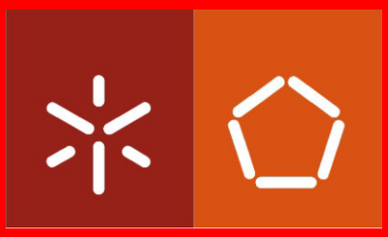

**Universidade do Minho**
Escola de Engenharia

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# Development and characterization of novel edible films with antimicrobial properties for food packaging

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Doutoramento em Engenharia Química e Biológica

Trabalho efetuado sob a orientação do
**Professor Doutor Fernando Octávio de Queirós Dourado**
**e da**
**Professora Doutora Lígia Raquel Marona Rodrigues**

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**Título da tese**

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Universidade do Minho, 31 de Outubro de 2014

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# DECLARAÇÃO DE INTEGRIDADE

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**Development and characterization of novel edible films with antimicrobial properties for food packaging**

# Abstract

Active packaging is an increasingly reliable technology for assuring the safety and maintenance/improvement of the organoleptic properties of the enclosed food products. Bio-based materials with improved characteristics were herein assembled through a simple and effective methodology to produce affordable edible antibacterial films. Bacterial cellulose (BC) and electrospun fish gelatine (FG) were used as film structures. BC possess noteworthy properties for food casing, namely high toughness. FG is a protein source that has not been much explored as film substrate. Both BC and electrospun FG were functionalized with bovine lactoferrin (bLF), a recognized broad spectrum bactericidal glycoprotein. Thin (approx. 25.75 µm) BC films were prepared form two sources i. static culture fermentation of *Gluconacetobacter xylinus* ATCC 53582 and ii. commercial BC product (*nata de coco*). BC films were functionalized with bLF through absorption, and were extensively characterised regarding their physicochemical characteristics, absorption/de-absorption profiles (mean absorbed amount 37.5 mg $mL^{-1}$ $g^{-1}$ and mean de-absorbed amount 12.6 mg $mL^{-1}$ $g^{-1}$), mechanical properties (approx. 42 MPa max. tensile strength), bactericidal efficiency *per s*e against *Escherichia coli* (approx. 69%) and *Staphylococcus aureus* (approx. 97 %), and in a food case study using fresh sausages (94% *E. coli* and 36% *S. aureus*). Moreover, cytotoxicity was determined before and after digestion using an *in vitro* gastrointestinal tract model and displayed no relevant cytotoxicity. The herein developed FG electrospun films presented interesting properties, such as resilience, biocompatibility and stability in aqueous solutions following glutaraldehyde vapour crosslinking. Therefore a nanocomposite was developed comprising FG and bLF as filler, followed by glutaraldehyde vapour crosslinking. Fourier transform infrared spectroscopy showed that the structure of both nanocomposite proteins remained intact through the electrospinning blending and crosslinking procedure. FG fibres with absorbed bLF were used as control and displayed similar bactericidal efficiency to as-spun FG (91% *E. coli* and 94% *S. aureus*). The nanocomposite FG electrospun films containing 10 wt% bLF as filler displayed 99.9999 % of contact killing capacity against *E. coli* and *S. aureus.* In conclusion, these new nano bio-based edible films, BC with bLF and FG with bLF, present a high active potential to control the food commodities' endogenous microflora, and therefore are expected to extend their shelf-life and enhance their safety.

**Desenvolvimento e caracterização de novos filmes edíveis com propriedades antimicrobianas para aplicações em produtos alimentares**

# Resumo

As embalagens ativas são cada vez mais uma tecnologia fiável para assegurar a segurança alimentar, bem como a manutenção/melhoramento das suas propriedades organoléticas. Nesta tese desenvolveram-se materiais com base em fontes naturais mas com características melhoradas recorrendo a uma metodologia simples e eficaz para produzir filmes ativos com propriedades antibacterianas e comercialmente acessíveis. Filmes de celulose bacteriana (BC) e filmes eletrofidados de gelatina de peixe (FG) foram usados como suportes estruturais. A BC possui um grande potencial para formar filmes alimentares de alta performance, nomeadamente uma notável resistência mecânica. FG é uma fonte proteica que não tem sido muito explorada como suporte estrutural de filmes. Os filmes de BC e electrofiados de FG foram funcionalizados com lactoferrina bovina (bLF), uma glicoproteína detentora de um reconhecido largo espectro antibacteriano. Os filmes finos (aprox. 25.75 µm) de BC foram obtidos de duas fontes distintas i. cultura estática de *Gluconacetobacter xylinus* ATCC 53582 e ii. BC comercial (*nata de coco*). Posteriormente filmes foram funcionalizados com bLF por absorção e as suas propriedades físico-químicas, perfis de absorção/de-absorção (em quantidade média absorbida 37.5 mg $mL^{-1}$ $g^{-1}$ e quantidade média de-absorvida 12.6 mg $mL^{-1}$ $g^{-1}$), propriedades mecânicas (força de tensão máx. de aprox. 42 MPa), eficiência antibacteriana *per se* contra *Escherichia coli* (aprox. 69%) e *Staphylococcus aureus* (97%), e num caso de estudo usando salsichas frescas (94% *E. coli* e 36% *S. aureus*). Foi avaliada a citotoxicidade dos filmes antes de depois da sua digestão *in vitro* num sistema modelo do sistema gastrointestinal, não se tendo detetado uma citotoxicidade relevante. Os filmes resultantes da electrofiação de FG apresentaram propriedades interessantes, tais como resiliência, biocompatibilidade e estabilidade em soluções aquosas após reticulação com vapor de glutaraldeído. Assim, desenvolveu-se um nanocompósito constituído por FG e bLF como enchimento, seguido de reticulação com glutaraldeído. Por espectroscopia no infravermelho por transformada de Fourier demonstrou-se que não há alterações estruturais nas proteínas do nanocompósito após produção e reticulação. Filmes eletrofiados de FG foram adsorvidos com bLF par uso como controlo, demostrando uma atividade bactericida semelhante às fibras de FG (91% *E. coli* e 94% *S. aureus*). Os nanocompósitos de FG contendo 10% (p/p) de bLF demonstraram uma atividade antibacteriana de 99.9999% de morte por contacto contra *E. coli* e *S. aureus*. Em conclusão, os novos filmes nano e edíveis obtidos a partir de fontes naturais, BC com bLF e FG electrofiada com bLF, apresentam um grande potencial para controlar as comunidades microbianas endógenas de produtos alimentares, bem como para reforçar a sua segurança.

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# Abbreviations

AATCC - American Association of Textile Chemists and Colourists

ATCC - American type culture collection

$a_w$ - Water activity

BC - Bacterial cellulose

BC 1 - Bacterial cellulose synthesized by Gluconacetobacter xylinus ATCC 53582

BC1+bLF - Processed bacterial cellulose synthesized by Gluconacetobacter xylinus ATCC 53582 with absorbed bovine lactoferrin

BC2 - Processed commercial bacterial cellulose

BC2+bLF - Processed commercial bacterial cellulose

bLF - Bovine lactoferrin

CFU - Colony forming units

DAPI - 4',6-diamidino-2-phenylindole

DMEM - Dulbecco´s modified eagle´s culture medium

DMF - N,N-dimethylformamide

DSC - Differential scanning calorimetry

E - Elastic modulus

EC - European Commission

EDTA - Ethylenediamine tetraacetic acid

EFSA - European Food Safety Authority

EPA - Environmental protection agency

EU - Endotoxin units

FA - Formic acid

FG - Fish gelatine

FPLC - Fast protein liquid chromatography

FTIR-ATR - Fourier transform infrared spectroscopy with attenuated total reflection

hLF - Human lactoferrin

HS - Hestrin Schramm culture medium

IL - Interleukin

Kd - Dissociation constant

LF - Lactoferrin

LF - Lactoferrin

LPS - Lipopolysaccharide

MAP - Modified atmosphere packaging

MH - Mueller Hinton agar culture medium

MIC - Minimal inhibitory concentration

MLA - Mouse lymphoma TK assay

n - Number of independent assays

NB - Nutrient broth culture medium

NCS - Newborn calf serum

NHN - Ninhydrin

NK - Natural killer

NO - Nitric oxide

OFW - Ozawa-Flynn-Wall method

PAMP - Pathogen associated molecular pattern

PAMPS - Pathogen associated molecular patterns

PBS - Phosphate buffered saline

pI - Isoletric point

PI - Propidium iodide

RH - Relative humidity gradient

SEM - Scan electron microscopy

SIES - Small intestinal electrolytic solution

TC - Terminal complex

TGA - Thermogravimetric analysis

TIM - Gastrointestinal tract model

TNO - Netherlands Organization for Applied Scientific Research

WVP - Water vapour permeability

WVT- Mass variation rate

ZoI - Zone of inhibition

FDA – Food and drug administration

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# Chapter 1

## Thesis motivation and outline

# 1. Thesis motivation and outline

Currently, food is much more than an item to insure nourishment. Food may be an important ambassador of a region, a country, a continent or even a culture. Therefore, the maintenance of the food organoleptic traits, like the flavour, texture, smell, quality assurance and food safety are crucial for the food industry. Food products *per se* possess highly complex properties, which may be controlled or manipulated to delay the deterioration of its properties. Understandably, the optimal scenario is to maintain intact all food traits, from the moment they are harvested or processed to the moment they reach the consumer's plate, no matter how much distance exists between them. In this sense, active food packages represent a powerful tool towards that end.

Food packaging is the encased food first line of defence against environmental aggressions. Its constant evolution is grounded on the continuous enhancement of safety and the need to adaptat to modern consumer demands. Foodborne infection and intoxication account for more than 300.000 reported cases of foodborne illness, in the European Union each year [1]. Also, currently the consumer requirements include sustainability and fresher food products, thus pressing the food packaging industry to use eco-friendly materials, as well as minimal food processing procedures and with minimal use of synthetic additives [2, 3]. Additionally, food packaging technology must evolve to provide consumers an increased convenience, and time saving alternatives, such as online food shopping. The food industry maintains its share on the worldwide food packaging market, which was estimated as €580 billion in 2014 and over than €628 billion in 2016 [4-6].

This thesis envisaged the development of novel bio-based nanomaterials with antimicrobial activity, towards potential application as active packaging that can address the following concerns:

**Item #1:** To use of eco-friendly materials – solely bio-based sources were exploited in this thesis, namely bacterial cellulose (BC), fish gelatine (FG) and bovine lactoferrin (bLF).

**Item #2:** To avoid antimicrobial resistance – currently, antimicrobial resistance is a huge concern that urges the search for alternative agents [7]. In this work, the antimicrobial agent (bLF) is known to possess a low probability to induce antimicrobial resistance [8].

**Item #3:** To enhance food safety – the antimicrobial activity of the designed materials was intended to eliminate or significantly reduce the major etiological agents of foodborne diseases, namely bacteria as for example *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. In order to study the contamination of fresh sausage (as a model food product), *E. coli* and *S. aureus* were used as contaminant microorganisms.

**Item #4:** To diminish food spoilage – the reduction of the bacteria population in a food product may extend its shelf-life, thus contributing to diminish the loss of food commodities and of economic value.

**Item #5:** To provide added value – although this item was not fully addressed in this thesis, the use of a functional ingredient such as bLF, may bring additional beneficial effects to the microbial flora and gastrointestinal epithelium cells.

This thesis is organized in 6 chapters as follows:

**Chapter 2** comprises an overview of the state of the art on active and intelligent packaging, as well as of the bio-based materials herein used namely BC, FG and bLF. Besides, the European Union and national legislation framework concerning the active packaging materials is revised.

**Chapter 3** describes the production of BC by two different strains, and exploits its modification through periodate oxidation. The bactericidal activity of pristine and oxidized BC was tested against *E. coli*, *S. aureus* and *S. epidermidis*. The results presented in this chapter resulted in two poster communications in the following conferences:

- Padrão, J.,Rodrigues, L., Dourado, F. "Prospective of the use of bacterial cellulose as an antimicrobial edible film" (2011) MICRO-BIOTEC - Instituto de Ciências da Vida e da Saúde, Universidade do Minho, Braga, Portugal
- Padrão, J. Rodrigues, L., Dourado, F. "The potential of bacterial nanocellulose as an antimicrobial edible film" (2012) 11° Encontro de Química de Alimentos - Instituto Politécnico de Bragança, Portugal

**Chapter 4** presents the study of two different types of BC blended with bLF. The physicochemical and mechanical properties and the antimicrobial activity against *E. coli* and *S. aureus*, as well as the cytotoxicity before and after digestion in an *in vitro* gastrointestinal track model (TIM), were

assessed. The results presented in this chapter comprise the contents of two manuscripts to be submitted to international peer reviewed journals and one poster communication in a conference:

*Papers in international scientific periodicals with referees:*

- Padrão, J., Gonçalves, S., Silva, J.P., Sencadas, V., Lanceros-Méndez, S., Rodrigues, L.R., Dourado, F. "The potential of edible bacterial cellulose films functionalized with lactoferrin" – to be submitted
- Padrão, J., Gonçalves, S., Silva, J.P., Pinheiro, A.C., Vicente, A.A., Rodrigues, L.R., Dourado, F. "*In vitro* gastrointestinal tract model of an edible antibacterial nanocomposite – cytotoxicity assessment" – to be submitted

*Poster communication in conference:*

- Padrão, J., Gonçalves, S., Silva, J.P., Sencadas, V., Lanceros-Méndez, S., Pinheiro, A.C., Vicente, A.A., Rodrigues, L.R., Dourado, F. "Edible lactoferrin bacterial cellulose films as an effective and low-cost antimicrobial active packaging" (2014) 1st Congress on Food Structure Design – Porto, Portugal

Chapter 5 includes the production and characterization of random and aligned fish FG fibre mats obtained by electrospinning, as well as the design and evaluation of a protein electrospun nanocomposite composed of FG and bLF. The cytotoxicity of the FG electrospun mats and the bactericidal efficiency of the nanocomposites were evaluated. The results presented in this chapter were published in two peer reviewed papers, and an additional manuscript was prepared to be submitted, as well as in a poster communication in a conference.

*Papers in international scientific periodicals with referees:*

- Correia, D.M., Padrão, J., Rodrigues, L.R., Dourado, F., Lanceros-Méndez, S., Sencadas, S. "Thermal and hydrolytic degradation of electrospun fish gelatin membranes" (2013) Polymer Testing 32:995-1000
  DOI:10.1016/j.polymertesting.2013.05.004
- Padrão, J., Silva, J. P., Rodrigues, L. R., Dourado, F., Lanceros-Méndez, S., Sencadas, V. "Modifying fish gelatin electrospun membranes for biomedical applications: cross-linking and swelling behavior" Soft Materials (2014) DOI:10.1080/1539445X.2013.873466
- Padrão, J., Machado, R., Sencadas, V., Lanceros-Méndez, S., Casal, M., Rodrigues, L.R., Dourado, F. "Antibacterial performance of bovine lactoferrin-fish gelatine electrospun nanocomposites" – to be submitted

*Poster communications in conferences:*

- Padrão, J., Machado, R., Casal, M., Rodrigues, L.R., Dourado, F., Lanceros-Méndez, S., Sencadas, V. "Antibacterial performance of bovine lactoferrin-fish gelatine electrospun nanocomposites" (2014) International Conference on Antimicrobial Research – Madrid, Spain

**Chapter 6** comprises the general conclusions of this work and provides future perspectives of the current work.

# Chapter 2

## General introduction

# 2. General introduction

The preservation of the food's organoleptic characteristics from "farm to fork" is a highly challenging procedure due to the both numerous and highly complex variables in play.

## 2.1. Food spoilage and foodborne illness

Food spoilage occurs throughout the entire food chain including farmers, food industry, retailers and consumers. The massive amount of food wasted due to spoilage is a worldwide problem. Indeed, it is estimated that t 1.3 billion tonnes of food products year are spoiled, which represents a third of the worldwide food production [9]. Several factors contribute for such alarming figure, including retailers rejecting visually unattractive foodstuffs, representing an annual loss of 1.6 million tonnes, as well as inadequate consumer habits, comprising the purchase of excessive number of food commodities, usually compelled by retailer promotions, inadequate expiring dates, ineffective storage and retail logistics [10]. According to Mena and collaborators [11] the main reasons for the food waste generated include inappropriate packaging, poor handling and transportation, along with failures on forecasting and storage.

Inadequate storage conditions favour the two main types of food spoilage, *i.e.* microbial and physicochemical. Both processes are dependent on the food's intrinsic, extrinsic and implicit traits, as well as prior preservation treatments and package properties [12]. Food's intrinsic characteristics comprise its formulation, ingredients, pH, redox potential and water activity ($a_w$); whereas the extrinsic parameters include the environmental storing conditions such as temperature, light and humidity. On the other hand, the implicit traits are centred on the behaviour of the food's native microbial population. The nutrient composition, as well as the pH of the food matrix may favour the growth of both native and exogenous microorganisms [12, 13]. However, the endogenous microbes may also adopt a competitive behaviour secreting antimicrobial agents or shifting the pH to an unfavourable value, thus promoting an antagonistic effect. Preservation treatments will also substantially affect the microbial population and the inherent biochemical reactions [12, 14]. Meat, seafood, poultry and dairy products are food commodities with a particularly perishable profile due to their high protein content, nutrient availability, high moisture level and neutral or slightly acid pH [12].

Every year bacteria cause more than 300.000 reported cases of foodborne diseases in the European Union, which probably implies the existence of a considerable number of unreported cases [1]. The European Food Safety Authority (EFSA) identified the most relevant foodborne microorganisms. Based on all reported cases between 2006 and 2010, *Listeria monocytogenes* is the bacteria that induced the higher mortality rate in the European Union (17 to 20%). *Clostridium botulinum* exhibited an average mortality of 2.5%, while lower mortality rates (≤0.2) were observed for *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli*, *Yersina* spp., *Campylobacter* spp. and *Clostridium* spp. (not including *C. botulinum*). *Bacillus* spp. and viruses did not showed death counts [15].

Moreover, EFSA conducted an evaluation of the positive reported cases in specific groups of food commodities between 2004 and 2009, which demonstrated that approximately 20% of the reported illness cases resulted in a hospitalization (Table 2.1.1.).

**Table 2.1.1.** Confirmed food outbreaks in European Union from 2004 to 2009 collected by the European Food Safety Authority (EFSA). The values were obtained from the cumulative reported cases foodborne illness that resulted in disease, in severe disease that justified the hospitalization, and in the death of the consumers. The cases were identified in the following food products: bakery, desserts with and without raw eggs, confectionary (chocolates and sweets), pasta and noodles cooked alongside other foodstuffs [15].

| | Ill | Hospitalized | Death |
|---|---|---|---|
| *Bacillus* spp. | 105 | 22 | 0 |
| *Salmonella* spp. | 4667 | 1095 | 9 |
| *Staphylococcus aureus* | 228 | 26 | 1 |
| *Clostridium perfringens* | 43 | 0 | 0 |
| *Caliciviridae* | 850 | 5 | 0 |
| Other / unknown | 57 | 0 | 0 |

Active and intelligent food packaging may assist the food industry to reduce the cases of foodborne illness. Additionally active food packaging may greatly contribute to reduce the worldwide food waste, by extending the products shelf-life, but also by buffering non-optimal food storage and distribution conditions.

## 2.2. Food packaging

The main role of food packaging is to provide a suitable shelf-life, achieved by protecting the encased food from external aggressions and offering the required physical strength and physicochemical conditions, to extend the food preservation [16]. Therefore, food packaging

materials must provide the appropriate mechanical resistance and optimal gas and water vapour permeability [17]. These parameters are difficult to control when using classic packaging materials, since all direct food contact materials have to be as inert as possible [18, 19]. The use of innovative materials is envisaged to extend food's shelf-life without compromising its organoleptic properties [20]. Additionally, food organoleptic "live" status may be monitored by intelligent packaging, whereas the optimal preservation conditions may be optimized and extended by the use of active agents [17]. Indeed, active packaging is an attractive technology due to its "hands on" food overall conditions, in opposition to the simple passive monitoring of the intelligent packaging.

### 2.2.1. Intelligent packaging

Intelligent packaging is the definition for all packages that possess an external or internal quality indicator, that inform the retailer or the consumer about the quality of the encased food [21]. Some of the developed intelligent packages include time-temperature indicators, oxygen and carbon dioxide indicators, and microbial growth indicators. The indicators *modus operandi* may be based on dyes that react on specific redox, pH, temperature conditions, ripening factors and seal leak due to atmosphere changes, or in the presence of metabolites, originated by autolysis or microbial activity [2, 17]. Therefore, intelligent packages possess responsive components that are included in the package structure formulation or as an adjunct, such as sachet, patch or a label, that contain the responsive dye or physicochemical agent [22].

### 2.2.2. Active packaging

The definition of active packaging consists in every package that directly influences the environmental conditions within and/or exerts a continuous direct action on the encased food product during the storage period, with the objective of maintaining or improving the food organoleptic properties, safety and extend its shelf-life [2, 17, 20].

Food active packaging formulation may be tailored-made for each food specification or particular parameter that must be controlled for an effective food protection or maintenance [2]. A plethora of active packages have been reported and are currently available as briefly described below:

#### 2.2.2.1. Oxygen scavengers

Oxidation of food commodities leads to the off flavour generation, unpleasant odour formation and degradation of colour. The main driving source of food oxidation reactions in food it is the available

atmospheric oxygen, which is also a vital parameter for the growth strictly aerobic bacteria. However, the introduction of a modified atmosphere during packaging of the so called modified atmosphere packages (MAP) may not be sufficient for a prolonged effect. Furthermore, the oxygen may permeate into the package, or it may be released by biochemical reaction undertaken in the food product *per se*, for instance the generation of oxygen due to autocatalytic reactions [23]. Therefore, the use of an oxygen scavenger agent or material within the package may guarantee the extended oxygen entrapping, since it enters in the package headspace and remains during the storage period. One of the first successfully commercialized active agents used for headspace oxygen scavenging was Ageless®, developed by the Japan's Mitsubishi Gas Chemical Co. in 1977 [24], consisting on a sachet containing iron, which reduces the available oxygen. Active systems using iron are both simple and effective, thus they are still currently used and are the subject of further developments [25]. Other oxygen scavenging systems include ascorbic acid, unsaturated lipids, catechol, immobilized enzymes such as glucose oxidase, alcohol oxidase and catalase, and immobilized yeasts [21, 24, 26-29]. Due to the vast existing commercial options of oxygen scavenging systems allied to the food industry increasing demand for this technology, its global market value was estimated as € 720 million in 2010 [2, 24].

