obtained after P recovery, showed high concentration of biodegradable compounds being subsequently fed as substrate in a microbial fuel cell (MFC). Microbial acclimation to urine was performed in a MFC resulting in an anaerobic community successfully enriched in "urine-degrading" electroactive microorganisms. When compared to the control assay operated without preliminary microbial enrichment (81±9 mA m^{-2}), the acclimation method achieved significantly higher current density (455 mA m^{-2}) ($p<0.05$). *Tissierella* and *Paenibacillus* were the dominant genus identified in the adapted microbial community. *Tissierella* can convert creatinine to acetate, whereas bacterial species belonging to the *Paenibacillus* genus are known to function as exoelectrogens. *Corynebacterium* that comprise urea-hydrolysing bacteria was also detected in the developed biofilms.
The potential of urine obtained after P recovery for energy production in a microbial electrolysis cell (MEC) was evaluated using three different carbon anode materials, phenol-based (Keynol), cellulose-based (C-TEX) and polyacrilonitrile-based (PAN). The MECs were inoculated using the previously acclimated microbial community. MEC using C-Tex generated the highest current density (904 mA m^{-2}), which was almost 3-fold higher than the MEC with Keynol (338 mA m^{-2}) and almost 8-fold higher than the MEC with PAN (118 mA m^{-2}) at an anode potential of -0.300 V vs. Ag/AgCl. The higher percentage of bacteria belonging to *Lactobacillales* and *Enterobacteriales* identified on C-Tex, suggest that microbes assigned to these orders were the responsible for the higher current generation.
In conclusion, this work contributed with new insights on the degradation of organics in urine, changes in anodic bacterial community and the effect of anode materials, aiming at improving anode performance on BES operating with urine.

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Magnetic carbon composites as recycling electron shuttles on anaerobic biotransformations

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The unique properties of magnetic nanoparticles (MNP), such as high surface area, magnetic, sorption and catalytic characteristics, make them very versatile for many applications in different areas including environmental remediation, as catalysts, adsorbents, immobilising agents for microorganisms and enzymes, and as supports for biofilm growth and water disinfectants. In order to improve their stability and to introduce additional surface properties and functionalities, MNP can be coated with carbon materials (CM) due to their chemical stability, biocompatibility and possibility of tailoring their textural and surface chemical properties for specific applications [1]. We have previously proved that various CM, including activated carbon, carbon xerogels and carbon nanotubes (CNT), can be used as redox mediators (RM) in anaerobic biotransformation, accelerating the electron transfer and, consequently, the reduction rates of organic compounds [1,2]. The combination of CM with MNP offers the possibility of creating magnetic carbon composites with synergistic properties: the adsorptive and catalytic properties of both and the magnetic character of MNP, improving the material performance and rendering it easier to be retained and recovered, by applying a magnetic field.
A set of core(ferrite, FeO)-shell(carbon, C) composites, C@FeO, C@MnFeO, C@CoFeO, and CNT impregnated with 2% of Fe (CNT@2%Fe) were prepared and tested as RM in the biological reduction of the azo dye Acid Orange 10 (AO10). In the absence of RM, the AO10 decolourisation after 24 h of reaction was only 30% at a rate of 0.2 d^{-1}. In the presence of the core-shell composites, the extent of AO10 decolourisation was above 90% and rate improved circa 29-fold. With CNT@2%Fe, (98 ± 3%) of AO10 decolourisation was achieved at a 79-fold higher rate than the decolourisation in the absence of materials. Catalytic effect was also observed in abiotic reactors in the presence of composites, though at lower extent, likely due to the transfer of electrons from nanoscale iron to carbon and then to the dye. Owing to their magnetic character, the proposed materials were removed from the media and successfully applied in successive cycles. The high efficiency of proposed materials as RM, at low concentrations, and the possibility of easily recover with a magnet and being reused, make these materials preferential as compared with other soluble RM, which need to be continuously added and will be mixed with the treated solution. They are also advantageous than other non-magnetic insoluble materials to which costly techniques such as filtration need to be applied for recovery.