#### 2.2.2.2. Antioxidant agents

The antioxidant agents act against one of the most important factors of food spoilage, *i.e.* lipid rancidity. The oxygen scavengers already provide an answer to minimize lipid oxidation, however, reactive oxygen species, namely oxygen superoxide, generated during muscle food autolysis may only be neutralized immediately after their formation by antioxidant substances [23, 30]. Natural antioxidants are increasingly popular for food applications, such as vitamin E and spices like cinnamon, rosemary and oregano due to their high content of phenolic acids and flavonoids [2, 30-32].

#### 2.2.2.3. Ethylene scavengers

If the active package desired action is to delay the ripening of fruits, one of the most effective option is the use of ethylene scavenger, which will reduce the concentration of this ripening promoter phytohormone [33, 34]. The pioneering ethylene scavenging systems were based on palladium chloride, active carbon and potassium permanganate, using different immobilizing materials due to their toxicity when ingested [35-37]. Potassium permanganate commercialized solutions such

as Retarder® possess the additional advantage to provide a visually indication of its remaining efficiency, since the ethylene oxidation leads to the colour shift of purple to brown [38].
The use of zeolites and minerals to diminish the ambient ethylene is exploited by PEAKfresh® [39]. Fairly new oxygen scavengers use palladium based materials, that permits ethylene oxidation reaction even in high moisture environments [40].

#### 2.2.2.4. Moisture regulators

Food presentation is a crucial parameter for consumer acceptance, which in case of several food commodities, may be easily compromised by the formation of condensation water, or excess of liquids originated by the food's dripping of blood, juice or water [6, 41]. The use of desiccants reduce the excess of liquids, safeguarding food's presentation and most importantly, hinder microbial growth, by reducing the $a_w$. The use of sachets containing desiccants such as silica gel and calcium oxide, or the use of patches of highly hydroscopic materials, such as cellulose, are the two most common classes of commercial applications. More advanced solution have been developed, such as the presented by Hekal [42] consisting of a composite material comprising a hydrophobic polymer matrix, a hydrophilic agent (namely polyglycols) and a deliquescent material to control the headspace relative humidity. This triple component concept is also used by the commercial application Activ-Pak™ [39].

#### 2.2.2.5. Antimicrobial

The use of antimicrobial agents to reduce or even eliminate all the food's microbial population in order to insure its safety and extend its shelf-life has been a highly exploited concept during over last decades.
The list of potential antimicrobial agents to be used in active packaging is quite vast, due to their proven biocidal activity, and apparent absence of cytotoxicity [6]. Several antimicrobials were tested individually or in combination against relevant bacteria associated with food spoilage and foodborne illness. Indeed, it has been found that the combination of several antimicrobials shows the most promising results [43]. Some active packages are based on the use of antimicrobial ingredients, such as spices [44], essential oils [45], nisin [43, 46], lysozyme, protective cultures (bacteriophages or bacteriocin producing microorganisms) [47, 48] among others. In summary, the antimicrobial agents may be present in the packages as:

1) Sachets that release the active agent in the package headspace, such as the commercial application Ethicap® (Freund Industrial Co., Ltd., Japan) that is an ethanol emitter [22];

2) Dispersion of the active agents within the food, such as the use of a formulation of an active solution in constant contact with food, namely sodium acetate and sodium diacetate or glucono-δ-lactone or sodium lactate for an enhanced safety against *Listeria monocytogenes* in frankfurters [49];
3) Films or coatings embedded with antimicrobial agents, intended to come into direct contact with the food, that may or may not be edible (Table 2.2.1.);

**Table 2.2.1.** Examples of direct contact antimicrobial active packaging for food applications, including its antimicrobial agent, support material, the inhibited microorganisms, and in which food commodity these active packaging has been tested. "n.d." stands for not described.

| Antimicrobial agent | Support material | Effective against | Food commodity | Reference |
|---|---|---|---|---|
| Chitosan | Chitosan film | *Listeria monocytogenes*, *Listeria innocua* | Emmental cheese | [50] |
| Cilantro essential oil | Gelatine gell | *Listeria monocytogenes* | Vacuum packed ham | [51] |
| Nisin | Polyethylene and cellulose film | *Listeria monocytogenes* | Tofu | [46] |
| Silver (Ag) | Polyethylenoxide film | *Alicyclobacillus acidoterrestris* | Apple juice | [52] |
| Chitosan | Chitosan coating | Endogenous flora | Grilled pork | [53] |
| Lysozyme | Polyvinylalcohol film | *Micrococcus lysodeikticus* | n.d. | [54] |
| Oregano essential oil | Chitosan film | n.d. | Bologna slices | [55] |
| Cinnamon essential oil | Paper and wax | *Rhizopus stolonifer* | Sliced bread | [56] |
| Nisin | Bacterial cellulose film | *Listeria monocytogenes* | Frankfurters | [57] |
| Chitosan and/or clove essential oil | Fish gelatine film | *Lactobacillus acidophilus*, *Pseudomonas fluorescens*, *Listeria innocua*, *Escherichia coli* | n.d. | [58] |
| Triclosan | Polyethylene film | *Escherichia coli*, *Staphylococcus aureus* | Ham | [59] |
| ε-Polylysine | Bacterial cellulose film | *Escherichia coli*, *Staphylococcus aureus* | Sausages | [60] |
| Sodium lactate and ε-polylysine | Whey films | Endogenous flora | Fresh beef | [61] |

Even though the successful application of experimental packages has been widely reported in the literature, and several commercial applications are used for decades in Japan, United Sates of America, and Australia; in Europe the support materials for packages aren't based on essential oils or spices see their commercial application hindered by legal restrictions [2, 62]. However, some amends were recently implemented in the European Union regulation to facilitate the integration of novel active packaging technologies in the market towards the enhancement the food industry competitiveness, and new amends are expected (see Section 2.5.).

## 2.3. Support materials for food packaging

Food packaging may be formulated from natural materials, which present the advantages of renewability, biodegradability and edibility, which are absent concepts in the petrochemical-based food packages [16, 63-66]. Bio-based materials may also enhance the nutritional value, organoleptic traits and even the presentation of the food commodities. Moreover, if the bio-based packages are designed to be ingested together with the food product, such as a wrapping film or coating, they may exert a more efficient action, while reducing the generated waste thus following the recommendations from the Environmental Protection Agency (EPA) [67]

The main sources for bio-based materials are: proteins, polysaccharides, lipids, and their composites [63, 68-70]. However, the commercial implementation of the bio-based materials by the food industry is hindered by some characteristics of these "green" materials, such as limited mechanical properties, and usually high water vapour permeability (WVP), namely in case of polysaccharide and protein-based application to their inherent hydrophilicity [71-73]. However, many novel developments on the nanotechnology fields enable the enhancement of the bio-based materials favourable characteristics and provide solutions to overcome their limitations [34].

The term "nano" implies the use of materials at a scale of 1 to 100 nanometers (1 nm = $10^{-9}$ m) in at least one of the material's dimensions [74]. Smaller sizes mean higher surface area, which may improve both the activity and responsiveness of active and intelligent food packages respectively [74, 75]. Additionally, nano-sized materials may also improve tensile strength, gas and moisture permeability properties [75]. The development of nanocomposites is a straightforward strategy to enhance the material properties and/or to maximize the efficiency of active packages [16, 76].

The nano scale of the bioactive agents permits a higher targetability and bioavailability of antimicrobial impregnated in the nanopackage [16, 77]. In the case of intelligent food packaging,

the use of nanosensors allows the monitoring of the temperature fluctuations, humidity, oxygen and/or even detect the presence of microbial metabolism by-products, thus, providing more accurate expiration dates [74]. Additionally, the development of fully bio-based nanomaterials and nanocomposites, for superior food packaging manufacturing, allows the exploitation of green material sources, representing a viable possibility to reduce the over 12 million tonnes of plastic used every year for packaging purposes [16, 78].

The blending of nanostructures with nano reinforcements or bioactive agents, such as antimicrobial effectors, already proved a high efficiency in several applications [76]. For instance the use of starch nanocomposites reinforced with nanoclays presented significant enhanced mechanical properties [79] and the nano cellulose reinforcement of manga puree nanofilms also incremented mechanical resilience and decreased the WVP [80]. Protein-based composites exploit several protein sources such as collagen, casein, soybean protein, and whey protein [77]. For example, whey protein films were blended with ZnO to develop an antimicrobial film [81].

The most exploited bio-based materials used in food applications fall into one of three below mentioned categories [82]:

1) Bio-based polymers extracted from biomass: several natural polymers that are commonly used possess interesting properties for the development of innovative packages; these include for example plant cellulose, gelatine, starch and collagen.
2) Bio-based polymers obtained from chemical polymerization of renewable bio-based monomers – the most notable example is polylactic acid, recovered from the fermentation of carbohydrate feedstock. However, this methodology is limited by the high prices associated the monomers synthesis.
3) Bio-polymers naturally synthesized by microorganisms, or obtained using genetically engineered microorganisms – such as bacterial cellulose, or elastin elastomers obtained through heterologous production [83, 84].

In this work, two bio-based materials were used due to their high potential to be used as innovative high-end supportive materials for active food agents. Bacterial cellulose is an extremely pure source of cellulose, with enhanced properties in comparison to its plant sibling, such as absence of allergic reactions, remarkable mechanical properties and promising loading capacity. Fish gelatine is a highly unexploited protein source analogous to one of the most exploited biopolymers in the world, gelatine.

### 2.3.1. Cellulose

The French chemist Anselme Payen, was the first to describe, in 1838, the most abundant biopolysaccharide on planet Earth, i.e. cellulose [85, 86]. Cellulose elemental chemical composition is $C_6H_{10}O_5$, it is composed exclusively of D-glucose units that are covalently bounded by acetal functions formed between the C1 atom carbon and the equatorial OH group of C4, and therefore commonly named as β-1,4 glycosidic links [86-88]. Cellulose worldwide biological synthesis is estimated to be $10^{11}$ to $10^{12}$ tonnes per year [87, 88]. This homopolymer is only considered cellulose once the glucan chains reach at least 30 kDa, in other words, when it is composed of roughly 90 repeating units of cellobiose (two D-glucose units bounded by β-1,4 glycosidic bounds) [89]. The glucan chain aggregation leads to the formation of an insoluble polymer since the degree of its polymerization is superior to 6 [89].

Cellulose possesses two crystallization forms, cellulose I and cellulose II. Cellulose I is comprised by parallel cellulose polysaccharide chains, whereas cellulose II is constituted by anti-parallel polysaccharide chains. The cellulose I is defined as thermodynamically metastable, although cellulose II is even more thermodynamically stable, due to the existence of one extra hydrogen bound per glucose residue [85, 89, 90]. Cellulose II synthesis in nature is rare, generally only being synthesized by some algae and bacteria [91]. Cellulose I is the most common allomorph present in nature, and it may present one of two distinctive suballomorphs, $I_\alpha$ and $I_\beta$. The $I_\alpha$ structure is compose by single chain triclinic cell unit [92]. On the other hand $I_\beta$ is more thermodynamically stable than its suballomorph counterpart composed of a two-chain monoclinic cell unit, and is only synthesised in its pure form in tunicates [86, 90, 93]. The ratio between $I_\alpha$ and $I_\beta$ present in cellulose is defined by the organism of origin [94].

Cellulose-based products are used in a wide array of applications, including in the food industry as an ingredient, due to its biodegradability, edibility and its importance for a healthy diet [77, 95]. Higher plants are the main source of cellulose, since cellulose is the major constituent of the plant cell wall (approximately 35 to 50% of the total cell dry weight). Cellulose may be retrieved in almost pure form from cotton seed hairs. Otherwise, pectin, lignin and hemicelluloses are always present in the plant cell walls structure representing approximately 5 to 35% of the plant cell dry weigh [96]. Therefore, chemical purification procedures must be undertaken to obtain pure plant cellulose. Usually the first delignification step implies the use of high temperatures (130 and 180 °C) and aggressive chemicals ($NaHSO_3$ or NaOH and $Na_2S$), followed by a pulp bleaching step that

includes chlorine chemicals ($Cl_2$, NaClO, $ClO_2$) or peroxides. Then the pulp is washed, dried and packed, and finally bleached into cellulose with 90 to 95% of purity [97]. The required man-hours, electricity, chemicals acquisition and their subsequent waste treatment, represent an inevitable economic burden concomitant with these purification treatments. There are three main alternative sources to plant cellulose. Cellulose chemical synthesis from modified glucose units, the *in vitro* production of cellulose using anabolic enzymes, and cellulose produced by prokaryotic organisms [86, 98].

Both chemical synthesis and *in vitro* production are cost ineffective if the intended production requires large cellulose volumes. Therefore the microbial production may present itself as a viable alternative to plant cellulose. Several genera of strict aerobe Gram-negative bacteria are able to produce cellulose, such as *Agrobacterium*, *Acetobacteria*, *Alcaligenes*, *Rhizobia*, *Pseudomonas*, *Achromobacter*, *Aerobacter*, *Azotobacter* and *Alcaligenes*. Moreover, *Sarcina* which is an anaerobic Gram-positive bacteria can produce [89, 99, 100].

#### 2.3.1.1. Bacterial cellulose or bacterial nanocellulose

Since firstly scientifically characterized by Brown [101] in 1886, bacterial cellulose also known as bacterial nanocellulose (BC) was the subject of extensive research specially in the mid 80's, owed to the identification of the BC's remarkable mechanical properties [95]. Even though several bacteria possess the enzymatic machinery to produce cellulose, its production is by far most thoroughly studied in the bacteria *Gluconacetobacter xylinus* [83, 100]. Formerly named *Acetobacter xylinus*, *G. xylinus* is grouped with the acetic acid bacteria, it is a non-pathogenic Gram-negative, α-proteobacter, rod shaped, strictly aerobic without flagellum [86, 88]. *G. xylinus* is found in nature in fruit, rotting fruit and products that are sugar rich and/or possess alcohol (namely wine). The production of BC by *G. xylinus* may occur in static or agitated culture conditions.

#### 2.3.1.2. Bacterial cellulose synthesis, structure and properties

Each *G. xylinus* cell produces a cellulose ribbon composed of 10 to 100 microfibrils at a rate of 0.2 μm $min^{-1}$ [89, 102]. The cellulose producing sites were identified in the *G. xylinus*' longitudinal axis, with each site forming a perpendicular pore in the cell envelope [102]. Each cellulose anabolic site is formed of a protein cellulose synthesizing complex, named as terminal complex (TC) subunit, which comprises several cellulose synthase catalytic sub-units (BcsAs). The β-D-glucopyranose chains, after being assembled in the BcsAs, start to be pushed into the extracellular medium by newly added D-glucose units. Several individual chains are separately "guided" through the protein

complex until they reach the extracellular medium where they self-assemble through hydrogen bounding with each other, and/or with other cellulose nanofibrils, ultimately forming a single cellulose I ribbon, typically possessing 20 to 80 nm in width, 1 to 9 μm in length and 3 – 8 nm in thickness [78, 103-105]. The BC is 80% composed of $I_{\alpha}$, a high degree of polymerization of approximately 2000 to 8000, a 60 to 90% of crystallinity of and its architecture may be controlled by culture conditions, namely the concentration of available oxygen [78, 86, 106, 107].

In static cultures, the clustering and entangling of cellulose ribbons, eventually, forms a macroscopic nanogel composed of 99% water and 1% of cellulose, possessing a remarkable water holding capacity [95, 108-111]. Furthermore, even though BC is composed solely of cellulose, the extensive hydrogen bounds in its fibrils lead to mechanical properties quite distinct from its plant counterpart and uneasily matched by biological polymers [83, 95, 112]. The reported values of tensile strength reported in the literature are distinct, most likely due to the use of different culture conditions, different *G. xylinus* strains, and different experimental conditions (Table 2.3.1.) [113]. The determined pore size of air dried BC membranes was found to be less than 0.1 μm [114].

**Table 2.3.1.** Bacterial cellulose mechanical properties "*E*" elastic modulus, "$\sigma_{max}$" maximum stress and "$\varepsilon_{break}$" elongation at break. "n.a." stands for not available.

| *E* (MPa) | σ (MPa) | ε (%) | Reference |
|---|---|---|---|
| 4900 | 85 | n.a. | [108] |
| 16900 | 256 | 1.7 | [95] |
| 2700 | 92 | n.a. | [115] |
| 2.9 | 2.2 | 0.21 | [116] |
| 25000 | 250 | 1.75 | [86] |
| 5.2 | n.a. | 3.75 | [117] |
| 30.0 | 12.5 | 3.4 | [118] |
| 286 | 2.8 | 0.8 | [119] |
| 6500 | 241 | 8 | [120] |

### 2.3.1.3. Bacterial cellulose large scale production and its commercial applications

Due to its notable properties, such as purity, high surface area and water binding capacity, impressive mechanical properties and its improbability to trigger allergic reactions, BC possess a great application potential, already extensively exploited by a wide spectrum of industries. For instance, in the early 90's six Japanese companies, Ajinomoto, Shimazu Construction, Nakamori Vinagar, Mitsubishi Paper, Nikki and Nikiso alongside with the Japan Key Technology Center, which

was jointly managed by the Japanese Ministry of International Trade and Industry and the Ministry of Post and Telecommunications, founded an approximately € 35 million joint venture named as Biopolymer Research Co., Ltd. From its intensive BC research and development activities several patents and scientific manuscripts have been published [121].

The viable implementation of BC in commercial materials is always dependent on a highly efficient large scale production, which inevitably demands an adequate optimization of both culture medium composition and fermentation parameters, in order to obtain BC with the required standard quality and properties for the desired application, since its properties vary according to the culture conditions. Hence, several fermentation techniques were developed and optimized to increase productivity [122]. The fermentation methodologies studied for obtaining pebble like BC include stirred cultures [119], non-static culture [115], airlifts and bubble-column-type [123], with the last two being successfully scaled-up to pilot scale [123, 124]. However to obtain BC foils or sheets, static culture conditions is the most efficient methodology [122].

*Static culture*

The traditional and most widely used form of BC production is the static culture, in which BC is synthesized in the air to medium interface, due to the higher oxygen availability [95, 125]. In static culture, the BC membrane ultimately adopts the shape of the culture recipient surface. In an agitated culture, *G. xylinus* produces cellulose in the form of small pebble-like or star-like shapes, and the BC yield is usually considerably lower due to the higher generation of mutants [126, 127]. These spontaneous mutants are generally named as Cel- due to their restrict and residual production of BC in the form of cellulose II [88].Static culture is still currently seen as the standard method for BC production, mainly because it is highly reproducible, and it can be cost effective, since it requires minimum operational costs, especially when using low-end or by product culture medium. It is important to mention that this method is limited in terms of oxygen concentration, which is an essential parameter for the strict aerobe *G. xylinus*, however the optimal BC production occurs at limited concentrations of this gas, namely between 10 and 15% [107]. A large scale static production requires a large area for facilities, which can spike the production costs due to rents or other associated costs, namely in the expansion of the facilities. Thus, new methods to retrieve the BC pellicle without disrupting the culture were engineered [128]. Also, a bioreactor was designed to spray aerosolized culture in the surface of the BC membrane, which allows it to grow vertically [129]. A pilot semi-continuous automated static culture bioreactor named as HoLiR®, was designed to harvest BC foils produced in static culture using conveyor, allowing a new BC sheet to form in

the medium surface, while the produced BC is submitted to an automated chemical cleansing [130]. This production method provided highly promising results that, in a near future, may represent enough BC production to meet the industrial demands, which include food, biomedical, electronic, textile, pulp and paper among other specialized companies.

*Food industry*

The first known application of BC was in the production of a food commodity in the Philippines, where it has been used as a desert for centuries. The fermentation of BC producing bacteria in coconut water gives rise to *Nata de Coco*, or *Nata de Pina* if the fermentation occurs in pineapple juice [95]. The classic production of this desert is quite simple; briefly the fruit juices are collected, poured into vessels and left to ferment. Due to its smooth texture richly embedded with the fruit flavour, the popularity of this desert was propelled overseas reaching southeast countries such as Japan, China and Indonesia. *Nata de Coco* also showed a high potential as a functional food since it lowers the cholesterol levels once ingested, which boosted the Japan trend of including BC into diet drinks [131]. BC has shown a great potential as food additive, namely BC retrieved from agitated cultures, as it showed an enhanced emulsifying effect [132]. Moreover, BC is used as a food thickener, bulking agent and stabilizer [77, 110, 121, 133].

A promising and still unexploited application, is the use of BC as an active packaging material. BC besides being edible has an enormous potential to be a carrier of antimicrobial agents or antioxidant ingredients [134]. Some studies suggested the efficiency of BC as an antimicrobial package to be used in processed meats, however results on such applications are still preliminary (Table 2.2.1.).

*Biomedical industry*

Biocompatibility is a key feature of any material that is ought to be applied in a medical device or biomedical product. The *in vivo* subcutaneous implantation of a BC membrane into mice corroborated the *in vitro* biocompatibility tests, where BC showed no cytotoxicity [117, 135, 136]. Additionally, BC membranes flexibility and shape retention, good permeability to gases, and high wettability favoured its use as a temporary skin replacement, after skin injury or burn, as proven by the successful commercial applications such as Xcell®, Gengiflex®, Biofill® [137]. Based on the referred BC properties, BASYC® was developed to replace damaged blood vessels. It consists in a 5 mm tube comprised mainly of BC with, an inner diameter of 1mm and wall thickness of 0.7 mm. Even so, it possess enough mechanical strength to withstand the blood pressure, while at the same

time it promotes cell adhesion without coagulation, thus showing good results when compared to the most used materials for the same application (Goretex® and Dacron®) [106, 138].

### 2.3.2. Fish gelatine

Proteins present themselves as biomacromolecules with an overwhelming array of properties and biological activities, due to their diverse amino acid composition and architectural conformation [139]. Protein-based materials that, at first glance, could be regarded as waste, may be transformed into high value products with remarkable active properties, besides their low cost [140]. The interesting barrier properties alongside with their eco-friendly origin and degradability, lead to exploitation of several protein-based polymers [141]. Among them is gelatine, which represents one of the most intensively used protein sources, with an increasing demand worldwide [142].