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Combining high-value biotechnological processes: from wastewaters bioremediation to bacterial bioenergy feedstock production

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The significant increase of global industrialization has been promoting the generation of large amounts of residues and wastewaters. In particular, oily wastewaters (contaminated with hydrocarbons) must be considered, since their disposal into the surrounding environments can represent a serious threat to several types of environmental resources. Simultaneously, the drastic depletion of fossil fuel resources demands for search of alternative feedstocks with environmental and economic advantages. Therefore, the production of bacterial lipids using inexpensive substrates, as wastes, has attracted much attention. Hydrocarbonoclastic bacteria are important players in bioremediation of hydrocarbon contaminated wastewaters with additional capacity for the accumulation of storage lipids such as triacylglycerols and wax esters [1, 2]. These compounds are relevant raw materials for biofuels and oleochemicals production. The present work aims at developing an indigenous hydrocarbonoclastic bacterial community able to produce storage lipids using a lubricant-rich wastewater and identify the influence of several cultivation parameters on storage compound accumulation. The obtained community was mainly composed by members of the genera *Rhodococcus*, *Acinetobacter* and *Pseudomonas* which are known for their ability to produce TAG, WE and PHA, respectively. In the applied conditions, the enriched community was able to fully degrade short chain hydrocarbons, while longer chain hydrocarbons were also degraded, but at a lower extent. By applying a five-level-three factor central composite circumscribed design based on surface response methodology it was found that nitrogen concentration and the interaction between carbon and nitrogen concentrations positively influenced neutral lipids production. Neutral lipids produced were essentially triacylglycerol (TAG) (33 % cdw), presenting a highly diversity of chemical structures composed by a narrow range of fatty acids. Therefore, the obtained mixed microbial community enriched in hydrocarbonoclastic and storage compound accumulating bacteria can be an effective inoculum to establish a more cost-effectively and ecofriendly biotechnological process combining valuable compounds production and treatment of hydrocarbon contaminated wastewater.

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Optimizing lab-scale wastewater treatment reactors operation for enhanced assays

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Wastewater treatment plants (WWTP) comprise a complex set of sequenced operations that ensure the safe discharge of water, previously contaminated by anthropogenic activities, into the environment. Roughly, these operations are divided in: preliminary treatment, primary treatment and secondary treatment. The secondary treatment is the most critical operation, encompassing a feeble equilibrium between physicochemical conditions and biological processes. It commonly consists in an aeration tank and a clarifier [1].

The microbial community present in the aeration tank is responsible for metabolizing most of the influent nutrient load. Pure oxygen, or air, is injected in this process to guarantee an adequate concentration of dissolved oxygen, in order to promote a rapid aerobic metabolism. Simultaneously preventing anoxic conditions, which denote a slower nutrient consumption and the generation of foul substances. Worldwide, the most commonly used microbial community for this process in the WWTP is activated sludge. Consisting of a highly complex community comprising bacteria, fungi, small protozoa and protozoa, the activated sludge "healthiness" is a critical factor for the efficiency of the wastewater treatment process. In addition, "healthy" activated sludge also possess a key physicochemical property for the downstream process of the aeration tank, namely, flocculation. In the clarifier the activated sludge flocs will sediment by the sole action of gravity, thus preventing a high microbial load in the effluent [2].

As a critical step of WWTP, the secondary treatment process must be thoroughly analysed. This work envisages the optimization of two laboratory scale reactors to accurately mimic the physicochemical and biological parameters regularly observed in a full scale WWTP. The reactors of approximately 4 L, each comprising an aeration tank a clarifier, and were feed with a real influent collected form a municipal WWTP. The physicochemical and biological parameters analysed include: pH, sludge volume index (SVI), total suspended solids (TSS), volatile suspended solids (VSS), fixed suspended solids (FSS), dissolved oxygen (dO_2), food to microorganism ratio (F/M), solid retention time (SRT), and characterization of the bacteria community through Gram and Neisser coloration. Moreover, the removal efficiency of chemical oxygen demand, biological oxygen demand, total nitrogen, total phosphorous and ammonium where also determined.

After optimization the reactors exhibited parameters equivalent to WWTP. Therefore, the reactors are now ready for the accurate characterization of tailored made influents. Particularly, influents loaded with toxics, biocides, nanomaterials or dominant bacterial species, among other factors, in order to predict, and solve operational problems in WWTP.

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Use of biocides in the control of filamentous bulking in activated-sludge

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Filamentous bulking can be controlled by specific and/or non-specific methods. Specific methods intend to recognize and resolve the major causes of filamentous bacterial proliferation and are preferred because they are selective for the target microorganism and cause limited damage to the remaining biomass. In what concerns non-specific methods, chlorination was one of the first methods to be used to control filamentous bulking and is still used, but its action is only temporary and tend to damage floc-forming bacteria, leading to floc and process breakdown [1]. The use of alternative biocides has attracted the attention of wastewater treatment technicians and researchers for the potential of its use in the control of filamentous overgrowth (ability to induce filamentous cell lysis) and is presently one of the most commonly used methods for the control of filamentous bulking under critical conditions [2].

Triclosan, cetyltrimethyl ammonium bromide (CTAB) and glutaraldehyde were used in a series of experiments to test its usefulness in the optimization of the performance of activated-sludge processes, with particular interest in the bulking filamentous overgrowth process. In a first set of assays, the referred biocides were tested in pure cultures of specific filamentous gram positive and gram negative bacteria, *Nocardia amarae* and *Sphaerotilus natans* respectively, through *in vitro* assessment methods: XTT reduction assay and live/dead viability assay (using epifluorescence microscopy and cytometry). For both filamentous bacteria, all the concentrations tested, the toxicity was found to be almost complete and the efficacy of the tested biocides varied between strains. The results showed a dose-dependent effect on bacterial viability, but a different mechanism of action for all biocides. In a second set of assays, the action of these biocides was studied in activated-sludge batch assays to assess the action in the community and in the overall performance of the treatment systems.

Results show potential in the use of these compounds in the cases of critical overgrowth of filamentous bacteria, but their effects on the protozoa and little metazoan as well as in the overall performance of the WWTP are non-negligible. The study also proved that the use of low concentrations of these compounds are not of significant interest.

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Eco-friendly process for the removal of Pb (II), Ni (II) and Zn (II) using different adsorbents: sepiolite and modified cedar

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Heavy metal contamination in groundwater and sediments is one of the most relevant threats to environmental quality and human health. Their presence in the aquatic environment has attracted global attention due to their toxicity, persistence in nature, non-biodegradability and ability to bio-accumulate in food chains. The traditional treatment methods for their removal have been used but chemical methods are often restricted due to the technical or economic restraints. Various biomaterials have been used to entrap those ions from water and wastewater such as clays, zeolites, industrial and/or agriculture wastes, becoming a good alternative to industrial wastewater treatment [1, 2].

The capacity of natural sepiolite (clay) and modified cedar (wood) sawdust for heavy metals adsorption, lead (Pb), nickel (Ni) and zinc (Zn), has been assessed using a batch method. Natural sepiolite was supplied by TOLSA and cedar was obtained from Morocco. Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) was used to characterize the morphology and chemical composition of both adsorbents. Cedar was subjected to an alkaline chemical pre-activation with of 0.2 M solution of potassium hydroxide, as suggested by literature.