#### 2.3.2.1. Properties and origin

Gelatine is easily available, transparent, flexible, resilient and low cost rendering it as a preferred base material vastly used by food, pharmaceutical, cosmetic, photography and several specialized industries [69, 143, 144]. Alternative sources of gelatine are being explored to allow increasing its production, and diminishing the use of mammalian origin gelatine due to the food borne transmission of bovine spongiform encephalopathy as well as other prion outbreaks [143, 145]. Gelatine is produced through the partial denaturation of collagen, one of the most prominent proteins found in mesodermal tissues and is mainly extracted from bones, skins, tendons and hides, which comprises all 20 amino acids in its sequence [146, 147]. Gelatine type A and type B are obtained by acid and alkali processing respectively [144, 148]. The origin and the processing methods of the collagen renders different gelatines with diverse physicochemical characteristics. All known 28 types of collagen comprise at least one helical block, consisting of three counter clockwise $\alpha$-chains that rotate around a single and common axis, in a clockwise fashion to form the characteristic triple helix [147, 149, 150]. This distinctive architecture is stabilized and interlaced by hydrogen bounds, formed by the amino acid triplet repeat of Glycine-X-Y; the imino acids proline and hydroxyproline are the most frequent amino acid residues found in this triplet [149, 150]. Although the secondary amino acid hydroxyproline is a relatively rare in organic tissue composition, it is found in high concentrations in collagen-like structures, and its prevalence is referenced as possessing a major role in the stability of the triple helix [151, 152], which ultimately

influences the gelling strength, viscosity and melting point of the downstream gelatine. The inedible fish constituents abundant in collagen such as, skin, fins, maws and bones may be processed into gelatine, and this "waste" accounts for approximately 30% of the processed catch and it was roughly estimated as 50 million tonnes of the worldwide fishery production in the year 2013 [153-155]. The typical amino acid profile of the fish gelatine (FG) is poorer in proline and hydroxyproline content (approx. 1 - 6% less) when compared to its mammalian counterpart [143, 156-158]. It is important to mention that using different approaches and technologies the melting point and bloom strength of FG can be improved [159-162].

Furthermore, bio-based polymers natively nanosized such as BC or with enhanced properties via "nanoconversion" by techniques such as electrospinning, may be further functionalized by [163]:

1) Coating – addition of a thin layer to the surface of the bio-based materials with a material that will improve, or will attribute an action/function to the films;
2) Blending –incorporation of functional nano elements into the bio-based polymer matrix, such as a functional protein;
3) Chemical or physical modification – chemical reactions may improve the properties of bio-based materials, namely their barrier properties. For instance, citric acid, once added to starch films enhanced its water vapour permeability barrier properties.

The active antimicrobial agent used was the protein lactoferrin isolated from bovine milk, which plays a key role in the host innate immune system, usually exerting their biocide action by interacting on the microorganism's surface, consequently destabilizing the membrane, increasing permeability and causing cell lysis. Bactericidal action in such conserved and unanimous cell structures confers to these proteins an high unspecific action and the ability to hinder the development resistance mechanisms [8].

## 2.4. Antimicrobial agent

### 2.4.1. Bovine lactoferrin

Glycoprotein lactoferrin (LF) was first reported in 1939 [164] and first isolated in 1960 [165, 166], and since then it collected a remarkable amount of roles and activities, inspiring Baker and Baker [167] to describe it as "classic multifunctional moonlighting protein". Expressed in a multitude of mammals, albeit it is also found in rainbow trout fish eggs, and is regarded as an important

member of the innate immune system [168-171]. Thus, as a member of the host first line of defence, LF is constitutively present in several exocrine secretions, such as milk, colostrum, saliva, tears, semen, uterine, bronchial and nasal fluids, secreted by epithelial cells, and it is also present in secondary granules of polymorphonuclear neutrophils [172-174]. Among its host protective effects are the antimicrobial activity, that include biocide actions against a broad spectrum of bacteria, yeast, virus, and protozoa, also bacteria anti-adhesion; immune regulation; antioxidant; adjuvant promotion of beneficial micro flora establishment; anti-tumour and prevention of tumour metastasis [171, 175-181]. LF bactericidal action is inherent to its aptitude to bind to (1) specific bacteria surface proteins present in a wide spectrum of bacteria, (2) lipotheichoic acid of Gram-positive, (3) porins and (4) lipid A component of lipopolysaccharide (LPS) of Gram-negative bacteria [182-185]. All these host safeguarding properties lead to the hypothesis that its concentration in colostrum and milk is directly proportional to the extent of the period of time in which the new-born is dependent on its progenitor [186]. The LF homology with serum transferrin (around 60%) included it into the transferrin family, alongside with ovotransferrin, carbonic anhydrase and melanotransferrin [168, 187, 188], albeit LF highly distinctive characteristics. LF has a high cationic net charge, and consequently a high isoelectric point (pI) of approximately 9 (the highest pI of the transferrin family). Also LF possesses an α-helix connective lobe linker (an exclusive trait within transferrin family), and is able to withhold its iron binding capacity at low environmental pH between 3 and 4 (while the lowest Fe binding pH of transferrin is 5 to 6) [167, 181, 189]. This last referred feature is an important immunologic reference, since during pathogen invasion, low pH values may be reached at infection sites as a consequence of the release of both active leucocytes products and pathogen's metabolite release [181].

All LF numerous roles and activities, together with its near omnipresence, including in the restricted list of proteins that are able to cross the blood brain barrier [190, 191], leads to its classification as an outstanding glycoprotein. This thesis explored the antimicrobial properties of bovine lactoferrin (bLF) for the development of active packages, therefore a description of the protein and properties is provided below.

#### 2.4.1.1. Structure

Synthesised in a singular protein containing a 689 amino acids chain with approximately 80 kDa from cDNA with 2.2 kb, bLF is architecturally arranged in two elliptic globular domains [189, 192]. The lobes share 37% amino acid homology and are connected by a 3 turn α-helix lobe linker

sequence (334–344 amino acid) and tightly packed together by hydrophobic interactions (Figure 2.4.1.) [187, 189, 192, 193].

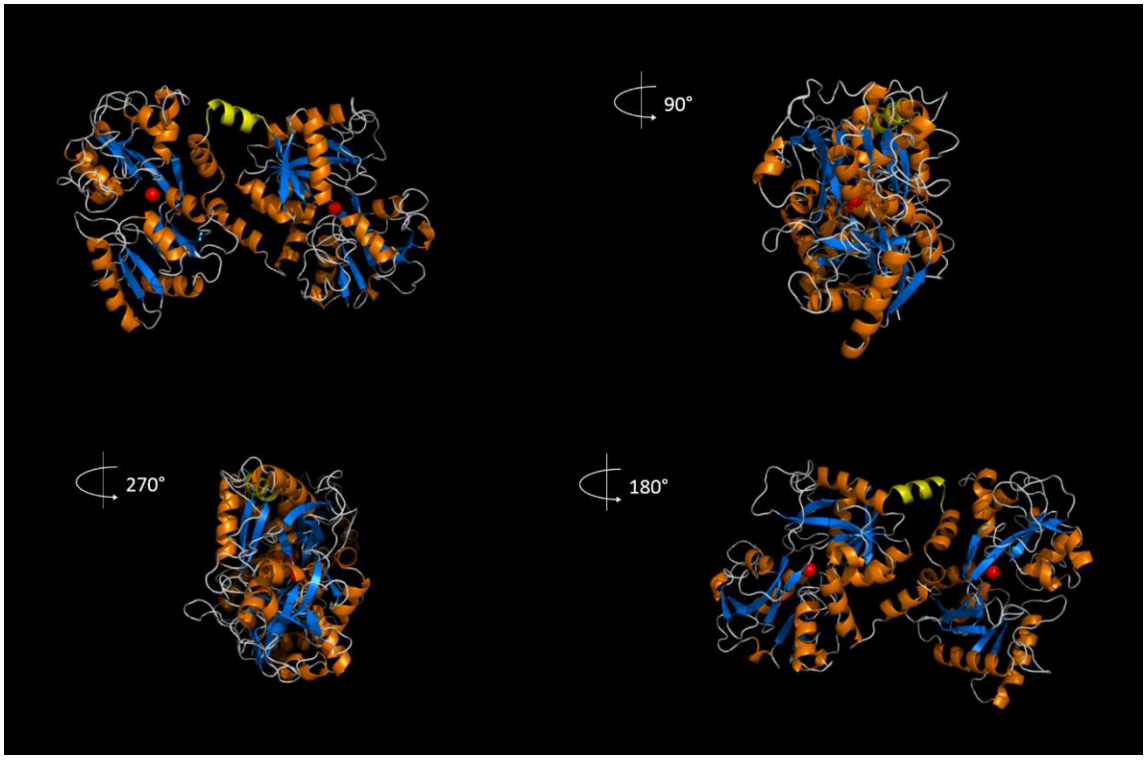


**Figure 2.4.1.** Bovine lactoferrin structure: β-sheets are highlighted in blue, α-helix are highlighted in orange, interlobe linking sequence is highlighted in yellow and iron atoms are represented as red spheres. Images obtained using Protein Data Bank file "1BLF" in PyMOL software (DeLano Scientific).

Each lobe is further divided into two domains: the N-terminal lobe is divided into N1 composed of 1 to 90 plus 251 to 333 amino acid residues and N2 domain comprising 91 to 250 amino acid residues; whereas the C-terminal halve is divided into the C1 region possessing 345 to 431 plus 593 to 676 amino acid residues and the C2 domain with 432 to 592 amino acid residues (Figure 2.4.2) [189]. Each lobe possess one conserved cleft with high affinity towards $Fe^{3+}$ composed of two tyrosines, one histidine and one aspartic acid residues [189]. The $Fe^{3+}$ chelating occurs with the synergistic binding of carbonate ion ($CO_3^{2-}$) and is completely reversible even though its high dissociation constant ($K_d$) ~$10^{-20}$ M affinity [167, 194]. Natural bLF possess 10-30% of iron saturation and hololactoferrin and apolactoferrin are the names used to describe iron saturated and iron free LF, respectively [172, 195]. bLF possess five typical n-glycosylation sequences composed of asparagine-X-serine-threonine, at asparagine: 233, 281, 368, 476 and 545 [189]. Unlike all the other four glycosylation sites, whose side chain are constantly bounded to a glycan, asparagine 281 glycosylation only occurs in 30% of bovine colostrum and 15% of bovine milk [196]. bLF-a is the bLF with the 5 sites glycosylated (Mw = 84 kDa) whereas bLF-b only has 4 glycosylation sites occupied (Mw = 80 kDa) [197]. Higher glycosylation levels lowers the susceptibility to enzymatic

and pH proteolysis [189, 196, 198]. Even when it is fully glycosylated, bLF is still 100 fold more susceptible to trypsin degradation than the human portein (hLF) [196]. Some of the carbohydrate groups identified in the glycosylation sites are N-acetyl-glucosamine and β-1.4 mannose, D-galactose and N-acetylneuraminic acid [199].

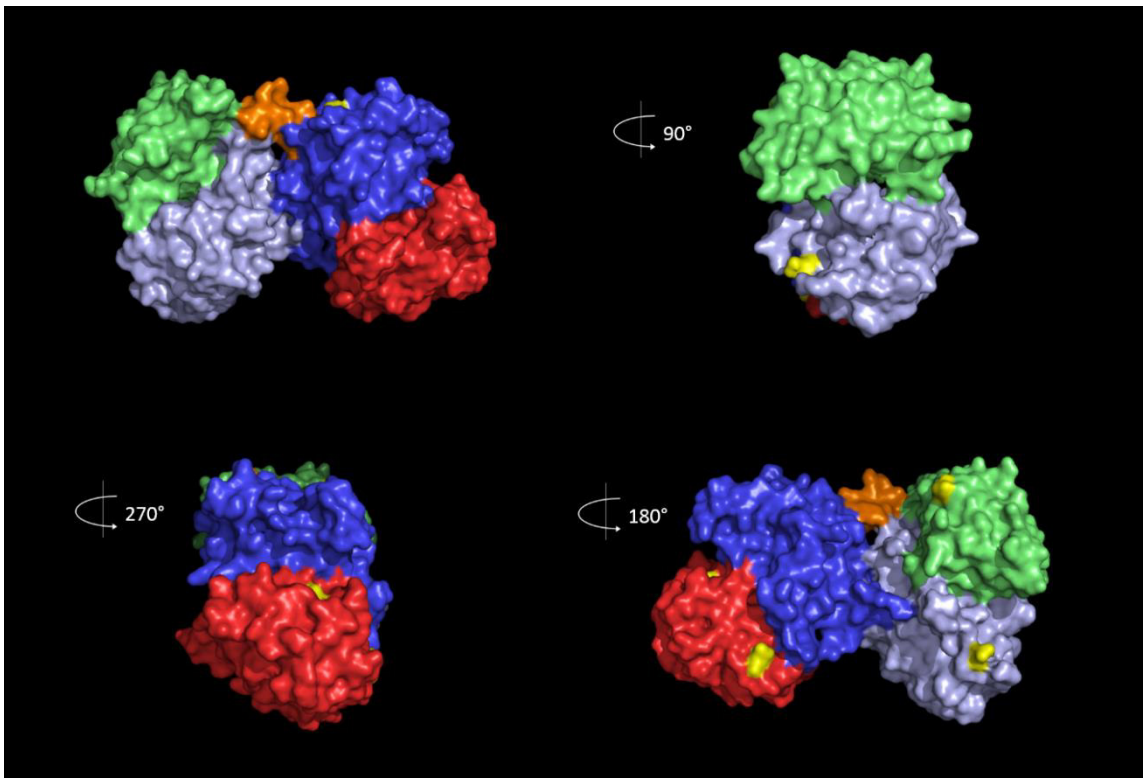


**Figure 2.4.2.** Bovine lactoferrin (bLF) domains: "N1" grey, "N2" green, "C1" blue and "C2" red. Highlighted: "linker" orange, "potential N-glycosylation site" yellow. Images obtained using Protein Data Bank file "1BLF" in PyMOL software (DeLano Scientific).

Figure 2.4.3. highlights the surface amino acid residues responsible for the +13 cationic charge of bLF. Although, bLF surface also possess a high propensity to amphiphilic behaviour, since its surface is heavily populated by hydrophobic residues (Figure 2.4.4.). Amphiphilic proteins with strongly cationic nature possess the essential traits for antimicrobial action [200, 201].

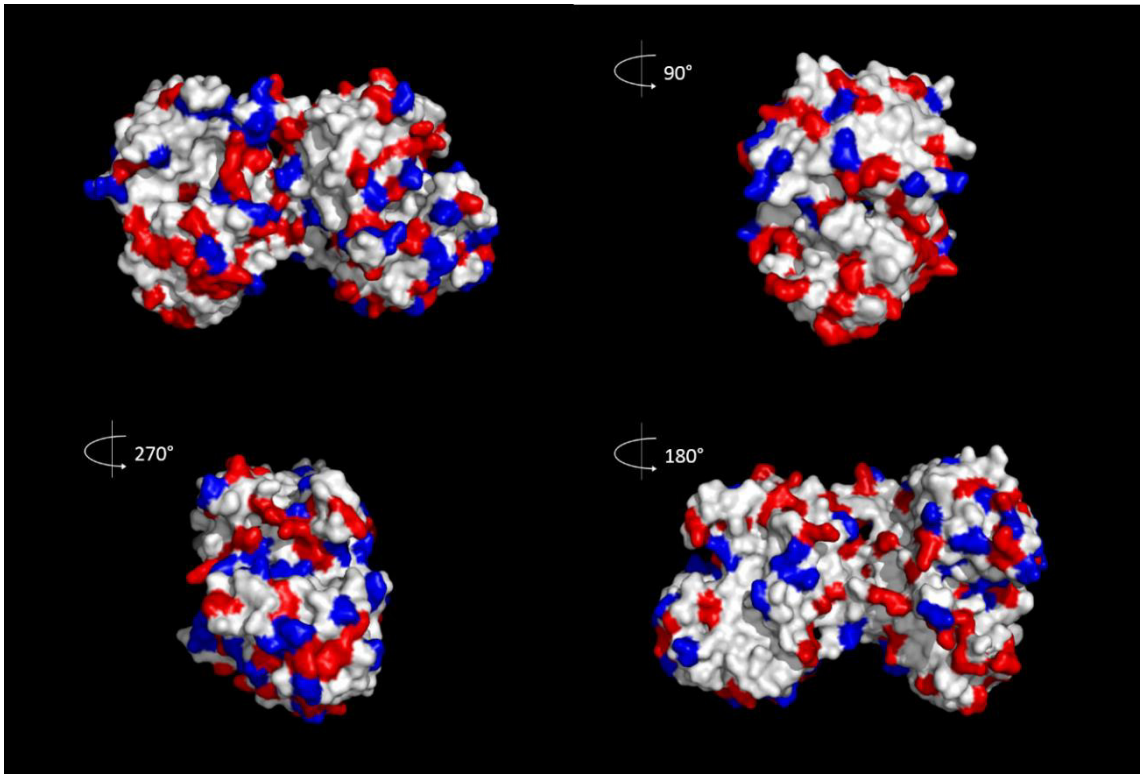


**Figure 2.4.3.** Bovine lactoferrin (bLF) polar amino acid residues at pH 7.4 [202]. Highlighted in red: positive amino acid residues (arginine, histidine and lysine), highlighted in blue: negative amino acid residues (aspartic acid and glutamic acid). Figure obtained using Protein Data Bank structure file "1BLF" in PyMOL software (DeLano Scientific).

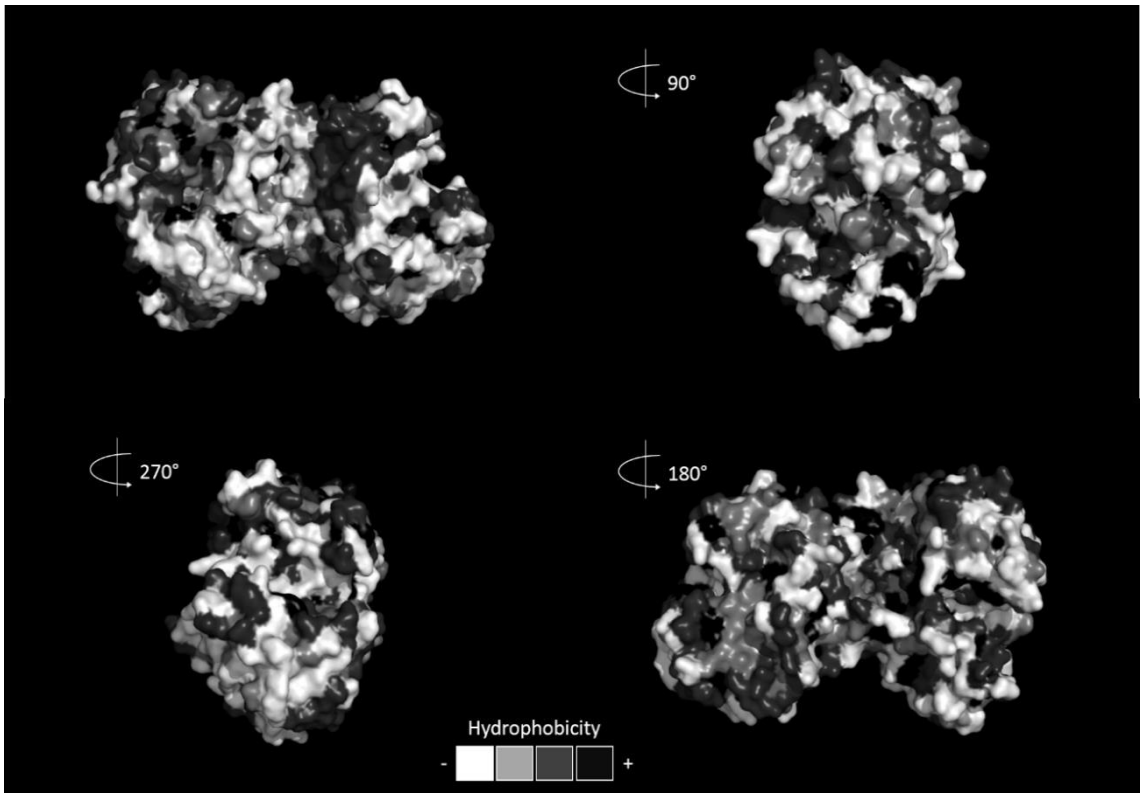


**Figure 2.4.4.** Bovine lactoferrin coloured using a hydrophobicity scale (increasing hydrophobicity): the polar residues (both positive and negative) where coloured in white, the neutral residues were coloured grey light, the hydrophobic coloured in grey and finally the aromatic residues were coloured in black. Images were obtained using Protein Data Bank file "1BLF" in PyMOL (DeLano Scientific).

#### 2.4.1.2. Active peptides

The highly cationic bLF´s N-terminal possess domains that when standalone, bear an even more potent bactericidal action. Peptide LF1-11: $^{1}$APRKNVRCTI$^{11}$, Lactoferrampin: $^{268}$WKLLSKAQEKFGKNKSR$^{284}$, Lactoferricin B: $^{17}$FKCRRWQWRMKKLGAPSITCVRRAF$^{41}$ (Figure 2.4.5. a)) [169, 203]. Interestingly, Bellamy and collaborators [204] corroborated the absence of bactericidal activity on the Lactoferricin B C-terminal corresponding sequence $^{356}$KKCQQWSQQSGQNVTCATAST$^{376}$ (Figure 2.4.5.b)), most likely due to the significantly distinct identity with a lower sequence percentage of cationic residues (38% vs 8%), and lower percentage of aromatic residues (19% vs 4%). The peptide release of these peptides may occur in the infection site, due to the presence of both pathogen and host proteolytic enzymes, or in the gastrointestinal tract and exocrine secretions due to pepsin activity [204-206].

#### 2.4.1.3. Activity

Universally, bLF is considered as a highly multi-functional glycoprotein, owning a number of regulating roles and direct actions that are discussed below.

*Antibacterial activity*

The primary bactericidal mode of action attributed to bLF is intrinsically related to its iron chelating activity. Its potent iron binding capacity may deprive the environment of this essential micronutrient, blocking several essential pathogen's biochemical reactions, namely ATP synthesis by oxygen reduction during the Krebs cycle, thus exerting a bacteriostatic action [207-210]. However, it is important to refer that unspecific iron independent bactericidal activity has been extensively reported [168, 192]. The bactericidal action of bLF may be distinct for Gram-negative and Gram-positive bacteria, however some actions are similar for both types.

The surface net charge of bacteria is unanimously negative. The major components of their plasma membrane are the anionic phosphatidylglycerol, and zwitterion phosphatidylethanolanine, thus the overall bacteria cytosol membrane charge is negative. [8, 211-213]. Additionally the cell envelope of Gram-positive bacteria possess negatively charged teichoic acid groups in its surface [214].

Due to the high cationic charge of bLF, this protein is attracted to bacteria surface by electrostatic tropism [213, 215]. Once bLF is in contact bacteria's surface, several events may unfold, namely the Shai-Matsuzaki-Huang mechanisms [8, 211, 216, 217]. The bLF amphiphilic nature, and/or bLF antimicrobial peptides that may be released by bacteria proteolytic action, interact with the bacteria membrane through two main possible mechanisms, carpet and "barrel-stave" [211, 216,

217]. bLF may bind to a bacteria surface, covering it like a carpet, thus causing a membrane charge shift, altering the cell homeostasis, and increase metabolic burden, moreover if the protein concentration is high it causes membrane permeation and consequent disruption [211]. On the other hand, the "barrel starve" mechanism consists in the formation of a transmembrane pore due to the bundle of amphiphilic domains [216, 217]. The N-terminal domain of bLF was described as highly flexible and with a remarkable cationic nature, thus being able to form transmembrane pores [180, 212].

One noteworthy action of this protein is its capability to bind specifically to the LPS hydrophobic and anionic component, lipid A. Two LPS binding sites were identified in bLF, a high affinity in the N-terminal and a low affinity in the C-terminal. Elass-Rochard and colaborators [218] reported the N-terminal bLF LPS binding loop, comprised in the sequence $^{28}$KLGAPSI$^{34}$, possessing $K_d$ equal to 4.5 nM (Figure 2.4.5.b)). Whereas its lower affinity C-terminal hold has a $K_d$ = 576 nM. Besides the bLF direct binding to LPS, Rossi and co-workers [219] demonstrated a remote action of bLF analogous to the mechanism exerted by ethylenediaminetetraacetic acid (EDTA) and polymyxin B, that ultimately leads to LPS release [220, 221]. LPS inner core is stabilized by $Ca^{2+}$, therefore its depletion via chelating by the N-acetylneuraminic acid present in the glycan moiety of bLF, compromises its integrity, ultimately causing an increase on permeability and consequently cell death [219]. So, the bLF's LPS releasing efficiency is dependent on the saturation of LPS binding sites and $Ca^{2+}$ saturation of N-acetylneuraminic acid.

Another important role of bLF is its ability to prevent the adhesion and invasion of several bacteria, through its catalytic action, or anti adhesive binding sites. bLF was found to protect intestinal epithelium cells by hindering the adhesion of the major foodborne *E. coli* O157:H7, probably through its proteolytic action over the products of type III secretory system [222]. Several active proteins that mediate the adhesion and invasion of host cells are secreted by Type III, a highly conserved active protein delivery mechanism in multiple Enterobacteriaceae [222-224]. A serine-like proteolytic activity, was found in less than 10% of bLF molecules, and, alike its human counterpart it is able to cleave arginine rich domains of two colonization factors of *Haemophilus influenzae* [225-228]. Sub-bactericidal concentrations of bLF inhibits the invasion of group A Streptococci, an infamous group of bacteria specialized in invading epithelial and endothelial cells [229]. Additionally, bLF's sequence 473-538 prevented the adhesion of *Streptococcus mutans* (Figure 2.4.5.a)) [230]. bLF's antibacterial activity depends on its concentration and inoculum size [222, 231]. bLF bactericidal activity has been proven against *Helicobacter pylori* [232], *Clostridium*

spp. [233], *S. aureus* [234] and *E. coli* [235]. bLF antibacterial activity is not unanimous nor indiscriminate, since this protein promotes the growth of *Bifidobacterium* spp., a beneficial member of the gut flora [178, 192, 195].

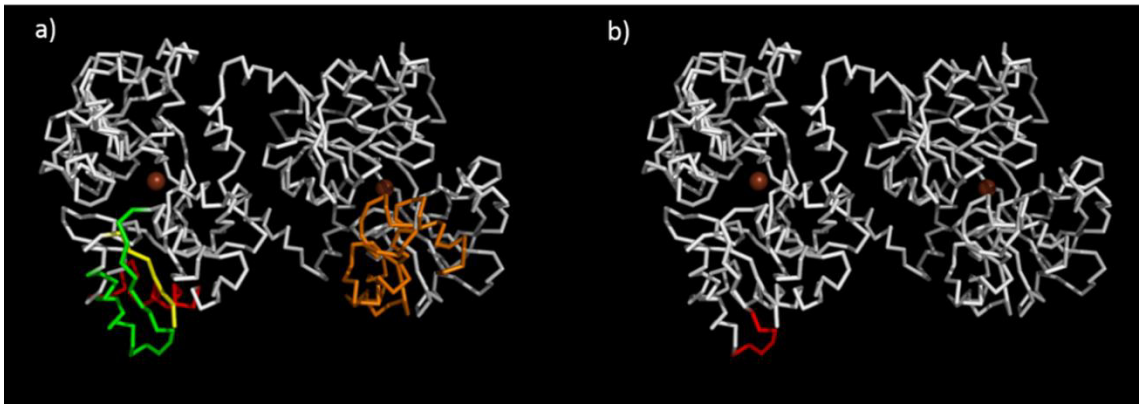


**Figure 2.4.5.** Schematic representation of bovine lactoferrin: **a)** notable antimicrobial peptides: lactoferricin B (green), bLF1-11 (yellow), lactoferrampin (red) (N-terminal) [169], and functional anti-adhesion domain towards *Streptococcus mutans* (orange) (C-terminal) [230]; **b)** lipopolysaccharide (LPS) high affinity loop (red) (N-terminal) [218]. In both images the iron atoms are represented as brown spheres. Images were obtained using Protein Data Bank file "1BLF" in PyMOL (DeLano Scientific).

*Antimycotic, antiparasitic and antiviral activity*

Protozoa bear negatively charged surfaces, and thus the highly cationic nature of bLF may be the mechanism underlying its biocide action, although it has also been reported to exert an indirect action against *Toxoplasma gondii* [236]. Even though with a direct low efficiency, bLF is able to reduce the viability of *Candida* cells, and once ingested bLF triggers a systemic effect in the host, upregulating the immunologic action against *Candida albicans*, tinea corpis and pedis promoting patients cure [237-241]. Two mechanisms have been proposed to explain how bLF hinders viral infection, the first is based on the bLF binding to viral receptors in host cells, impeding their access or recognition; and the second is based on bLF direct interaction with viral proteins [242, 243]. Several virus have been inhibited by bLF including human cytomegalovirus, Influenza A, human simplex virus, hepatitis C virus and rotavirus [242-248]. Besides its direct antiviral infection activity, the ingestion of bLF increases the population of lymphocytes $CD4^+$, $CD8^+$ and natural killer (NK) cells in the intestine [249]. These cells are vital for the prevention of early viral infection stages [250, 251].

*Antioxidant and immunoregulatory activity*

The iron binding capacity of bLF prevents lipid rancidity, since the chelation $Fe^{3+}$ prevents its subsequent reduction to $Fe^{2+}$ when in contact with ascorbic acid [252]. The presence of $Fe^{2+}$ in

contact with oxygen generates the radical $O_{2^-}$ and the reactive element $H_2O_2$ that produce lipid aldehydes. bLF immunoregulatory role is highly complex and yet not fully understood, although its active participation in the modulation of the immune system is unquestionable. Macrophages, mastocytes, NK cells, eosinophils, basophils and dendritic cells, members of the innate immunity, possess pattern recognition receptors that allow the identification of characteristic invading microbial elements, named pathogen associated molecular patterns (PAMPS), such as LPS, peptidoglycan, and lipoteichoic acid, moreover they are also able sense the presence nitric oxide (NO) and cell debris [179, 214]. Once these elements are recognized, immune response is triggered, pro-inflammatory cytokines tumour necrosis factor α(TNF-α) , interleukin 1 (IL-1) and interleukin 2 (IL-2) are secreted to recruit leukocytes, fibroblasts and endothelial cells. bLF may promote the inflammatory activity, recruiting NK cells, or it can down regulate the inflammatory process, by bind LPS, making it inaccessible for recognition, or it may stimulate the production of anti-inflammatory interleukins 4, 6 and 10 (IL-4, IL-6 and IL-10) [179, 253, 254]. Once ingested, bLF promotes the secretion of interleukin 18, IL-10 and interferon γ in intestinal epithelial and intraepithelial lymphocytes [255-257]. Moreover, bLF has been reported to activate the classical pathway of the complement system [258].

*Resistance mechanisms*

Some microorganisms may present defensive mechanisms towards bLF antimicrobial activity. To counter the environmental hypoferremia, some bacteria produce extremely potent $Fe^{3+}$ binding molecules named siderophores, which are able to re-supply the $Fe^{3+}$ needed for the normal functions of the microorganisms [210, 259]. Others microorganisms, such as *Neisseria meningitides, Neisseria gonorrhoeae* and *Moraxella catarrhalis* synthesize periplasmatic complexes that are able to recover $Fe^{3+}$ from bLF [260]. *S. aureus* and *Staphylococcus epidermidis* produce exopolysaccharides with high molecular weight (commonly known as slime) as a response to the hypoferremia [261].

#### 2.4.1.4. bLF gastrointestinal digestion

bLF is a constitutive protein of several food commodities, namely in beef, as in the polymorphonuclear neutrophils present in its residual blood (approximately 1.6 to 13.3 ng bLF $g^{-1}$), natural cheese (approximately 3 mg bLF $g^{-1}$) and raw milk (approximately 0.1 – 0.4 mg $mL^{-1}$) [168, 175, 262]. Due to the several, direct and systemic, health benefits exerted by the ingestion of bLF [171, 263], and the absence of negative effects when high concentrations were

administered (up to 2 g $kg^{-1}$ $day^{-1}$ in mice and 7.2 g $day^{-1}$ in humans) [248, 264], the protein was used in the formulation of a commercial infant supplement in Japan, almost 30 years before its approval by EFSA [175, 265]. 60% of bLF resists the proteolytic action of the gastrointestinal digestion, thus, once in the small intestine, it is able to exert unimpaired all its beneficial actions [171, 266, 267]. The protein possesses important antioxidant and immunostimulator functions once ingested [179, 268-271], and therefore a highly favourable element of functional foods [271]. Moreover, bLF has proven to be very useful in the anti-adhesion of microbial populations and direct antimicrobial activity [255, 262].

#### 2.4.1.5. Purification and applications

bLF is a milk serum protein and therefore, it can be found in high amounts in the whey fraction and its concentration in milk is low [272, 273]. Still, mature bovine milk possess between 10 – 200 μg $mL^{-1}$, although it may reach 1 – 5 mg $mL^{-1}$ in colostrum, and during mammary involution bLF concentration spikes to 20 – 100 mg $mL^{-1}$ [186, 274, 275].

An industrial scale high yield purification process for the recovery of bLF was first used in 1985 by the Belgium Company Oleofina [175, 189, 219, 255]. Skim milk, colostrum, milk whey and cheese whey are the main sources of bLF, typically purified as described by Wakabayashi and collaborators [175]. The first step comprises the bLF source filtration, followed by a cation-exchange chromatography (usually carboxymethyl-sephadex) without pH adjustment; subsequent column washing process with crescent concentrations of NaCl; bLF is eluted at 5% (w/v) NaCl; the NaCl is then removed using diafiltration, and finally bLF is sterile filtered and freeze dried. This purification process yields approximately 100 mg $mL^{-1}$ [276], however other purification methodologies can be used to recover bLF such as magnetic affinity separation, chromatography with hydrophobic affinity, ultrafiltration, chromatography using hydroxyapatite, Cibacron Blue affinity chromatography and adsorptive membrane chromatography [277-284]. As previously mentioned bLF is a safe, accessible and affordable polypeptide source, used mainly by nutrition, cosmetic and health industries, with a worldwide increasing production (Figure 2.4.6.) [175, 255, 285]. Applications of bLF include the development of products targeting the gut microbiota, and products that make use of its anti-infection and antioxidant properties, such as in infant formulas, tablets, yoghurt, skim milk, drinks and pet food. Antioxidant and antimicrobial properties of bLF are used to improve cosmetic formulas, and also tooth paste, mouth wash and tooth-friendly chewing gum [175].

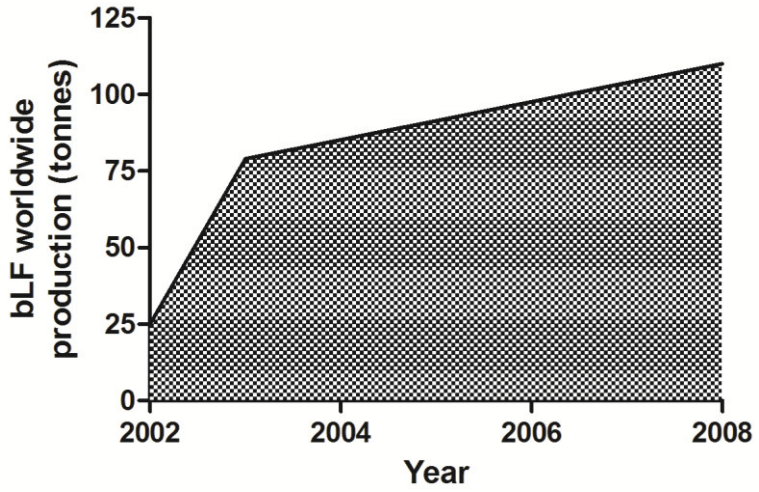


**Figure 2.4.6.** Bovine lactoferrin estimated worldwide production. Data collected from Tomita *et al.*, Wakabayashi *et al.* and Legrand *et al.* [175, 276, 286].

## 2.5. European Union legislation concerning active and intelligent materials and articles intended to come into contact with food

Prior compliance with European Union requirements for food and food contact materials is imperative for its entry in the European Union market. The European Union possesses strict regulations to prevent the transmission to food, of potentially hazard substances used in the manufacturing of food contact materials/products. Food commodities possessing a specific group of contact materials or ingredients will be rejected from the European Union market if a single of the requirements is not met. A summarised overview of the European Union regulation towards food contact materials and ingredients is described below.

The European Parliament Regulation (EC) N° 1935/2004 [18] presents the framework legislation for all items, ingredients and materials that are in direct contact, or are intended to be in direct contact, or have a probability to come into direct contact with food. This regulation does not apply to coating materials that are an intrinsic part of the foods constitution and may be consumed by it (for instance fruit rind). This framework legislation defines the general principles and groups of food contact materials and ingredients, the necessary procedure for the authorization of these materials and ingredients and also refers to the Community and national reference laboratories to test food contact materials. The reference laboratories insure the quality and uniformity of the scientific results, and are regulated by the framework described in the European Union Regulation (EC) N° 882/2004 [287].

The general principles required by the Regulation (EC) N° 1935/2004 [18] for food contact materials are the following: food contact materials must be safe; they cannot transfer their components into food that could jeopardise the consumer's health; or change in an unacceptable manner or degenerate the food's composition and organoleptic traits. Moreover, the food contact materials must be manufactured using good manufacturing practices, and bear a label that clearly states that it is intended for food contact or have the "glass-and-fork" symbol, and must also explain how the item is used if necessary. Also, the information present in the label as well as the overall presentation of the product cannot mislead the consumer. Finally, the materials must be traceable throughout the manufacturing and distribution chain. Seventeen groups of materials and articles are identified in the Regulation (EC) N° 1935/2004 [18]:

1) Active materials and intelligent materials and articles;
2) Adhesives;
3) Ceramics;
4) Cork;
5) Rubbers;
6) Glass;
7) Ion-exchange resins;
8) Metals and alloys;
9) Paper and board;
10) Plastics;
11) Printing inks;
12) Regenerated cellulose;
13) Silicones;
14) Textiles;
15) Varnishes and coatings;
16) Waxes;
17) Wood.

The Regulation possesses specific measures for active and intelligent materials and products, as well as for ceramics, regenerated cellulose, plastics, and recycled plastics.

Since active food contact materials and articles may change the organoleptic traits of the food, the extent of the food modification has to be within the limits allowed by the Council Directive 89/107/EEC [288] amended by Directive 95/2/EC [289], subsequently amended by Directive

2001/5/E [290] that states that the food additives may not endanger the consumer's health at the purposed level of consumption. More importantly, these additives may only be applied if there is a viable technological need, which cannot be replaced by other practicable technologies and economic investments.

If active materials release substances into the food product, these substances must comply with food legislation, for instance food additives framework legislation.

### 2.5.1. Petition for legal approval of new materials and/or substances to be used in direct contact with food

Prior to the application for authorisation of a new material and/or substance, proof that it satisfies the general principles stated before is imperative. If the Regulation general principles are met, an application can be submitted to a competent authority in any member state country that must include the name and address of the applicant, the technical documentation containing the material and/or substance information and its safety assessment data that will be published by the EFSA. A summary of this technical documentation must also be delivered. After receiving the application the competent authority must inform the EFSA and confirm the receipt of the application in a period of fourteen days. Additionally the competent authority must provide the application data and supplementary information to the EFSA, which in its turn will provide the application and supplementary information available for the other European Union Member States and for the Scientific Committee on Food. Then, the EFSA will publish the specific guidelines required for the approval, in the meantime, the applicant may confirm if the product is in accordance to the guidelines of the Scientific Committee on Food (SCF/PLEN/GEN/100). These guidelines state the conditions that establish the safety of migrating substances by assessing their potential hazard and toxicological information. These guidelines assumes that an individual consumer may ingest daily up to 1 kg of food in contact with the applicant material. The rate of migration and the level of exposure are directly proportional to the amount of toxicological information that will be required. For instance, if the applicant product possess a high migration (5 to 60 mg $kg^{-1}$ of food), a thoroughly and extensive toxicological study must be undertaken to determine its safety, whereas a low migration (< 0.05 mg $kg^{-1}$ food) only requires a reduced toxicological study. Briefly, some of the required information of the materials and/or substances that must be supplied to the Scientific Committee of Food by the applicant, includes the material/substance identity and physicochemical properties, a statement describing the intended use, and all the pertinent microbiological properties

of the materials/substance. The migration of the substance, is a key parameter, therefore the description of its impurities, its breakdown products and reactions if any, as well as the information concerning its endogenous concentration on the food products should be supplied. If the migration rate is known for non-food materials, this information must also be included. The migration study data may be supplied using standard conditions or under the worst case scenario conditions. If the substance is already approved and used in non-European Union Member States, such as Japan and United States of America, its approval information also be supplied to the Scientific Committee. All scientific toxicological data supplied to Scientific Committee for Food must be in accordance to the Good Laboratory Practice and according to the guidelines provided by the European Union or by the Organization for Economic Co-operation and Development. The toxicological data for low migration rate (<0.05 mg $kg^{-1}$ of food) only requires three mutagenicity tests:

1) Assessment of induction of gene mutagenesis in bacteria;
2) *In vitro* assessment of gene mutation in mammalian cells (preferably using mouse lymphoma TK assay (MLA) that includes a battery of tests to detect mutagenic and clastogenic events [291]);
3) *In vitro* assessment of the induction of chromosomal aberrations in mammalian cells.

The cases that possess a moderate migration (0.05 – 5 mg $kg^{-1}$ of food) require the 3 mutagenicity tests stated above, a 90 day oral toxicity assessment and scientific data proving the absence of accumulation in the human body. As for the products possessing a high migration rate (5 to 60 mg $kg^{-1}$ of food), these must provide the scientific data comprising the same 3 mutagenicity studies mentioned earlier, a 90 day oral toxicity assessment for two different species, the data comprising the absorption, distribution, metabolism and excretion profiles, studies on two different species concerning possible toxicity impacts on the reproduction and development, and finally long-term evaluation of the toxicity and carcinogenicity in two different species.

Based on the chemical nature of the material or substances additional assessment studies may be required for approval, namely immunotoxicity tests, peroxisomal proliferation evaluation, neurotoxicity or endocrinological assessment. Some of the substances that require these special tests include biocides, food additives and ingredients, polymeric additives and hydrolysable products. In the case of the use of biocides the applicant must prove that the use of the biocide substance is in no way a justification for the reduction in the standard hygienic protocols used in foodstuff handling. Also the applicant must provide information of the biocide intentional and non-

intentional release, the assurance of the non-generation of resistance and selectivity of the microorganisms present in the food.

If the materials are hydrolysable in contact with food, or during gastrointestinal digestion, the breakdown components, and the extent and ratio of the hydrolysis must be assessed. The toxicity of the non-hydrolysed material as well as of every individual hydrolysis products must also be evaluated.

Polymeric additives may require different sets of toxicological data, since fractions possessing a molecular mass below 1 kDa require the extensive toxicological evaluation whereas the fractions with a molecular weight above 1 kDa only require a limited set of toxicological assessment. As for the food ingredients and additives already approved by Scientific Committee for Food, it will probably only require providing information of the identification, properties and intended use. Additionally in the case of the additives, the information of its migration profiles is also required.

However, if the applicant possesses valid scientific knowledge of the identity, use and potential exposure to the petitioned substance, the applicant may deviate from these guidelines, if proper and solid scientific justifications are provided.

The authorization of the material/substance is granted once it is listed in the legislation. However, National legislation or specific industry codes of practice can be applied in materials that are not yet covered by a specific European Union legislation.

### 2.5.2. Additional legislation

The Regulation (EC) N° 1935/2004 [18] is supplemented by the European Commission Regulation (EC) N° 450/2009 [292] and amended by the Regulation (EC) N° 596/2009 [293], where some articles were revised to increase the agility and shorten the time limits of the regulatory procedures, in order to enhance competitiveness and innovation of the European industries, without compromising food safety.

The active and intelligent materials manufacturing must be in compliance with the European Commission Regulation (EC) N° 2023/2006 [294] that establishes the framework for good manufacturing practice for materials and products that are intended to come into contact with food listed in the European Commission Regulation N° 1935/2004 [18]. This regulation alerts for the implementation of good manufacturing practice in all industries of the European Union member States to ensure uniformity of products that are intended to come into contact with food. The good manufacturing practice define the quality and control actions required for achieving an overall

quality standard for food contact materials or products, and therefore insure the absence of substances that may jeopardize the consumer's health or cause an unacceptable change in the organoleptic properties or composition of the food. The material and products quality standards are maintained by a set of measures defined by the quality assurance system, which must be consistently applied. These measures include the hiring of skilled employees, the acquisition of the necessary equipment and the guarantee of the quality of the starting materials, in order to assure the final product quality standards. These measures also include the constant monitoring of the manufacturing line, to verify its compliance with the good manufacturing practices. Quality assurance system takes into account the size of the company, with a tighter control for bigger business.