The adsorption capacity of these two adsorbents was investigated at 0.1 g adsorbent dose (maximum uptake dose achieved in previous assays in this laboratory). Sorption assays were carried out in batch system at room temperature (25ºC±1ºC) and 160 rpm, for 24 h, with 200 mL of Pb (II), Ni (II), and Zn (II) solution with an initial concentration of 60 mg/L for each metal. Samples were analyzed for heavy metals concentration by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

The experiments demonstrated that Pb (II) was adsorbed more efficiently than Ni (II) and Zn (II) by both tested adsorbents. The maximum uptake was reached for modified cedar with 24.45 mg/L for Pb (II). For Ni (II) and Zn (II) the best adsorbent was sepiolite, with an uptake value of 7.04 mg/L and 8.03 mg/L, respectively.

The conclusion of this study is that both adsorbent can efficiently adsorb cationic species, which may have an interesting application such as the removal of those species from polluted waters, using a low cost and environmental friendly process.

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Untangling the role of facultative bacteria in LCFA conversion to methane

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Palmitate accumulation is frequently reported in continuous methanogenic bioreactors fed with lipid-rich wastewater, and facultative bacteria were suggested to be involved in this conversion. In this work, the possible effects of limited oxygen conditions in triggering palmitate formation from oleate were studied. Two bioreactors were operated in parallel, one under strict anaerobic conditions (AnR) and the other with the feeding tank open to the air (FR). Palmitate was the main LCFA in both reactors, but it reached approximately 7 times higher concentrations in the FR than in the AnR (16 and 2 $g \cdot L^{-1}$ in COD, respectively). Moreover, oleate was more abundant in the AnR than in the FR, presenting concentrations of 1.3 and 0.5 $g \cdot L^{-1}$, and oxidation-reduction potential values (ORP) of -366 mV and -255 mV were measured. Batch incubations of samples collected from the reactors showed that methanogens were completely inhibited in the AnR, while the FR sludge exhibited methanogenic activity, possibly due to the lower toxicity of palmitate when comparing with oleate. In a second experiment, hydraulic pressure was used to promote a selective washout of the microorganisms that did not perform oleate conversion to palmitate. A continuous stirred tank reactor (CSTR) and a plug flow reactor (PFR) were assembled in series and were fed with oleate from a tank open to the air. Biological activity occurred mainly in the biofilm, where palmitate accounted for up to 82 % of the LCFA and Pseudomonas was the predominant genus (42-58 % relative abundance), which highlights the important role of this genus in oleate to palmitate bioconversion. From the PFR biofilm, two different Pseudomonas sp. were isolated and further tested. These isolates were able to degrade oleate with oxygen as electron acceptor, but not in anaerobic conditions, and palmitate formation was not observed. Therefore, the formation of a biofilm and/or the presence of other microbial partners in a complex microbial community appear to be necessary conditions. All these results show that the presence of vestigial amounts of oxygen, directly related with the activity of Pseudomonas and higher redox potentials, are imperative for oleate conversion to methane.

Coupling a bioelectrochemical cell with a redox flow battery for sustainable energy production and storage

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Bioelectrochemical systems (BES) are devices capable to convert chemical energy into electricity through the degradation of different organic compounds using electroactive bacteria as biocatalyst. The ability of microorganisms to form biofilms in electrode surfaces allows the transport of electrons, resultant from the oxidation of carbon sources, to an terminal electron acceptor [1].

Redox flow batteries (RFB) are electrochemical systems applied in the conversion and storage of chemical energy in electricity. Redox chemical species (in soluble form) are the main responsible for the energy storage [2]. Quinones are electroactive molecules applied in RFB because of their chemical and physical properties.

The aim of this work is to develop an innovative technology to generate and storage the energy resultant from BES. The strategy outlined is coupling a BES with a RFB that present potential to combine bioenergy production and storage in a microbially charged redox flow battery.

Firstly, a BES was studied with *Geobacter sulfurreducens* as biocatalyst to convert a quinone (2,6-anthraquinone) in its respective reduced form, acetate being the carbon source used. The BES presented current intensities around 500 $mA.m^{-2}$ and power densities around 2 Wm^{-2}. The reduction was assessed visually by a typical colour change (from yellow to dark red) and by cyclic voltammetry. Simultaneously, as a control, the 2,6-anthraquinone was electrochemically reduced applying and controlling the cathode potential where the reduction was also observed by colour change and by cyclic voltammetry.