The guidelines of good manufacturing practice include the control the application of inks in the food package including in its labelling. The ink migration profiles in the non-food contact side, in order to assure the inexistent migration to the food contact side. However if ink migration does occur to the food contact side, it may never be in concentrations that will endanger the consumer's health or bring unacceptable changes to organoleptic traits of the food.

The labelling of active and intelligent materials and articles is controlled by the Directive 2000/13/EC [295], whose main objective is to assure the uniformity of the foodstuffs labelling, and therefore bring equal competitiveness of food products in all European Union Member States, since standardize labels insure their legal compliance. Briefly, this Directive states that the labelling advertising used in food material as articles must not mislead the consumer, including the use "DO NOT EAT" message and figure in all non-edible parts of the food article whenever these elements may be perceived as ingestible. The labelling must also refer:

1) The name of the product by which it is sold;
2) The complete list of ingredients;
3) Some categories of ingredients require the stating of their concentration. These ingredients include the ingredients present in the name of the product by which it is sold, or is emphasized on the labelling in words or by pictures, or when the ingredients are essential to characterize the food;
4) Pre-packaged food require the denotation of the net quantity;
5) Must state the expiration date;
6) The obligatory reference to the special storage conditions when required;

7) It must state the name, or brand, and address of the manufacturer, packager, or retailer belonging to the European Community;
8) The detailed place of origin is required;
9) If necessary, instructions of use must be included;

Since active and intelligent materials and products are used during the manufacturing of the food, and are present, in an intact or altered form, in the finished product, they have to be included in the list of ingredients.

Novel ingredients must be applied in accordance to Article 4 of the Regulation (EC) N°258/97 [296], just as bLF was submitted by Netherlands Organisation for Applied Scientific Research (TNO) on behalf of Friesland Campina (formerly known as DMV International) in 2009, which lead to the legal acceptance of bLF as a food ingredient [265].

Finally, the materials intended to come into contact with food must be in accordance with the Regulation (EC) N°178/2002 [297], which sets the general principles of food safety. This Regulation includes the definition of food, the general food law, and the general responsibilities of food business operators.

Unfortunately there isn't yet a European Community regulatory framework to control the resulting environmental impacts of the use of active and intelligent materials and products.

The equivalent Portuguese legislative framework of Regulation (EC) N° 1935/2004, are the Law Decree N° 175/2007 [298] defines the authority responsible for enforcing the food safety law and procedures in Portugal, while the Law Decree N° 378/2007 [299] of the Portuguese Official Journal states the responsible Portuguese Authority to whom the petition for a new food ingredient, or new material or article that comes into direct contact with food has to be delivered.

# Chapter 3

## Periodate oxidized bacterial cellulose films functionalized with lactoferrin

# 3. Periodate oxidized bacterial cellulose films functionalized with lactoferrin


## Abstract

The potential of bacterial cellulose (BC) to become an edible functionalized membrane for food packaging application is huge. In this work, we proved that the BC functionalization with the broad spectrum antimicrobial protein bovine lactoferrin (bLF) was successfully achieved. The uptake of bLF significantly inhibited *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* as assessed by the disk diffusion susceptibility test. The dried and never dried BC membranes functionalized with bLF showed similar bactericidal efficiencies. Aiming at both the immobilization and increase of bLF uptake in the BC membranes, these were oxidized with sodium periodate ($NaIO_4$). Periodate oxidation leads to the formation of aldehyde groups in the D-glucan units of BC, which subsequently may react of the ε-$NH_2$ lysine groups, thus forming a covalent bond. The bLF uptake in the oxidized samples increased, however contrary to what was expected, the antimicrobial activity was impaired, as well as the mechanical properties of the membranes.


## 3.1. Introduction

Nowadays, food industry faces several challenges imposed by an increasingly more informed consumer community, which demands the minimal processed food, minimal use of petrochemical constituents in the packaging formulation and additionally favours convenient and time saving purchasing methods. In order to meet all these demands food industry requires an effective development of packaging technology. Numerous commercial applications, patents and research studies have been presented revealing packaging systems that interact actively with the encased food, or package headspace in order to constantly maintain or even improve the foods organoleptic traits, during its shipping and storage [2]. The use of bio-based materials as part of active packaging composition formulation possesses several advantages. It implies the use of renewable components with minimal carbon foot print. Usually bio-based materials are edible, which may allow its close contact with food, enhancing its protective effect over the food product, as well as simultaneously reducing the generated waste [300].

Bacterial cellulose (BC) is a bio-polysaccharide with a remarkable set of characteristics, some of them ideal to be used as an edible active packaging. These include high wettability that may improve the stability allied to impressive mechanical properties. Additionally, and more importantly BC has an enormous potential to carry a multitude of antimicrobial agents, which may increase safety and extend the shelf-life of food products [134].

Bovine lactoferrin (bLF) is a glycoprotein usually isolated from the whey fraction of the milk. bLF is an important member of the innate immune system, besides a great number of relevant roles have been attributed to it. Wide spectrum antimicrobial activity is one of the major activities exhibited by bLF. The potent iron binding domain, present in each of its bilobar structure, may chelate the environmental iron, preventing its availability, thus inhibiting the further microorganism development [189, 208, 209]. Additionally, bLF is capable of exerting a broad spectrum bactericidal effect by fatal destabilization of the bacterial plasmatic envelops through interactions induced by the bLF cationic net charge alongside by its hydrophobic domains [211, 212]. bLF is also able to bind to lipotheichoic acid groups present on the surface of Gram-positive bacteria, and to porins and lipid A component of lipopolysaccharide present in Gram-negative bacteria [182, 184, 185, 301]. Therefore, bLF is a promising candidate to combine in active packaging bio-based materials, as for example the BC membranes. However, strategies to increase the bLF uptake by the BC membranes, by covalently binding the bLF protein to BC membranes, are required. An example of a possible strategy is through the oxidation of the BC membranes. When an individual glucan unit of BC is oxidized, an aldehyde is formed in C2 and C4, thus allowing subsequent nucleophilic attack by ε-NH2 attack to generate carbinolamines [302, 303]. These ε-NH2 are present in lysine residues, therefore the oxidation of polysaccharides, such as BC, is a simple method to achieve covalent bounds between proteins and polysaccharides. Sodium periodate is a superior oxidizing agent, additionally [304]. The immobilization of bLF in the BC membranes, besides allowing a higher protein uptake, may also permit a constant concentration of bLF, which can prolong the bLF antimicrobial activity in the contact interface.

## 3.2. Materials and methods

### 3.2.1. Microorganisms, culture medium, solutions and materials

The microorganisms used to produce BC were *Gluconacetobacter xylinus* American type culture collection (ATCC) 53582 and *G. xylinus* ATCC 700178. The culture medium used was Hestrin

Schramm (HS) [305]. Briefly, its composition comprised 2% (w/v) of glucose (Sigma-Aldrich), 0.5% (w/v) peptone (Sigma-Aldrich), 0.5% (w/v) of yeast extract (Himedia), 0.34% (w/v) of $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich), 0.15% (w/v) citric acid (Pronolab). The final pH was adjusted to 5.5 using HCl 18% (v/v) (Sigma-Aldrich); solid HS medium also includes 2% (w/v) of agar. The HS solid and liquid medium, and phosphate buffer saline (PBS) were autoclaved at 121 °C, 1 bar for 20 minutes before use. The composition of PBS (pH 7.4) was (w/v), 0.8% NaCl (Fisher), 0.02% KCl (Fischer), 0.14% $Na_2HPO_4$ (Sigma) and 0.03% $KH_2PO_4$ (Flücka). PBS was used in all inocula transfer and dilutions, as well as in the preparation of the bLF solutions. bLF was purchased from DMV International (USA) with a reported composition of 96% in dry weight percentage of protein comprising approximately 120 pp of iron, 0.5% (w/w) of ash and 3.5% (w/w) of moisture. All bLF solutions used to functionalize BC membranes were prepared immediately before use. The intended dry bLF mass was dissolved in PBS, using mild agitation at room temperature, and afterwards were filter-sterilized (0.22 µm). The bactericidal assessment assays were preformed using *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* cultured in Muller Hinton (MH) agar (Himedia).

### 3.2.2. Biomass and BC production by *Gluconacetobacter xylinus* ATCC 53582 and *G. xylinus* ATCC 700178

The most commonly used BC producing species is *G. xylinus* [88]. During the production of BC in static conditions, it is not possible to accurately determine the cell density without disturbing the BC pellicle being formed at the culture surface. Therefore, to evaluate the BC production of the two strains regularly used in our laboratory, ATCC 53582 and ATCC 700178, the biomass and the BC production were evaluated according to the methodology presented in Figure 3.2.1.. The pre-inoculum for each strain was prepared from a fresh HS agar plate. A McFarland 0.5 turbidity standard was used as a reference to inoculate a static culture with 72 hours at 30 °C, in a 1/5 HS culture medium to flask volume ratio. The assay was performed using several culture flasks, each with the same amount of HS culture medium (1/5; medium culture volume/flask volume), all inoculated using the same volume of pre-inoculum (200 µL). Each flask corresponded to a time point of the experiment, *i.e.* each flask was sacrificed at a given time point to enable the determination of the biomass and BC concentration without compromising the following time points. Once the culture medium was extensively agitated, and the BC membrane if any, was carefully removed, the BC membrane was washed thoroughly with sterile water, dried at 50 °C

and weighted. Both culture medium and washing water were filtered through a 0.45 µm filter, dried at 50 °C and afterwards weighted. After the BC membrane weighing, it was immersed in a 4% (w/v) NaOH solution for 24 hours, to remove the bacteria debris. Afterwards, the BC membrane was thoroughly rinsed with water until the membrane pH was equivalent to the water pH, thus removing the remaining NaOH. Then, the membranes were dried (50 °C) and weighed (Figure 3.2.1.). The total biomass for each time point was calculated using Equation 3.2.1.:

$$Total\ biomass = culture\ medium\ biomass + bacterial\ cellulose\ and\ biomass - bacterial\ cellulose$$

**Equation 3.2.1.**

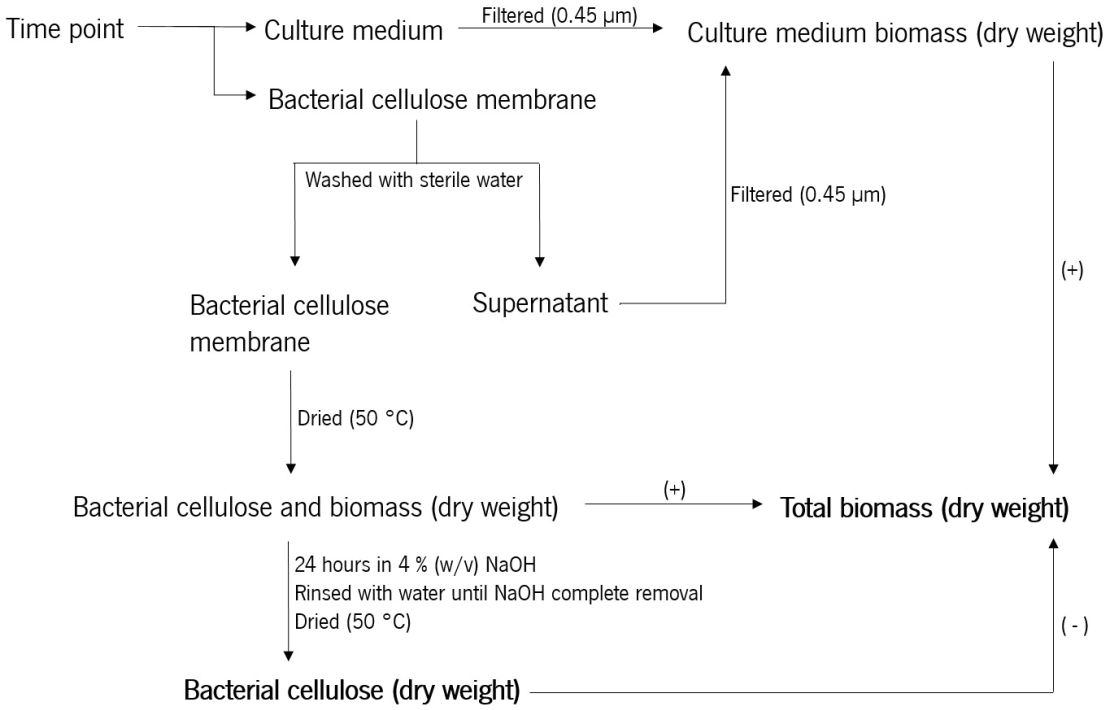


**Figure 3.2.1.** Methodology used to determine the biomass and bacterial cellulose production in *Gluconacetobacter xylinus*.

## 3.2.3. Bacterial cellulose periodate oxidation

Purified never-dried BC membranes were obtained by culturing *G. xylinus* ATCC5382, 10 mL of liquid HS medium per well in a 6 well-plate for 21 days, in static conditions at 30 °C (Memmert). At the end of the fermentation the BC membranes were retrieved and autoclaved in distilled water in order to disrupt the *G. xylinus* cells. Afterwards, the BC membranes were rinsed with tap water and submerged into 4% (w/v) NaOH (Fisher) for 24 hours, in order to leach from the membranes the remaining bacteria debris and culture medium residues. Next the BC membranes were rinsed with distilled water until the pH became equal to the water pH. Finally, the purified BC membranes

with 3.5 cm diameter and 0.5 cm thickness were autoclaved (AJC88) in distilled water and kept in sterile conditions until further use.

The oxidation of BC with sodium periodate forms two aldehyde groups per unit of glucose present in the BC fibrils, which are functional groups that may react with the lysine residue ε-$NH_2$ of bLF. ε-$NH_2$ reacts with the two aldehydes (dialdehydes) formed per each glucose unit through a nucleophilic attack originating a carbinolamines (Figure 3.2.2.) [302].

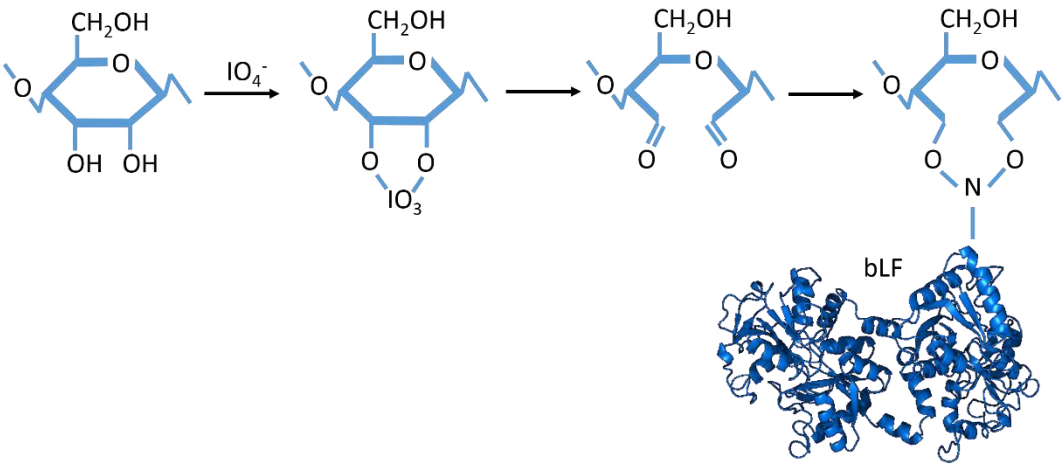


**Figure 3.2.2.** Schematic representation of the bacterial cellulose periodate oxidation and subsequent covalent bound formation with bovine lactoferrin, based on the protocol described by Sanderson and Wilson [302].

The oxidation reaction profile was determined in order to control the oxidation process. Never dried membranes (purified and sterilized as previously described) were immersed into a sodium periodate aqueous solution at 50 °C with mild agitation and protected from light. The ratio of BC and $NaIO_4$ was 1 mol of BC per 1 mol of $NaIO_4$. The $IO_4^-$ consumption and concomitantly the percentage of oxidation was followed by measuring the absorption at 290 nm using a UV-vis spectrometer (Jasco). The samples with 25, 50 and 75% of oxidation, after transfer from the oxidizing solution, were thoroughly washed in sterile distilled water and immediately functionalized.

### 3.2.4. Bacterial cellulose functionalization with bovine lactoferrin

Never dried BC and never dried oxidized BC membranes were immersed in bLF solutions (1 membrane per 10 mL of solution) with different concentrations (0.25, 0.5, 1 and 2 mg $mL^{-1}$), and subjected to mild agitation for 24 hours at room temperature. Non-oxidized never dried BC membranes were additionally functionalized in a bLF solution with 10 mg $mL^{-1}$ of concentration.

The concentration of absorbed bLF by the BC membranes was determined using the Bradford protein assay. The difference between the initial and final protein concentration in the solution, allowed assessing the loaded bLF in the BC membranes. The processed BC films were used immediately after preparation, except for the BC membranes exposed to the 10 mg $mL^{-1}$ solution, that were immediately used or vacuum dried for at least 72 hours at room temperature.

### 3.2.5. Disk diffusion susceptibility test

The antimicrobial properties of the BC processed membranes were evaluated through the disk diffusion susceptibility assay Its experimental procedure was described by Wiegand and collaborators [306]. *E. coli*, *S. aureus* and *S. epidermidis* were incubated separately in MH agar culture medium plates overnight at 37 °C. The McFarland 0.5 turbidity standard was used to determine the inoculum strength in PBS, which corresponded to 1–2 x $10^8$ colony forming units (CFU) $mL^{-1}$ of each bacterium, which were then evenly spread on fresh MH agar medium surface. Sample membranes with 5 mm diameter and 0.5 cm thickness were obtained using a puncher, and were immediately and carefully placed on the prior inoculated MH agar plates. Afterwards, the plates containing the samples were incubated for 24 hours at 37 °C. High resolution pictures were acquired and the zone of inhibition (ZoI) was determined using Image J software [307].

## 3.3. Results and discussion

Two of the most commonly reported *G. xylinus* strains (ATCC53582 and ATCC700178) for the production of BC were used. The goal was to assess which was the most productive strain to proceed with the following tasks of the thesis. For that purpose, biomass growth and BC production were evaluated for both strains (Figure 3.3.1.).

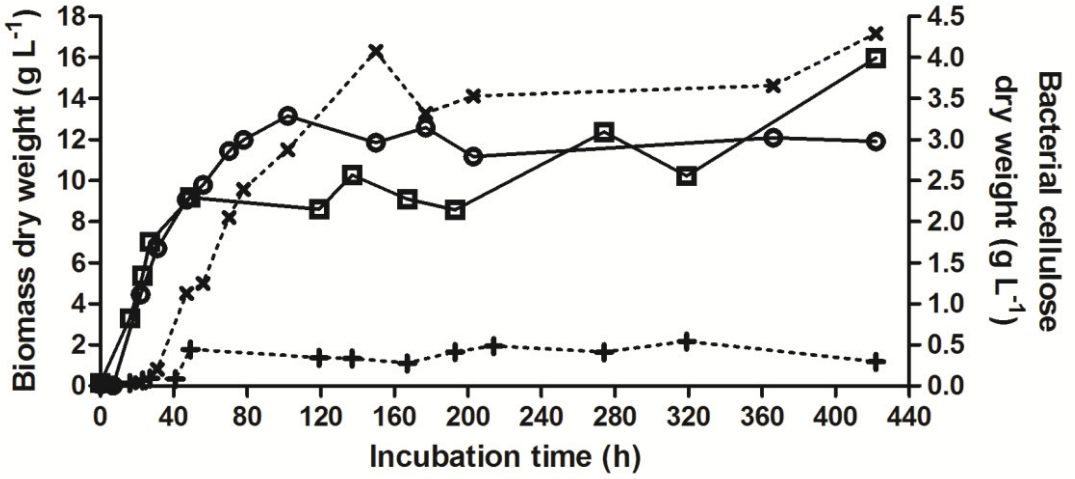


**Figure 3.3.1.** Dry weight variation of: "⊖" *Gluconacetobacter xylinus* ATCC 53582 biomass; "⊟" *G. xylinus* ATCC 700178 biomass, "-✖-" bacterial cellulose synthesized by *G. xylinus* ATCC 53582, "-✚-" bacterial cellulose synthesized by *G. xylinus* ATCC 700178. Static culture at 30 °C in culture flasks with Hestrin Schramm culture medium.

Although the biomass production profile was identical for both strains during the first 50 hours of incubation, it was found that the BC production was very distinct. After 24 hours of incubation the biomass and BC yields are within the same range for both strains, since 4.45 g $L^{-1}$ of *G. xylinus* ATCC 53582 biomass produced 40 mg $L^{-1}$ of BC, whereas the 5.37 g $L^{-1}$ of *G. xylinus* ATCC 700178 biomass synthesized 35 mg $L^{-1}$. Between the first and the fourth day (approximately 100 hours) of incubation, the increase of the ATCC 53582 strain biomass is followed by a steady increase of the BC production. In the case of ATCC 700178, the production of BC also increased during approximately 50 hours. Therefore, for both strains, the BC maximum production seems to be attained once the stationary phase is reached. However, it was found that at this stage the value of BC production by the ATCC 53582 strain is roughly ten times higher (3.77 ± 0.18 g $L^{-1}$) than by the ATCC 700178 (0.39 ± 0.03 g $L^{-1}$), being this difference statistically significant ($p < 0.05$). Indeed, *G. xylinus* ATCC 53582 has been reported to produce approximately 3.20 – 6.23 g $L^{-1}$ of BC in static HS culture [308, 309], whereas *G. xylinus* ATCC 700178 BC production in modified media has been reported to reach 1.75 g $L^{-1}$ [310]. These results corroborate the conclusions that ATCC 700178 strain synthesizes more BC in agitated cultures than static conditions [311]. However, in agitated cultures the BC is produced as small pebble-like shapes, which is not the intended BC profile for the envisaged application [126].

Finally, the specific growth rate for both ATCC 53582 and ATCC 700178 was approximately 0.08 $h^{-1}$, a lower level than the reported for *G. xylinus* ATCC 23769 in the same culture medium, which was approximately 0.12 $h^{-1}$, and this strain reached the stationary phase at approximately 60 hours

of incubation [312]. These differences are probably inherent to the strains and culture conditions, such as flask size and available oxygen in the medium.

BC oxidation with periodate is a straightforward approach to produce dialdehydes functional groups to covalently bind to the ε-NH2 groups of lysine residues present in bLF. This methodology was used to increase the bLF concentration embedded into the BC membranes. The periodate reaction can be controlled by monitoring the $IO_4^-$ concentration present in the solution and it can be easily removed [302]. The oxidation process was followed by the consumption of $IO_4^-$ (Figure 3.3.2.).

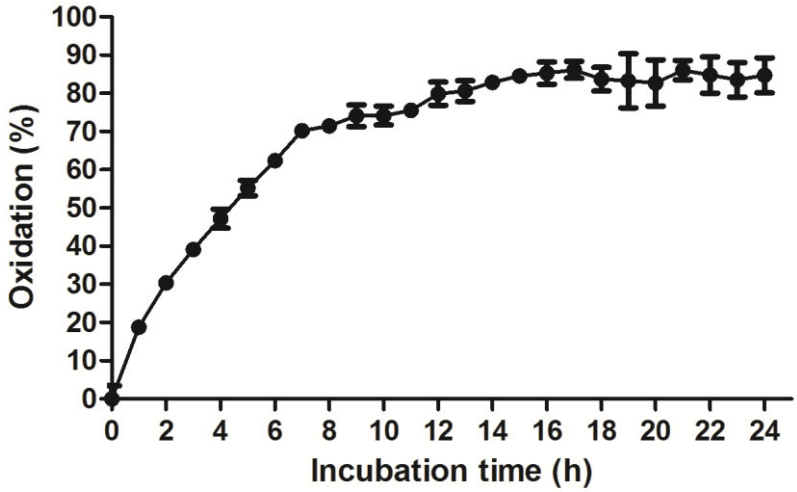


**Figure 3.3.2.** Percentage of bacterial cellulose oxidation by periodate reaction. The bacterial cellulose was produced by *Gluconacetobacter xylinus* ATCC 53582. Results are expressed in as mean ± standard error, obtained from three independent assays (n = 3).

The BC oxidation profile shows the existence of two phases, the first phase that corresponds to an oxidation rate of 9.1 ± 0.3% $h^{-1}$ during 7 hours of incubation, after which it is considerably reduced to 1.7 ± 0.2% $h^{-1}$ (second phase), until the maximum value of oxidation is reached (84.1 ± 1.5) at approximately 17 hours.

Figure 3.3.3. shows the bLF uptake by the BC membranes that may occur by protein absorption in its matrix and/or by adsorption by carbinolamine formation in the dialdehyde functional groups generated by periodate oxidation. The BC membranes with no oxidation, 25 and 50% of oxidation showed an increasing uptake of bLF proportional to the available bLF in the solution. This increasing uptake profile did not occurred in the sample corresponding to 0% of oxidation immersed in the bLF solution with 1 mg $mL^{-1}$ and the one corresponding to 50% oxidation resulting from the immersion in 0.5 mg $mL^{-1}$ bLF solution, as well as all BC membranes with 75% of oxidation. The BC membranes with 25% of oxidation immersed in the solutions containing 1 and 2 mg $mL^{-1}$ of bLF showed the expected results, *i.e.* a significant increase in the amount of bLF uptake. The BC

membranes with 75% oxidation showed a high load of the protein for all bLF concentrations tested, probably due to considerable BC morphological changes caused by the periodate oxidation. These membranes not only presented a substancial reduction in the dry weight (approximately 70%), but also became highly prone to brittleness, indicating the formation of dialdehyde groups, which consequently reduce the existing hydrogen bounds, impairing the BC mechanical properties. Therefore, the wide presence of dialdehydes functional groups may promote the covalent binding of bLF even at low concentrations. However, it is important to notice that the reduction in the number of hydrogen bounds, will turn the BC matrix less organized and with lower ability to retain the same bLF loading capacity through absorption. In opposition, the BC membranes with 50% of oxidation demonstrated an inconsistent uptake. These membranes showed an approximately 40% loss of dry weight and an impairment of the mechanical properties, although not as drastic as for the samples with 75% of oxidation. Still, the uptake is considerably lower than the observed in the control, mainly for the membranes immersed in the solution with 0.5 mg $mL^{-1}$ of bLF.

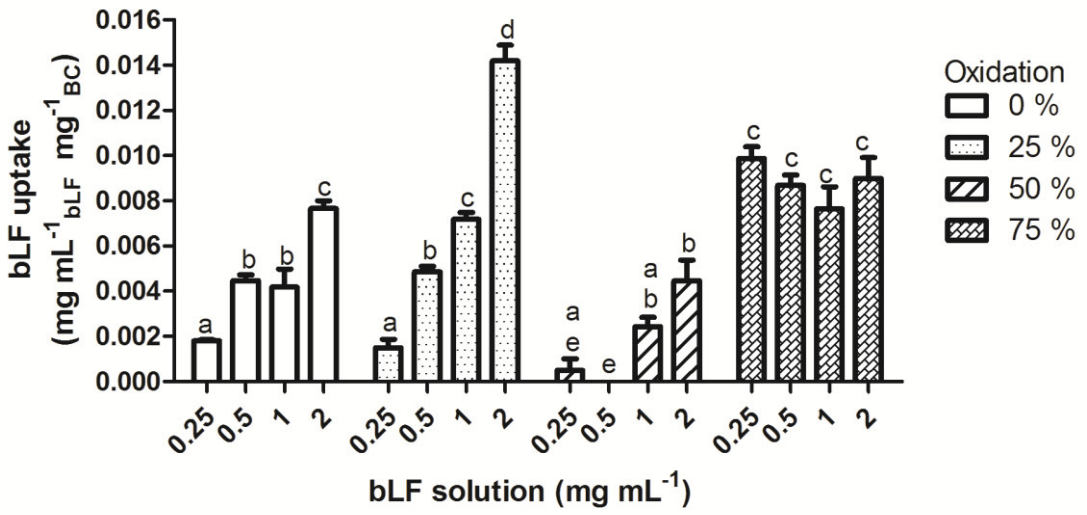


**Figure 3.3.3.** The bovine lactoferrin (bLF) absorption in bacterial cellulose membranes (3.5 cm diameter and 0.5 cm thickness) synthesized by *Gluconacetobacter xylinus* ATCC 53582 oxidized with periodate at :" ▭ " 0%, "▭" 25%, "▨" 50% and "▨" 75%; after 24 hours immersion in bLF phosphate buffer saline (pH 7.4) solution at 0.25, 0.5, 1 and 2 mg $mL^{-1}$. Different column letters denote significant differences determined by two-way ANOVA with Bonferroni post-hoc test ($p < 0.05$). Results are expressed in mean ± standard error, obtained from three independent assays (n = 3).

According to the results obtained, the oxidation percentage and concentration of bLF solution chosen for further studies was 25% and 2 mg $mL^{-1}$, respectively. The rationale of this choice was the fact that this conditions promoted the highest level of bLF uptake and the least dry weight loss (10%).

Afterwards new samples were prepared under those conditions to be tested for their antimicrobial potential. For that purpose the samples were challenged against three different microorganisms: *E. coli* (Gram-negative), *S. aureus* (Gram-positive) and *S. epidermidis* (Gram-positive) (Figure 3.3.4.). E. coli and S. aureus are recognized as food pathogens, while S. epidermidis is a universal colonizer of human skin, and thus a potential source of pathogenicity [1, 313].

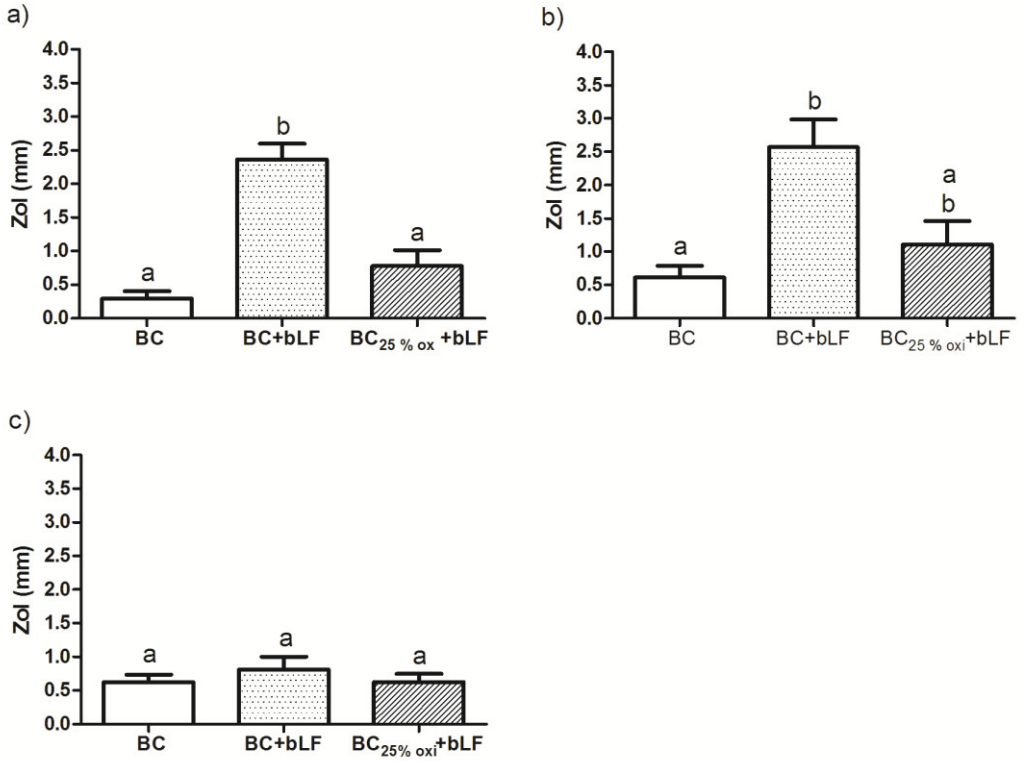


**Figure 3.3.4.** Zone of inhibition (ZoI) originated by: "▭" bacterial cellulose (control), "▭" bacterial cellulose with absorbed bovine lactoferrin, "▨" bacterial cellulose with 25 % of periodate oxidation loaded with bovine lactoferrin, after 24 hours of incubation at 37 °C, when challenged against: **a)** *Escherichia coli*; **b)** *Staphylococcus aureus* and **c)** *Staphylococcus epidermidis*., the statistical analysis used in **a)** One-way ANOVA with Tukey post-hoc test ($p < 0.05$), **b)** and **c)** Kruskal-Wallis with Dunn's multiple comparison test ( $p< 0.05$). Different column letters denote significant differences. Results are expressed in mean ± standard error, obtained from seven independent assays (n = 7).

Even though the BC oxidized with periodate possessed a significant higher load of bLF in comparison to the non-oxidized BC with bLF, its antimicrobial effectiveness was found to be lower. In fact, for all the bacteria tested, the ZoI obtained for the oxidized BC was not significantly different from the control. The ZoI of the control probably results from a dragging effect caused by the PBS present in the membrane that diffuses into the agar, consequently driving bacteria away from the membrane. Unlike the oxidized BC, the BC with absorbed bLF showed a significant ZoI, except against *S. epidermidis*. The *S. epidermidis* herein used produced high amounts of slime, and therefore this may have impaired the bLF antimicrobial effect [314]. As for the oxidized BC, its

lower antimicrobial efficiency may be due to the formation of carbinolamines with lysine residues. The bLF N-terminal is widely recognized to possess antimicrobial activity, mainly due to it highly cationic nature that can interact and destabilize the bacteria membranes and surface charge. Lysine is a positively charged amino acid, and it is present in abundance in this region of the glycoprotein, therefore its reaction with the dialdehyde functional group, and subsequent covalent binding, may ultimately suppress this important bactericidal mechanism, thus leading to an effect contrary to the envisaged one. The results herein gathered suggest that the uptake of bLF by non-oxidized BC the most effective strategy to obtain an antimicrobial edible film. Therefore, all the BC samples with embedded bLF were tested against the same microorganisms as before, namely *E. coli*, *S. aureus* and *S. epidermidis*. Moreover, the bLF concentration in the solution was increased to 10 mg $mL^{-1}$ to evaluate if it would lead to an enhanced antibacterial activity (Figure 3.3.5.).

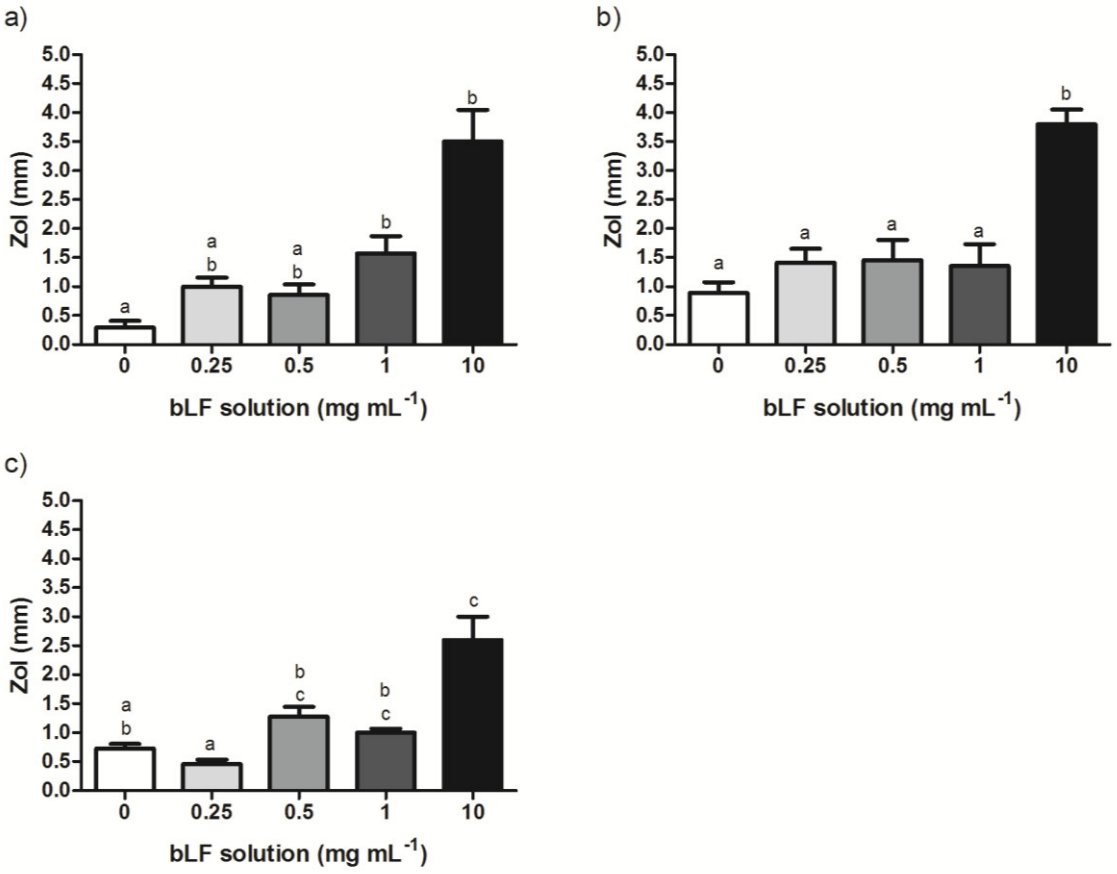


**Figure 3.3.5.** Zone of inhibition (ZoI) obtained by: "□" bacterial cellulose (control) and bacterial cellulose with absorbed bLF after contact with a bLF solution with: "□" 0.25 mg $mL^{-1}$, "□" 0.5 mg $mL^{-1}$, "□" 1 mg $mL^{-1}$ and "■" 10 mg $mL^{-1}$, when challenged against **a)** *Escherichia coli*, **b)** *Staphylococcus aureus* and **c)** *Staphylococcus epidermidis*.. The statistical analysis used: **a)** and **c)** Kruskal-Wallis with Dunn's post-hoc test ($p < 0.05$), and **b)** One-way ANOVA with Tukey's post-hoc test ($p < 0.05$). Different column letters denote significant differences. Results are expressed in mean ± standard error, obtained from seven independent assays (n = 7).

The BC samples exposed to 10 mg $mL^{-1}$ bLF, showed a significant bactericidal activity against the tested bacteria. The ZoI were similar for *E. coli* and *S. aureus* (3.5 and 3.8 mm, respectively), and a significant effect on the less susceptible *S. epidermidis* (ZoI of 2.6 mm) was found. As expected, BC samples with lower bLF uptake did not show a significant bactericidal effect, except for the BC with 1 mg $mL^{-1}$ bLF when tested against *E. coli*. Finally, in order to evaluate if the BC with embedded bLF maintained its bactericidal activity, dried films were also evaluated by the disk diffusion test (Figure 3.3.6.).

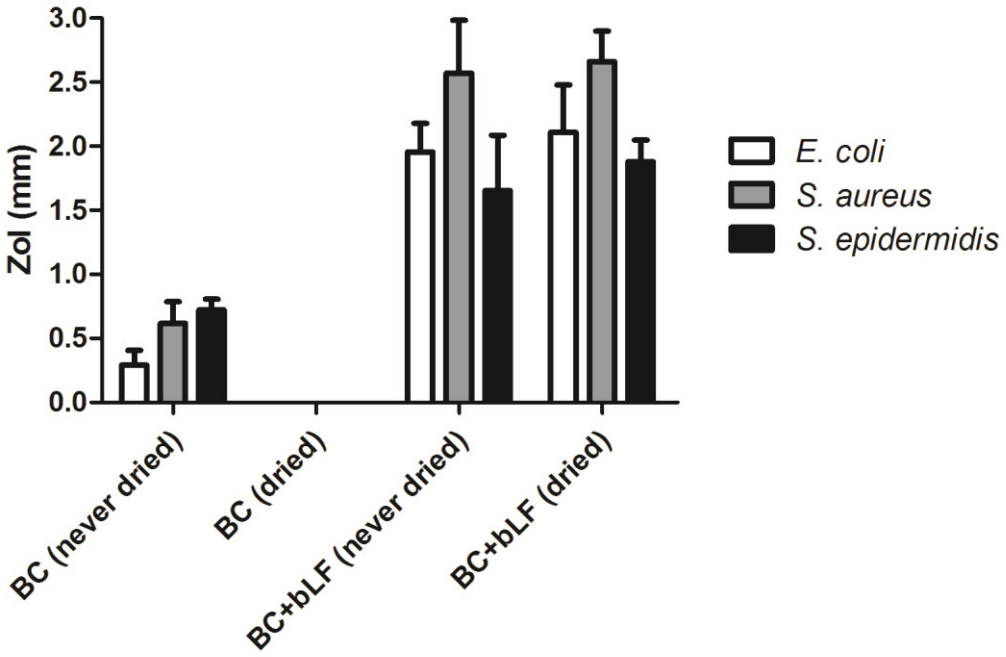


**Figure 3.3.6.** Zone of inhibition (ZoI) generated in: "□" *Escherichia coli*, "▒" *Staphylococcus aureus* and "■" *Staphylococcus epidermidis* by: "BC (never dried)" bacterial cellulose that was not submitted to drying process, "BC (dried)" bacterial cellulose that was dried, "BC+bLF (never dried)" bacterial cellulose loaded with bovine lactoferrin that was not dried and "BC+bLF (dried)" bacterial cellulose loaded with bovine lactoferrin that was dried. The bacterial cellulose membranes loaded with bovine lactoferrin were in contact with a solution with the concentration of 10 mg $mL^{-1}$ bLF. Vacuum drying at room temperature was used as the drying process. Results are expressed in mean ± standard error, obtained from seven independent assays (n = 7).

No significant statistical differences could be observed for each organism when comparing the never-dried with the dried results ($p < 0.05$), thus suggesting that the drying process does not interfere with the bactericidal activity of the BC membranes embedded with bLF.

## 3.4. Conclusions

A protocol to determine the exact biomass and BC production by two strains of *G. xylinus* was successfully designed and allowed highlighting the differences on BC production between the strains, using similar inoculum and culture conditions.

BC was oxidized by sodium periodate to increase the bLF uptake by the BC membranes. The bLF uptake by the never dried BC membranes with 25% of oxidation was found to be similar to the non-oxidized BC uptake when bLF solutions with lower concentrations are used. However, when immersed in bLF solutions at 1 and 2 mg $mL^{-1}$ concentrations, the uptake was found to be approximately two fold higher than the pristine BC. The samples with 75% of oxidation showed a considerable uptake even after immersion in low concentration bLF solutions, most likely due to the great availability of dialdehyde groups. However, the mechanical consistency of these membranes was considerably low.

The BC samples with 25% of oxidation surprisingly displayed a lower bactericidal activity as compared to the pristine BC, which led to the further evaluation of BC pristine membranes, since the later exhibited both mechanical integrity and a higher bactericidal effect.

The pristine BC was blended with a more concentrated bLF solution (10 mg $mL^{-1}$). The resulting membranes showed a significant bactericidal effect against *E. coli*, *S. aureus* and *S. epidermidis*. The BC membranes antibacterial efficiency was maintained even after the vacuum drying process.

# Chapter 4

## The potential of edible bacterial cellulose films functionalized with lactoferrin

# 4. The potential of edible bacterial cellulose films functionalized with lactoferrin


## Abstract

Active packaging is the technological answer for the maintenance or even improvement of the organoleptic traits of the enclosed food products, therefore prolonging the food product shelf-life and safety. Such active packaging, fully bio-based and edible can be obtained, as proposed in this work, by blending bacterial cellulose (BC), an extremely pure cellulose polysaccharide that presents remarkable mechanical properties, with bovine lactoferrin (bLF), a wide spectrum antimicrobial glycoprotein which also possess notable number of beneficial activities. Two different BC were evaluated, synthesised by *Gluconacetobacter xylinus* ATCC53582 (BC1) and commercially purchased "*nata de coco"* (BC2). BC1 and BC2 showed equivalent properties, namely thickness (approx. 25 μm), density (approx. 0.79 mg $mm^{-3}$), water vapour permeability (approx. 0.7 g mm $m^{-2}$ $h^{-1}$ $kPa^{-1}$) and maximum tensile strength (approx. 42 MPa). However, BC2 showed a higher bLF absorption (approx. 45 mg $mL^{-1}$ $g^{-1}$) and also higher bLF release (approx. 7 mg $mL^{-1}$ $g^{-1}$) than BC1. This difference was further noticed in the contact killing bactericidal assessment with both standalone films and fresh sausage conditioned with bLF were used, since both BC1 and BC2 with absorbed bLF (BC1+bLF and BC2+bLF, respectively) showed similar efficiency against *Escherichia coli* and *Staphylococcus aureus*. This efficiency was translated in a mean reduction of 69% for *E. coli* and of 97% for *S. aureus* counts when in contact with the films. In the case study, the mean percentage of reduction was 94 % and 36 % for *E. coli* and *S. aureus* respectively. Moreover, the BC1+bLF and BC2+bLF processed films significantly limited the specific growth rate of both bacteria. Finally, the cytotoxicity of the films against fibroblast 3T3 cells was evaluated, before and after digestion in the *in vitro* gastrointestinal tract model (TIM). No relevant cytotoxicity was found.