In an RFB (25 cm^2), the quinone bioreduced in the BES and electrochemically reduced in the electrochemical cell were studied using potassium hexacyanoferrate as the second redox chemical species for discharging/charging cycles, with a constant current density of 0.2 $mA.cm^{-2}$, where coulombic, voltage and energy efficiencies were observed, as the proof of concept of the microbially charged redox flow battery.

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Valorization of organic wastes through anaerobic digestion processes

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Increasing demand for sustainable development has stimulated political interest in measures to decrease pollution and greenhouse gas production by human activities. The greatest technological challenge for human society today is the replacement of fossil fuels by energy sources that are renewable and carbon neutral. One way to meet this challenge is trough biological processes, which has the potential to generate large flows of renewable energy, useful for decentralized systems. Wastewaters, sludges, residues, and other 'wastes of today' must be viewed as resources, within the concept of "waste-to-energy".

Anaerobic digestion (AD) is one of the answers to sustainable development since it reduces carbon emissions, provides clean fertilizers, and generates a green energy carrier (biogas), while concomitantly waste(water) treatment is performed. AD strategies have been implementing in order to maximize the energetic and economic value of recalcitrant wastes.

Harvesting residues, like shrub, could result in an average potential energy supply of 4.6 $EJ \cdot yr^{-1}$, only in Europe. Macroalgae, which do not compete with food crops for arable land and irrigation water, has shown promising results in terms of methane production. The co-digestion of *Gracilaria vericulophylla* (481 $L \cdot kg^{-1}$ (volatile solids – VS)) with sewage sludge and glycerol increased the methane production in 26 %. *Sargassum* sp. produced 91 L of hydrogen and 541 L of methane per kg (VS), yielding a theoretical potential energy supply of 600 EJ yr^{-1}, from the potential ocean area available for macroalgae production. Moreover, food-processing industrial waste(water) showed potential for methane production, i.e. AD of brewery waste can result in an energy production of 360 $PJ \cdot yr^{-1}$ worldwide. AD is thus a promising environmentally feasible alternative to create renewable energy.

Development of hemostatic materials made of electrochemically oxidized bacterial cellulose

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Cellulose is the most abundant polysaccharide in nature, being the main constituent of plant cell walls. It can undergo structural modification by oxidative methods, making it absorbable when implanted in the organism, contrarily to what happens with the non-oxidized cellulose. This way it can be used as raw material in medical devices, such as absorbable hemostatic materials and as a barrier to prevent post-operative adhesions. Cellulose can also be produced by bacteria (mainly from the species *Glucanocetobacter xylinus*), being this way known as bacterial cellulose (BC).
With the increasing use of the hemostatic materials based on oxidized cellulose in surgical procedures, there has also been an increase in the number of case studies that describe post-operative complications associated with the use of these materials. BC has improved characteristics and unique properties compared with polysaccharide derived from plants, namely a higher biocompatibility. It has therefore been the subject of increased research over the past years allowing its application in various fields, especially in biomedical applications [1].
This project aims to develop a hemostatic material to reduce post-operative complications, based on the oxidized BC, using electrochemical oxidation methods [2]. These methods are based on the stable nitroxyl radicals commercially available.
The oxidation of BC was investigated in aqueous medium using 2,2,6,6-Tetramethyl-1- piperidinyl-oxy (TEMPO) as redox mediator. TEMPO is a representative radical of the reagents used for these processes, occurring in a highly selective oxidation of C6 primary hydroxyl to carboxylic groups, which was verified by ^{13}C-NMR. After oxidation, samples were also analysed by ATR-FTIR technique indicating the successful oxidation of the hydroxyl groups. The degradation of the CB membranes is being studied and, to verify the hemostatic behaviour of the modified membranes some preliminary assays, namely whole blood coagulation tests, are ongoing.
One improvement in this field is the anodic regeneration of oxidizing species rather than using co-oxidants, considering a cleaner approach, which will highlight this project.