## 4.1. Introduction

Consumer standards towards food freshness and safety has increased exponentially over the last decades. Intensive research on food packaging is being developed to supply household tables with fresh, safe, minimal processed, easy to prepare products [2]. Moreover, the implementation of renewable and biodegradable materials must be adopted by the food industry in order to meet

environmental standards [315]. The use of bio-based materials represent a straight forward alternative to petrochemical-based polymers, by offering sustainability and edibility. The use of superior bio-based nanomaterials such as bacterial cellulose (BC), may represent an interesting technical advantage, due to its extreme purity, non-allergenic, high water holding capacity, and notable mechanical toughness [110, 111]. Moreover the interesting loading capacity of BC may be exploited to deliver active agents into food commodities. Food safety is a key priority for the food industry, which is particularly problematic when the food product is highly perishable. Meat, seafood's, poultry, and dairy commodities are highly prone to spoilage and foodborne illness transmission [22]. Antimicrobial active packaging aims at preventing food borne illness eradicating or inhibiting the food existing microflora, thus increasing the product safety and promoting the extension of its shelf-life [2, 316].

Bovine lactoferrin (bLF) is considered a safe ingredient with an increasing industrial scale production between 80 - 100 tonnes a year [175, 265, 317]. bLF is glycoprotein with approximately 80 kDa and possess a wide role of activities, such as immunoregulatory, anti-cancer and antimicrobial. bLF antimicrobial action has been reported in foodborne pathogens such as *Staphylococcus aureus* [234], *Escherichia coli* [235, 318] and *Clostridium* spp. [233]. This chapter describes the functionalization of two types of BC with bLF, as well as its further evaluation regarding their potential as edible antimicrobial films, intended to be used in direct contact with high perishable foods, namely meat products.

## 4.2. Materials and methods

BC from two different origins were used in this study: i. "BC1" produced in the laboratory by static cultures *Gluconacetobacter xylinus* American type culture collection (ATCC) 53582, ii. and a purchased "BC2" commercial grade 2 raw *nata de coco*, from HTK Food CO. Ltd. (China). bLF protein powder was obtained from DMV International (USA) with a reported composition of 96% dry weigh percentage of protein, approximately 120 ppm of iron, 0.5% (w/w) of ash and 3.5% (w/w) of moisture. The BC films containing bLF were named BC1+bLF and BC2+bLF, for the laboratory produced and the commercially acquired BC, respectively. A fresh sausage, bought in a local butcher shop (and produced in the northwest region of Portugal), was used as a substrate for the case study of antimicrobial tests. Its label information was the following: "pig's meat and fat, pepper, salt, spices, soy protein, emulsifier (E450 and E451), antioxidant (E316 and 331), colorant (E316) and stabilizer (E250), it contains soybean, milk and celery. Maintain between 0 and 5 °C".

Its casing, composed of pig small intestine, was used in the assessment of the mechanical properties described below.

### 4.2.1. Microorganisms and eukaryotic cells

As previously referred, *G. xylinus* strain ATCC 53582 was used for the synthesis of BC. The antibacterial properties of films with and without bLF were determined using *Escherichia coli* and *Staphylococcus aureus*, kindly provided by the Faculty of Pharmacy of University of Porto (Porto, Portugal). Cytotoxicity tests were performed using 3T3 mouse embryo fibroblasts ATCC CCL-164.

### 4.2.2. Culture medium, conditions and solutions

Hestrin-Schramm culture medium (HS) [305] in solid state with 2% (w/v) agar (Himedia) was used to maintain *G. xylinus*. BC1 production occurred in static culture conditions using liquid HS with a flask volume ratio of 1/4, at 25 °C for at least four weeks. The reagents and quantities (w/v) used for HS medium preparation were 2% of glucose (Sigma-Aldrich), 0.5% peptone (Sigma-Aldrich), 0.5% of yeast extract (Himedia), 0.34% of $Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich), 0.15% citric acid (Pronolab), and the final pH was adjusted to 5.5 using HCl 18% (v/v) (Sigma-Aldrich). Mueller-Hinton (MH) agar (Himedia) was used for *E. coli* and *S. aureus* maintenance and colony forming units (CFU) determination. The antibacterial assays liquid culture media was Nutrient Broth (NB) (Himedia), and all liquid media pre-inocula and experiments were carried out using a 1/5 culture medium/flask volume ratio in an incubator at 37 °C with 145 rpm of agitation (Heidoph). Prior to any experiment using *E. coli* and *S. aureus*, these microorganisms were incubated overnight, to produce the inocula. Phosphate buffer saline (PBS) (pH 7.4) composition (w/v) was 0.8% NaCl (Fisher), 0.02% KCl (Fischer), 0.14% $Na_2HPO_4$ (Sigma), 0.03% $KH_2PO_4$ (Flücka). HS (solid and liquid), MH agar, NB and PBS were autoclaved for 20 minutes, at 120 °C and 1 bar (AJC 88) before use. PBS was used in all inocula and sequential dilutions to avoid osmotic lysis. The bLF solutions were prepared immediately before use, by dissolving the intended dry mass of bLF in PBS, using mild agitation at room temperature. Once dissolved the bLF solution was filter-sterilized (0.22 µm). Animal cells were incubated in Dulbecco's Modified Eagle's medium (DMEM) (Biochrom), with 10% (v/v) new-born calf serum (NCS) (Invitrogen) and 1% (v/v) of penicillin/streptomycin at 37 °C in a 5% $CO_2$ humidified atmosphere. Electrolyte gastric solution composition was NaCl 0.48% (w/v), KCl 0.22% (w/v), $CaCl_2$ 0.02% (w/v), $NaKHCO_3$ 0.15% (w/v))

with pepsin 600 U $mL^{-1}$ and lipase 40 U $mL^{-1}$, whereas the small intestinal electrolytic solution (SIES) formulation was NaCl 0.5% (w/v), KCl 0.06% (w/v) and $CaCl_2$ 0.025% (w/v).

### 4.2.3. Bacterial cellulose processing

The same purification procedure was undertaken in both BC1 and BC2 membranes. Once the BC1 production was terminated, and immediately after unpacking BC2, the membranes were autoclaved, in order to disrupt the *G. xylinus* cells in BC1, and to eliminate potential contaminants in BC2. Once cooled, the membranes were rinsed with tap water and submerged into 4% (w/v) NaOH (Fisher) for a 24 hour period to promote the leaching of the bacteria debris and remaining culture medium residues. Afterwards, the BC membranes were abundantly rinsed with distilled water until the pH became neutral. Finally, the never dried BC membranes were cut into 1 mm thick slices and subsequently autoclaved and stored in aseptic conditions until further use. Several geometries were chosen for the different experiments, namely discs (with 5, 9 and 22 mm of diameter in average) and patches (25x70 mm and 30x80 mm in average).

Absorption of bLF onto BC nanofibres was achieved by immersing the never dried BC films into a 10 mg $mL^{-1}$ bLF solution (0.4 films $mL^{-1}$), for 24 hours with a mild agitation at room temperature. Afterwards, BC films were vacuum dried at room temperature for at least 48 hours. Control BC films were submitted to the same treatments in PBS but without bLF absorption. All assays were performed using the vacuum dried films, except for the absorption assays.

### 4.2.4. BC edible films physicochemical characterization

#### 4.2.4.1. Endotoxin level determination

*G. xylinus* is a Gram-negative bacterium, therefore, in order to assure the efficiency of the BC purification procedure, the levels of endotoxin were quantified. The endotoxin determination was performed using the limulus amebocyte lysate test Pierce® LAL Chromogenic Endotoxin Quantitation kit. The optical density was measured at $\lambda$= 405 nm, with a linear range set between 0.1 and 1.0 endotoxin units (EU) $mL^{-1}$.

#### 4.2.4.2. Determination of the absorption and de-absorption profile

A qualitative assay was performed to assess the bLF behaviour when in contact with BC films. Never dried BC1 and BC2 films were separately immersed into PBS with 10 mg $mL^{-1}$ of bLF, and for control purposes into PBS only. After a 24 hour of exposure at room temperature under mild

agitation, the films were removed and the excess of solution was removed using Whatman 1 filter paper. Next, samples were immediately immersed in a Prussian blue solution for 30 minutes at room temperature. The Prussian blue solution composition was 5% (w/v) of $K_4Fe(CN)_6$ mixed in a proportion of 1/1 with 5% (v/v) of HCl [319, 320]. The films were then rinsed with distilled water, transversely cut using a plastic scissor and placed on a microscopy slide. Afterwards, the samples were analysed in a stereo microscope (Olympus). $[Fe(CN)_6]^{4-}$ reaction with $Fe^{3+}$ (Equation 4.2.1.), gives origin to an intense blue coloration [321, 322].

$$4Fe^{3+} + 3[Fe^{II}(CN)_6]^{4-} + 14\ H_2O \longrightarrow Fe^{III}4[Fe^{II}(CN)_6]_3 \cdot 14H_2O \qquad \textbf{Equation 4.2.1.}$$

To assess the bLF loading capacity of the BC, films (25 x 170 mm) were immersed into a PBS solution with 10 mg $mL^{-1}$ of bLF (50 mL per sample) under mild agitation at room temperature. After a period of 24 hours, the films were vacuum-dried at room temperature for 48 hours in a desiccator. Once properly dried, the films were again immersed in a PBS solution at 25 °C with mild agitation to determine the release rate of bLF from the BC films. In both assays (absorption and de-absorption), aliquots were regularly collected and the bLF concentration in solution was quantified using a fast protein liquid chromatography (FPLC) apparatus (Pharmacia) with a UV-detector (λ = 280nm) (Knauer). Three film replicates of each type of BC were used in this assay, and each sample retrieved was measured three times in the FPLC.

The bLF release profile was adjusted to a modified Gompertz Equation 4.2.2. [323].

$$X(t) = X_{max}\ exp\left[-exp\left[\frac{R_r e}{X_{max}}(\psi + t) + 1\right]\right] \qquad \textbf{Equation 4.2.2.}$$

where, "$X(t)$" represents the cumulative bLF release at time (t) (mg $mL^{-1}$ $g^{-1}$), "$X_{max}$" the maximum release of bLF (mg $mL^{-1}$ $g^{-1}$), "$R_r$" the bLF release rate (mg $mL^{-1}$ $g^{-1}$ $min^{-1}$), "$e$" the constant equal to 2.7182818 and "$\psi$" the latency phase (min).

#### 4.2.4.3. Surface free energy

The surface free energy was determined through sessile drop technique at room temperature using the Data Physics OCA20 equipment. A 3 µL liquid drop was placed on the surface of the different BC films. The Van Oss Chaudhury Good (Equation 4.2.3.) was used to estimate the surface free energy, from which the surface thermodynamic properties were determined using Equations 4.2.4., 4.2.5. and 4.2.6. [324, 325]. The probe liquids used for the calculation of the polar

component of the surface energy were: water, glycerol and formamide, whereas 1-bromonaphthalene was used to determine the dispersive component.

$$\gamma_l(1+\cos\theta) = \left(2\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + 2\sqrt{\gamma_s^{+}\gamma_l^{-}} + 2\sqrt{\gamma_s^{-}\gamma_l^{+}}\right) \quad \textbf{Equation 4.2.3.}$$

$$\Delta G_{lwl}^{LW} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_l^{LW}}\right)^2 \quad \textbf{Equation 4.2.4.}$$

$$\Delta G_{lwl}^{AB} = -4\left[\left(\sqrt{\gamma_s^{+}\gamma_s^{-}}\right) + \left(\sqrt{\gamma_l^{+}\gamma_l^{-}}\right) - \left(\sqrt{\gamma_s^{+}\gamma_l^{-}}\right) - \left(\sqrt{\gamma_l^{+}\gamma_s^{-}}\right)\right] \quad \textbf{Equation 4.2.5.}$$

$$\Delta G_{lwl}^{TOT} = \Delta G_{lwl}^{LW} + \Delta G_{lwl}^{AB} \quad \textbf{Equation 4.2.6.}$$

where, " $\gamma_l$" represents the surface free energy of the probe liquid, "$\theta$" the contact angle obtained from the interaction between the solid and the measuring liquid (°),"$\gamma_s^{LW}$" the Lifshitz-van der Waals component of the solid, "$\gamma_l^{LW}$" the Lifshitz-van der Walls component of the liquid, "$\gamma_s^{+}$" the Lewis acid component (electron acceptor) of the solid, " $\gamma_l^{-}$" the Lewis base component (electron donor) of the liquid, "$\gamma_s^{-}$" the Lewis base component of the solid, and "$\gamma_l^{+}$" the Lewis acid component of the of the liquid. "$\Delta G_{lwl}^{LW}$" represents the Gibbs energy of the Lifshitz-van der Walls component (mJ $m^{-2}$), "$\Delta G_{lwl}^{AB}$" the Gibbs energy of the Lewis acid base component (mJ $m^{-2}$) and "$\Delta G_{lwl}^{TOT}$" total Gibbs free energy (mJ $m^{-2}$).

#### 4.2.4.4. Ultraviolet – visible spectroscopy

The ultraviolet-visible spectroscopy allows the identification of the characteristic absorbance profiles of proteins, thus the confirmation of their presence [326]. The registered absorbance profiles were within the wavelengths ranging from 200 nm to 700 nm (Jasco).

#### 4.2.4.5. Fourier transform infrared – attenuated total reflectance

BC and BC+bLF films were analysed at room temperature using Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR, Alpha-FT-IR spectrometer (Bruker)). The spectra were collected with a resolution of 4 $cm^{-1}$ after 32 scans that ranged from 4000 -400 $cm^{-1}$.

#### 4.2.4.6. Swelling behaviour

A gravimetric method based on the methodology described by Sangsanoh and Supaphol [327] was used to determine the degree of swelling in BC samples. Circular BC films with 5 mm of diameter were used in these assays. Samples were immersed in PBS solutions at room temperature and

were collected at regular intervals, placed between two sheets of tissue paper to remove the excess of liquid, and weighed (ADA 100). The swelling degree was calculated using Equation 4.2.7.:

$$Degree\ of\ Swelling\ (\%) = \frac{(W_s - W_d)}{W_d} \times 100 \qquad \textbf{Equation 4.2.7.}$$

where, "$W_s$" represents the weight of the membrane after it is submersed in the PBS solution (mg) and "$W_d$" the weight of the sample in is dried state (mg).

#### 4.2.4.7. Dry weight and thickness

Macroscopic physical characteristics of the processed films were calculated by determining the dry weight of 50 samples of each film, and by recording the thickness of 30 replicas of each film using a digital micrometre (Mitutoyo).

#### 4.2.4.8. Water vapour permeability

The water vapour transmission of all processed films was assessed gravimetrically, using an adaptation of the methodology described in the ASTM E96-95 [328]. The processed films were placed on top of glass vials containing $1/3$ of ultrapure water, and fixed using parafilm. The samples were equilibrated overnight in a desiccator containing silica gel at room temperature. Afterwards the vials were placed in a desiccator with silica gel, and fans in its interior to maintain homogenous conditions, as described by Cerqueira and co-workers [329] at 25 °C. Thus, the relative humidity gradient (RH) was 0/100 (RH outside/RH inside). The glass vials were weighed within a 10 hour period. The WVP was calculated according to Equation 4.2.8.:

$$WVP = \frac{WVT \times x}{A \times P_0 \times (RH_1 - RH_2)} \qquad \textbf{Equation 4.2.8.}$$

where, "$WVP$" is the water vapour permeability, "$WVT$" the mass variation rate (g/h); "$x$" the film thickness (mm); "$A$" the film membrane area exposed to the permeant ($m^2$); "$P_0$" the vapour pressure of pure water (1.359 kPa) and "$(RH_1 - RH_2)$" the relative humidity gradient used in the experiment at 25 °C.

#### 4.2.4.9. Mechanical properties

The proposed final objective for the BC films containing bLF is to encase meat products, which inherently possess high water activity [330]. Therefore, the mechanical properties were evaluated under wet conditions, using a Shimadzu Universal Testing Machine (AG-IS - load cell of 50 N). Immediately prior to the stress strain tests, the processed films were immersed in distilled water

for no less than 5 minutes. The defined strain rate was 0.5 mm $min^{-1}$ at room temperature. The maximum stress ($\sigma_{max}$), the elongation at break and the modulus of elasticity ($E$) were determined, with the latter being calculated in the linear zone of elasticity, between 0 and 1% of strain using Hook's law (Equation 4.2.9.) in 5 samples of each processed film [331].

$$\sigma = E\varepsilon \qquad \textbf{Equation 4.2.9.}$$

where, "$E$" is the elastic modulus (MPa), "σ" the strain (MPa) and "ε" the stress of uniaxial deformation (%).

## 4.2.5. Bactericidal activity characterization

The antibacterial properties of the processed edible films were assessed using *E. coli* and *S. aureus* in a series of experimental methodologies.

### 4.2.5.1. Specific growth rate impact analysis

Culture flasks containing NB with 0, 0.25, 0.5, 1, 2.5, 5 and 10 mg $mL^{-1}$ of bLF were inoculated with approximately $1.5x10^6$ CFU $mL^{-1}$ of either *E. coli* or *S. aureus*, and were subsequently incubated at 37 °C with 120 rpm of orbital agitation, in order to evaluate the influence of bLF in the specific growth rate of the microorganisms. Moreover, processed bLF-BC circular films with 22 mm diameter were aseptically introduced in culture flasks containing NB medium (0.6 circular films per mL of medium) and were incubated using equivalent inoculum and culture conditions, as previously stated. For all the tested flasks the optical density (λ = 600 nm) was registered every hour.

### 4.2.5.2. Live/dead viability and scanning electron microscopy (SEM) analysis

NB inoculated with approximately $1.5x10^6$ CFU $mL^{-1}$ of either *E. coli* or *S. aureus*, was poured on top of circular shaped processed edible films, with 5 mm and 22 mm diameter, inside 96 and 24 well titration plate respectively, and afterwards incubated at 37 °C, 150 rpm of orbital rotation, for a period of 4 hours. The 5 mm diameter films were used in a live/dead viability assessment according to the following procedure: the culture medium was removed, the films were gently rinsed with PBS, and afterwards the films were immersed for 10 minutes in PBS containing propidium iodide (PI) (0.33% (w/v)). Subsequently, the films were gently rinsed with PBS and then, with the purpose of bacterial cell fixation, a formaldehyde solution (3.67% (v/v)) was poured on the films and maintained at room temperature for 10 minutes. Next, the films were carefully rinsed

with PBS, and immersed into a PBS solution with 4', 6-diamidino-2-phenylindole (DAPI) (0.01% (w/v)) for 10 minutes and subsequently rinsed twice prior to the observation on the fluorescent microscope (Olympus BX51). Images were obtained with different fluorescence filters and were further merged using ImageJ software [307].

Scanning electron microscopy (SEM) was performed on 22 mm diameter BC disks, after cell fixation according to the procedure described by Andrade *et al.* [332]. Briefly, the culture media was removed and 1 mL of 2.5% (v/v) of glutaraldehyde in PBS was dispensed in each sample. The films were maintained for 1 hour at room temperature to promote the fixation of the bacterial cells on the films. Immediately after, the films were gently rinsed with distilled water and submitted to a dehydration process using a series of water – ethanol solutions, with increasing percentages of ethanol (55, 70, 80, 90, 95, 100% v/v). Samples were submerged in each solution for a period of 30 minutes at room temperature. Finally, the remaining ethanol in the films was left to evaporate at room temperature. The BC films were coated with gold-sputtered (Polaron) and were observed in SEM (LEICA) with a magnification of 20,000x using an electron accelerating voltage of 5 kV.

#### 4.2.5.3. Evaluation of contact bactericidal effectiveness (contact killing)

The American Association of Textile Chemists and Colourists (AATCC) test method 100-TM100 [333] was herein adapted to evaluate the contact bactericidal efficiency of the processed edible films. Briefly, circular film samples with 9 mm diameter were placed in a 48 well titration plate and were inoculated with either 9.2x105 CFU $mL^{-1}$ of *E. coli* or 9.4x105 CFU $mL^{-1}$ of *S. aureus*. After 2 hours of incubation at 37 °C, the samples were covered with 500 µL of PBS, and carefully stirred for no less than 1 minute, to homogenise the bacterial population. Afterwards the CFU $mL^{-1}$ was determined in MH agar plates incubated for 24 hours at 37 °C. The percentage of CFU reduction was determined using the Equation 4.2.10.:

$$R = \frac{A-B}{A} \times 100 \qquad \textbf{Equation 4.2.10.}$$

where, "$R$" is the percentage of CFU reduction, "$A$" the CFU $mL^{-1}$ of the inoculum, and "$B$" the CFU $mL^{-1}$ obtained after 2 hours of contact with the films at 37 °C. Three film replicas were used for each bacteria.

#### 4.2.5.4. Case study

Fresh sausage is highly prone to contamination during all the steps of its production until it reaches the final consumer. These include the handling, production, packaging, storage and transportation,

and therefore itwis a good model to test the antibacterial efficiency of the obtained films. A similar adaptation of the AATCC test method 100-TM100 [333] was also used for the fresh sausage samples. Circular films with 22 mm of diameter were individually placed at the bottom of sterilized flasks, under aseptic conditions, and were further inoculated with $9.5x10^5$ CFU $mL^{-1}$ of *E. coli* and $1.0x10^6$ CFU $mL^{-1}$ of *S. aureus*. Immediately after inoculation, a fresh sausage piece with 12 mm of diameter and 10 cm of height was carefully placed on top of the inoculated area. The fresh sausage cylinder was prepared immediately before use, being exposed to UV-light for 15 minutes to minimize the hypothetical endogenous contamination. As a control, the same procedure was employed in a circular patch of 22 mm diameter of the casing removed from the fresh sausage. After 24 hours of incubation at 37 ºC, 5 mL of PBS were poured into the flasks and submitted to a vigorous agitation. The obtained CFU $mL^{-1}$ was determined and the percentage of reduction was calculated using Equation 4.2.11.:

$$R(\%) = \frac{A-C}{A} \times 100 \qquad \textbf{Equation 4.2.11.}$$

where, "*R*" is the percentage of CFU reduction, "*A*" is the CFU $mL^{-1}$ obtained immediately after inoculation, and "*C*" is the CFU $mL^{-1}$ achieved after 24 hours of incubation at 37°C.