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Development of bacterial cellulose wound dressings with controlled delivery of vitamin D_3

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Wounds, in particular traumatic (e.g. burns) and chronic ones, are a major cause of morbidity and impaired life quality. They often result in long hospitalization stays, taking up substantial health resources in developed countries. This proposal aims at developing a safe, easy-to-use and non-expensive approach to efficiently address this problem, by attaining faster and proper wound healing.
Recent studies showed that an antimicrobial peptide (AMP), LLKKK18, released from conjugates with dextrin embedded in a Carbopol hydrogel significantly improved burn wound healing. In addition to antimicrobial activity, this peptide stimulates vascularization, thus supporting a faster healing and tissue regeneration[1]. As such, one can hypothesize that a hydrogel comprising drugs that stimulate the expression of LL37 will improve wound healing while keeping the wound area infection-free.
This work comprised the approach towards the development of a novel bacterial nanocellulose (BNC) dressing. BNC, already used clinically for the treatment of burn wounds due to the unique properties like high water holding capacity, high crystallinity, ultrafine fiber network, high resistance, high moldability and biocompatibility[2]. In this work BNC will be used as drug carriers for the controlled release of drugs, namely of vitamin D_3, an inducer of an endogenous expression of AMP LL37, known for accelerating the wound healing process, and as a protective barrier against exogenous agents (dust, microorganism) that can impair wound healing.
Since vitamin D_3 is poorly water soluble, and thus not easily incorporated in the highly hydrophilic environment of the BNC membrane, vitamin D_3 was loaded in to a newly developed hyaluronic acid (HA)-based amphiphilic nanogel and then incorporated in different types of BNC membranes. Such, nanogel was attained by conjugating a hydrophobic molecule to the HA chain. In aqueous environments, it self-assembles in nanosized structures with a hydrophilic shell and a hydrophobic core, able to incorporate hydrophobic molecules.
The new HA nanogel was successfully produced with a degree of substitution of 10.8±0.9% (out of 15 %, the maximum possible). Several vitamin D_3 concentrations were loaded with high stability into the nanogel (VitD_3-HA), successfully achieving an encapsulation efficiency between 70-91%, with better results at small drug concentrations. Two wet BNC membranes, with different initial thickness were evaluated in terms of swelling and vitamin D_3 *in vitro* release rate. Wet membranes with lower thickness revealed the best swelling results with a maximum water absorption after 8 h. Vitamin D_3 was released from both wet BNC membranes gradually, starting 2 h after the initial contact with the release medium. The thinner BNC membranes had a faster release profile than the thicker membranes. The HA conjugate and vitamin D_3 were tested for cytotoxicity, revealing their safety for *in vivo* applications.
These study revealed a great potential of BNC as natural drug-delivery system of vitamin D_3, but further research and development is necessary to explore its full potential on wound treatment still has to be confirmed *in vivo*.

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Delivery of Nanogel Formulations with Antimicrobial Peptides for the Treatment of Mycobacteriosis

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Mycobacterium tuberculosis is the human pathogen that causes Tuberculosis (TB). In 2015, 10.4 million TB cases and 1.8 million deaths were reported, placing this disease alongside HIV/AIDS as the deadliest infectious diseases. Current treatments rely in the administration of a cocktail of four first-line antibiotics during 6 months and, in the worst case scenario, a long-lasting treatment (24 months) with second-line drugs. The overuse or misuse of antimicrobial agents decreases the success of treatments and increases emergence of Multi-drug resistant (MDR) strains. Therefore, the development of new strategies for TB therapy is urgently needed. In this scope, antimicrobial peptides (AMPs) arise as promising candidates for TB treatment since they present high spectrum of antimicrobial activity, high efficacy at low concentrations and low propensity for bacterial resistance. Nevertheless, the low capacity of AMPs to reach the infected site and the use of high concentrations to overcome this problem limits its clinical application - this can be circumvented using a drug delivery system [1].
Recently, in a versatile, easy and reproducible manner, we developed a promising delivery system by grafting a hydrophobic molecule to Hyaluronic acid (HA). The amphiphilic conjugate self-assembles in aqueous environment, allowing the entrapment of bioactive molecules. We showed that LLKKK18-loaded nanogels cause no BMMΦ death and no significant effect in terms of the number of apoptotic cells, regardless the treatment or whether the BMMΦ were or not infected with *M. avium*. Additionally, *in vitro* incubation of macrophages with LLKKK18-loaded nanogel reduced the intracellular levels of both *M. avium* and *M. tuberculosis*. Most importantly, the capacity of macrophages to internalize HA was confirmed by *in vivo* results obtained in a model of TB infection, where just a few drug administrations over a short period yielded a promising 1.2-log reduction of the microbial burden [2].
In future work we intend to optimize the nanogel formulations, by promoting the encapsulation of LLKKK18 or LLKKK18/antimicrobial drugs in the nanocarriers, and unravel the *in vitro* immunomodulatory and metabolic effect promoted by the formulations in different cell models of mice and human. Since thorough optimization of the formulations and process will be carried out, the *in vivo* efficacy of an aerosol delivery of the formulations to infected mice will be assessed using the inEXPOSE inhalation system during one month. The susceptibility of MDR mycobacteria strains, and the eventual resistance mechanisms, that arise upon long *in vitro* treatments with LLKKK18 will also be studied.

References

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Bacterial cellulose as a novel stabilizer and texturizer for cosmetic and food applications

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Bacterial nanocellulose (BNC) is a sophisticated material produced biotechnologically by different microorganisms, but most efficiently by acetic acid bacteria from the genera *Gluconacetobacter*. While chemically identical to plant cellulose, BNC is chemically pure. Each BNC nanofiber is a bundle of cellulose nanofibrils. Due to their nano-size, these aggregates of extended cellulose chains have a rather large surface area. The unique properties of BNC account for an extraordinary physico-chemical and mechanical behaviour.

For industrial applications, hydrocolloidal microcrystalline cellulose from vegetable sources is widely used to regulate the texture, rheology, stability and organoleptic properties of the formulations [1]. Several studies are being carried out to investigate the technological role of BNC. Preliminary results already showed that BNC is technically superior to these vegetable celluloses, and can outperform plant celluloses in several applications within the food industry. As a novel hydrocolloid, BNC presents important features such as the stabilization of heterogeneous systems (air-liquid, solid-liquid and liquid-liquid): it is able to stabilize aerogels, increasing the incorporation of air in the liquid matrix (overrun), so it can be used as an additive in ice cream, smoothies and whipped cream; it can stabilize solid particles in a liquid matrix (e.g. cocoa particles in chocolate milk); BNC also stabilizes of oil-in-water emulsions, in spoonable and pourable dressings, without the need to add any other emulsifying agents.

Likewise, the stabilizing/thickening properties of BNC offer a similarly huge potential for application in the field of cosmetics. Regarding these exceptional functional properties, the effect of BNC was also assessed in a generic cosmetic cream (oil in water emulsion with other basic cosmetic ingredients), and compared to the effect of other commonly used additives in cosmetic formulations [2]. BNC can be used to stabilize active compound particles and oil droplets, as well as improving texture and other sensorial properties. Moreover, it may allow to reformulate the percentage of surfactants used on a liquid matrix without changing the rheological properties.

The incorporation of BNC in replacement of other polymers in food and cosmetics may bring environmental and economic advantages and reduce the use of plant cellulose and their (synthetic) derivatives in the above-mentioned applications.

References

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