## 4.2.6. Cytotoxicity

### 4.2.6.1. Cell viability assessment

In order to evaluate the direct cytotoxicity of the edible films, 3T3 mouse embryo fibroblasts (ATCC CCL-164) were seeded in DMEM enriched with NCS, on top of circular edible films, with 9 mm diameter. The initial cell density used was 1.3 x $10^5$ cells per well, and the incubation lasted for 72 hours at 37 °C in a 5% $CO_2$ humidified atmosphere. The total number of fibroblasts adhered to the membranes were quantified in the Neubauer chamber, using trypan blue (Flücka) to distinguish viable and non-viable cells.

### 4.2.6.2. Mimetic and dynamic *in vitro* gastro-intestinal model

The processed edible films were submitted to a dynamic *in vitro* gastrointestinal tract model digestion using a gastrointestinal tract model (TIM) similar to the model developed by TNO (Nutrition and Food Research, Netherlands). This system mimics the gastrointestinal peristaltic movements, allied to a dynamic transfer between the stomach, duodenum, jejunum and ileum compartments. The purpose of using the TIM model was to determine if the edible films would

generate cytotoxic elements during gastrointestinal digestion. To simulate the mouth digestion, the films were submitted to a protocol described by Sessa and co-workers; [334]. Briefly, a volunteer ingested 250 mL of milk, waited for 5 minutes, and afterwards collected 10 mL of the saliva produced. Edible films with 3 x 8 cm were shredded with scissors, immersed in the saliva solution and incubated at 37 °C for 10 minutes. Afterwards, 30 mL of PBS were added to the mixture, and all contents shredded using a blender. Finally, the mixture was inserted in the TIM gastro-intestinal system. The digestion conditions were described by Reis *et al.* [335]. In the stomach compartment, the sample was in contact with gastric solution added with a flow rate of 0.33 mL $min^{-1}$. The pH was gradually dropped, as shown in Table 4.2.1., until the value of 1.7 was reached. The stomach was gradually emptied, approximately 20 mL, every 30 minutes after the first 90 minutes of digestion. In the duodenum compartment, the samples were exposed to SIES enriched with 7% (w/v) of pancreatin and 4% (v/v) of bile with a flow rate of 0.6 mL $min^{-1}$. The pH was maintained at 6.5 and the duodenum was gradually emptied (approximately 20 ml) every 30 minutes after the first 120 minutes of digestion. When the sample reached the jejunum, it was mixed with SIES with 10% bile (v/v) added with at a flow rate of 2.13 mL $min^{-1}$, and the pH was maintained at 6.8. The jejunum compartment was gradually emptied after 150 minutes. In the ilium, the added solution consisted in solely SIES, added at 2.0 mL $min^{-1}$ and the pH value was maintained at 7.2. After 3 hours, all the volume present in the ilium was collected for further analysis. In all gastrointestinal compartments the pH was drop-wise adjusted by adding HCl (37% (w/v)) or $NaCO_3$ (10.6% (w/v)) when required.

**Table 4.2.1**. pH variation in the stomach compartment of the *in vitro* gastrointestinal tract model (TIM).

| Digestion time (min) | pH in the stomach compartment |
|---|---|
| 0 | 7.4 |
| 5 | 4.5 |
| 20 | 4.2 |
| 40 | 2.8 |
| 60 | 2.1 |
| 90 | 1.8 |
| 120 | 1.7 |

## 4.3. Results and discussion

The physicochemical, mechanical, rheological and thermal properties of BC are known to differ depending on the production strain, as well as on the composition of the culture media and operating variables (such as agitation, aeration, pH and temperature control) [113]. In this work, two types of BC were used to verify if the differences in their properties would affect the absorption and saturation limit of bLF, and also the functionality of the surface-modified films. *G. xylinus* ATCC 53582 was used to prepared BC films under static culture conditions (BC1). Also, (BC2), commercial grade 2 raw *nata de coco* from HTK Food CO. Ltd (China) was used. *Nata de coco* is bacterial cellulose obtained in Asian countries using the traditional tray culture fermentations [122].

### 4.3.1. BC edible films physicochemical characterization

#### 4.3.1.1. Endotoxin level determination

Lipopolysaccharide (LPS) is a characteristic pathogen associated molecular pattern (PAMP) present in outer membrane of all Gram-negative bacteria. LPS is widely known for triggering an extremely violent and uncontrolled immune response. Once LPS is recognized in the blood stream, it causes severe cytokine-mediated damage, ultimately leading to death [214, 336]. Even though the produced films in this work are solely intended to be applied in food commodities, the absence of LPS must be assured, since the BC membranes are produced by a Gram-negative prokaryote. Also, while BC is not absorbed by the intestinal tract, LPS may be released and absorbed, thus entering the blood stream. The measured endotoxin values on the processed BC membranes were 0.851±0.292 and 2.027±0.068 EU $L^{-1}$ for BC1 and BC2 respectively. These values are considerably below the maximum limit of acceptance determined by the Food and Drug Administration (FDA) for general medical devices materials, which is 500 EU $L^{-1}$ [337].

#### 4.3.1.2. Determination of the absorption and de-absorption profile

A simple methodology to functionalize the BC films consists on allowing bLF to absorb onto BC nanofibrillar network. Determining the concentration of bLF in the supernatant, although it allows to quantitatively inferring about the amount of absorbed protein, it does not provide information regarding the distribution profile of protein within the 3D nanofibrillar BC matrix. Thus, a Prussian blue formation analysis was performed on both BC1 and BC2 films to qualitatively ascertain about the distribution of the adsorbed protein within the 3D matrix. The bLF used possesses 120 ppm of

$Fe^{3+}$ in its iron binding, clefts, therefore its presence should lead to the synthesis of Prussian blue according to Equation 4.2.1.. The transversal cuts analysed in stereo microscopy showed that bLF was well absorbed throughout all the BC matrixes, Figure 4.3.1., c) and d). Contrarily, BC films solely exposed to PBS did not showed the characteristic coloration originated by the Prussian blue (Figure 4.3.1. a) and b)).

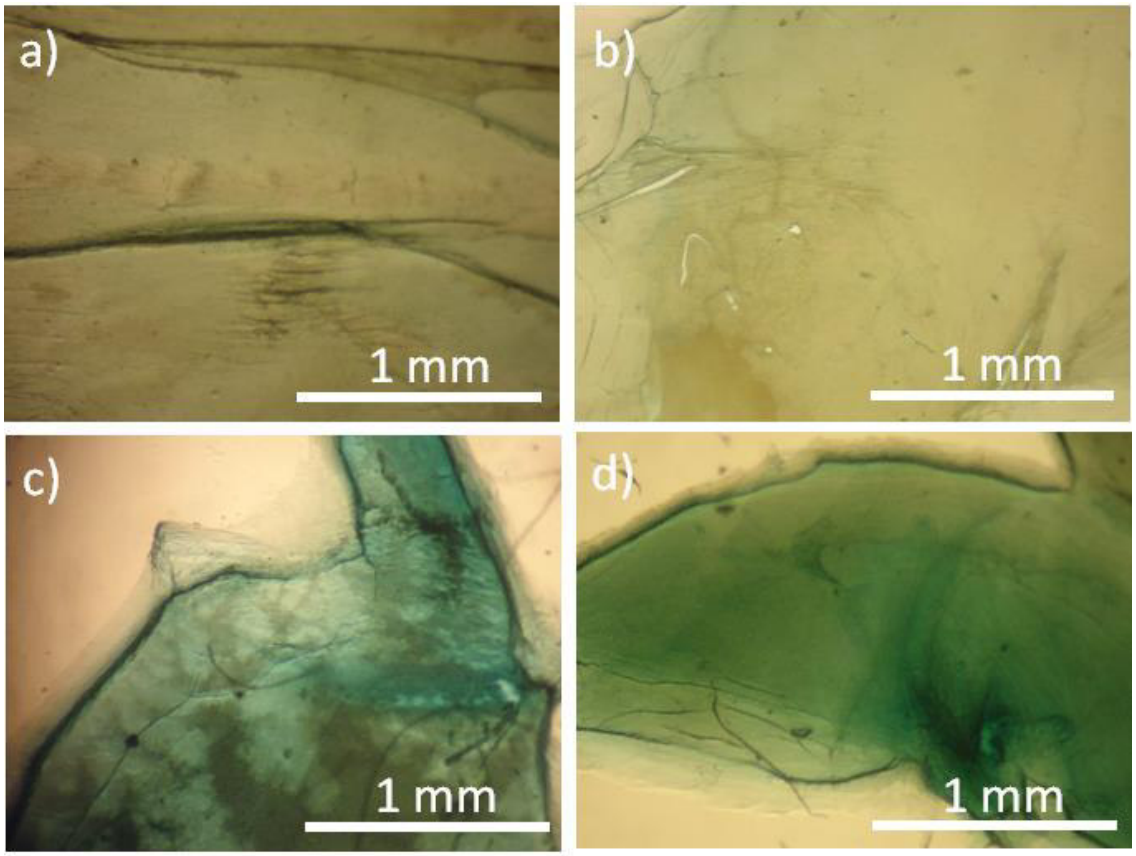


**Figure 4.3.1.** Analysis of Prussian blue formation in the processed bacterial cellulose (BC) films. **a)** bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), **b)** commercial bacterial cellulose (BC2), **c)** bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and **d)** commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF). The images were obtained using a stereo microscope (Olympus).

Once the absorption of bLF was confirmed, further analysis on the absorption/de-absorption profile allowed estimating the saturation limit of bLF. Figure 4.3.2. shows that both BC films present a similar absorption profile that may be divided in 3 phases. The initial absorption phase, comprised between 0 and 1 hour of incubation for BC1 and between 0 and 0.33 hours for BC2; followed by a step increase until the highest concentration plateau is achieved after approximately 4 hours of incubation, which is reached simultaneously by both types of BC films. The sharp bLF absorption rate was roughly estimated as 4.4 and 6.4 mg $mL^{-1}$ $h^{-1}$ for BC1 and BC2 respectively. After 24 hours of incubation, the absorbed bLF per gram of BC1 is 31.8 mg $mL^{-1}$; while BC2 absorbed 40.6 mg $mL^{-1}$. This difference between the two BC was found to be statistically significant (t test, $p < 0.001$).

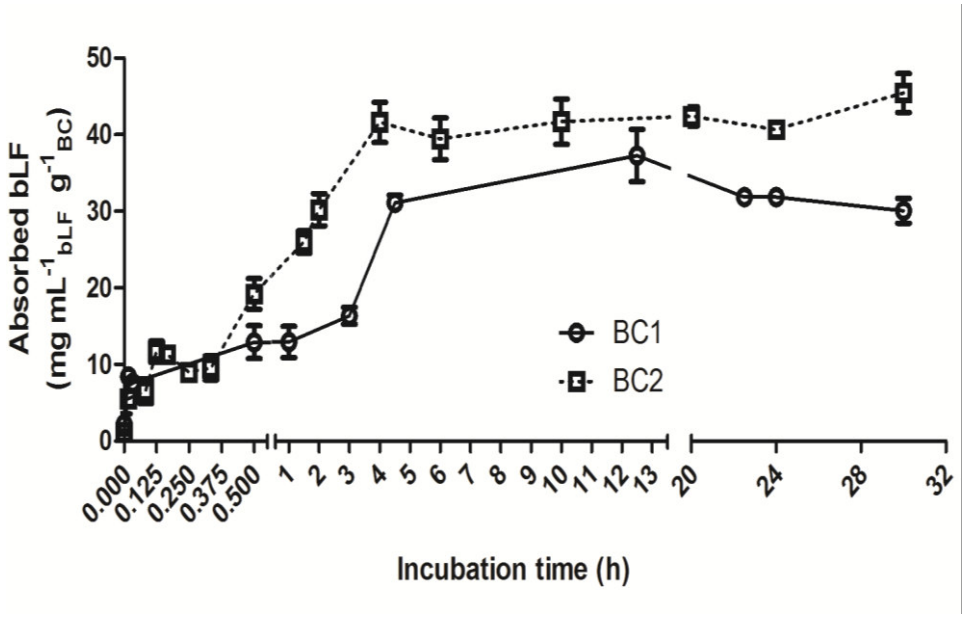


**Figure 4.3.2.** Absorption profiles of bovine lactoferrin (bLF) by: "-o-"never dried bacterial cellulose membranes obtained from *Gluconacetobacter xylinus* ATCC 53582 (BC1), and "-□-" never dried commercially acquired bacterial cellulose BC2. The presented values represent the mean ± standard error, obtained from nine independent assays (n = 9).

The release (de-absorption) profile of LF from BC samples is characterized by an initial steep increase followed by an asymptotic maximum of release. The obtained *in vitro* release profiles were adjusted with a modified Gompertz equation (Figure 4.3.3.).

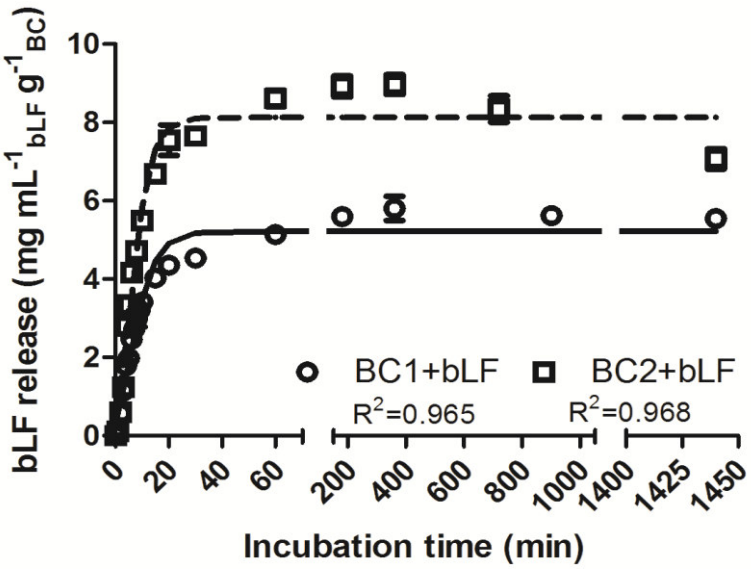


**Figure 4.3.3.** Cumulative release profiles of bovine lactoferrin (bLF) from BC samples: "o" bLF cumulative release from processed bacterial cellulose films synthesized by *Gluconacetobacter xylinus* ATCC 53582 with absorbed bLF (BC1+bLF), "□" cumulative release of bLF from processed commercial bacterial films with absorbed bLF (BC2+bLF), "–" bLF release model for processed *G. xylinus* ATCC53582 bacterial cellulose with absorbed bLF and "–" bLF release model for processed commercial bacterial cellulose with absorbed bLF. All values are represented by the mean ± standard error, obtained from nine independent assays (n = 9).

The release values retrieved from the model are presented on Table 4.3.1.. A significant difference is registered in the cumulative release of bLF at minute 20, as well as in the end of the experiment, at minute 1440, with the higher value corresponding to BC2 (t test, $p < 0.001$). BC2 seems to uptake a higher amount bLF and also it releases a higher concentration of bLF. However, both BC films released almost the same percentage of its absorbed bLF, 18.5% and 17.3% for BC1 and BC2, respectively. The release rate of BC2 is slightly higher as compared to BC1probably due to the higher uptake of bLF by the former BC. BC1, even though possessing a lower release rate, immediately begins to release bLF once immersed in the PBS solution, whereas BC2 takes approximately 1 minute to start releasing bLF.

**Table 4.3.1.** Bovine lactoferrin (bLF) release values estimated using modified Gompertz model (Equation 4.2.2.), "$X_{max}$" maximum bLF cumulative release, "$R_r$" rate of bLF release and "$\psi$" lag period prior to the bLF release.

| | BC1+bLF | BC2+bLF |
|---|---|---|
| $X_{max}$ (mg $mL^{-1}_{bLF}$ $g^{-1}_{BC}$) | 5.2 | 8.1 |
| $R_r$ (mg $mL^{-1}_{bLF}$ $g^{-1}_{BC}$ $min^{-1}$) | 0.4 | 0.7 |
| $\psi$ (min) | 0.0 | 0.8 |

#### 4.3.1.3. Surface free energy

The surface free energy results are gathered in Table 4.3.2.. Lifshitz Van der Waals dispersion forces component, which describes the electrodynamics interaction, was found to be similar in all membranes, whereas the Lewis acid base component, which is related to the interaction involving electron density and ultimately the polarity, showed that all processed BC are nearly exclusive electron donors or basic surfaces, presenting a very low Lewis acid component. [324, 338]. The energy value may give an indication of a possible mechanism for the bLF de-absorption. In order to desorb the bLF from the BC matrix, it is necessary to apply energy that can displace the bLF molecules. If the material is hydrophobic a higher energy will be required for the water molecules to occupy the bLF space. However, if the material is hydrophilic, less energy will be required. A negative value of Gibbs energy indicated the hydrophobic nature of BC1+bLF (-10.32 mJ $m^{-2}$). In opposition, BC2+bLF possess a hydrophilic nature, which may imply a more favourable release of bLF molecules from the matrix into the solution, thus explaining a higher release. It is also worth mention that the BC1 surface is considerably more hydrophilic than BC2 surface.

**Table 4.3.2.** Surface free energy and Gibbs energy components of the processed bacterial cellulose (BC) films: "$\gamma^{LW}$" Lifshitz-van der Waals component," $\gamma^{+}$ " Lewis acid component, " $\gamma^{-}$ " Lewis base component, "$\Delta G_{lwl}^{LW}$ " Gibbs energy of the Lifshitz-van der Walls component," $\Delta G_{lwl}^{AB}$" Gibbs energy of the Lewis acid base component, "$\Delta G_{lwl}^{TOT}$" total Gibbs energy, "BC1" bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin and "BC2+bLF" processed commercial bacterial cellulose with absorbed bovine lactoferrin.

| | $\gamma^{LW}$ | $\gamma^{+}$ | $\gamma^{-}$ | $\Delta G_{lwl}^{LW}$ (mJ m$^{-2}$) | $\Delta G_{lwl}^{AB}$ (mJ m$^{-2}$) | $\Delta G_{lwl}^{TOT}$ (mJ m$^{-2}$) |
|---|---|---|---|---|---|---|
| BC1 | 37.54 | 1.14 | 50.71 | -4.25 | 32.99 | 28.74 |
| BC2 | 40.05 | 0.07 | 26.11 | -5.51 | 1.14 | -4.36 |
| BC1+bLF | 39.28 | 0.48 | 22.57 | -5.11 | -5.21 | -10.32 |
| BC2+bLF | 33.54 | 2.16 | 32.23 | -2.53 | 8.98 | 6.45 |

#### 4.3.1.4. Ultraviolet – visible spectroscopy

The presence of bLF into the processed BC films was further analysed using UV-vis and FTIR spectrometry. UV-vis spectrometry reveals the presence of proteins in the films with absorbed bLF, due to absorption of in UV light in the range of 250 – 290 nm, by the aromatic amino acid residues of phenylalanine, tryptophan and tyrosine [326]. Thus, in both BC1+bLF and BC2+bLF, the typical absorbance peak of bLF is easily identifiable at 295 nm, due to its tyrosine residues involved in metal complexation (Figure 4.3.4.) [194]. After showing that bLF is well distributed within the 3D nanofibrilliar matrix (Figure 4.3.1.), it can be assumed that the higher absorption peak observed in the BC2+bLF denotes a higher uptake of bLF by BC2. Additionally, the high absorption peaks visible between 230 and 200 nm in the BC films with absorbed bLF are due to the absorption of UV light by histidine, cysteine, methionine and also by the aromatic amino acid residues [326].

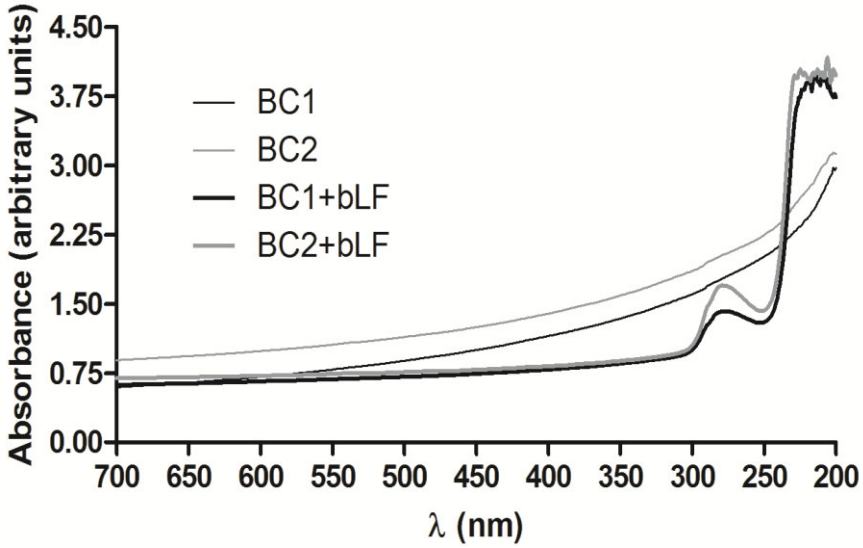


**Figure 4.3.4.** Ultraviolet – visible spectra of the processed bacterial cellulose films: "——" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), "——" processed commercial bacterial cellulose (BC2), "——" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and "——" processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF).

#### 4.3.1.5. Fourier transform infrared – attenuated total reflectance

FTIR-ATR spectra showed the characteristic BC bands in all processed edible films (Figure 4.3.5.). The sharp and steep band found at approximately 1060 $cm^{-1}$, suggests the C-O-C stretching. The low intensity band found neat the 2890 $cm^{-1}$ corresponds to the aliphatic C-H stretching, and finally, the broad band of O-H stretching vibration is present ~3350 $cm^{-1}$ [339]. BC1+LF and BC2+LF possess two characteristic peptide bound peaks: ~1530 $cm^{-1}$ and ~1640 $cm^{-1}$. These sharp peaks correspond to the Amide II (C-N bending and N-H bending), and Amide I (C=O stretching), respectively. The Amide III (~1240 $cm^{-1}$) and the C-O stretching from the $COO^-$ are not identifiable in the BC films with absorbed bLF samples. Moreover, Amide A (~3280 $cm^{-1}$) band is probably masked by the cellulose O-H stretching [191]. Albeit the indication of bLF presence in the BC films, the characteristic peaks of BC apparently are not affected by the absorption of bLF, probably due to the absence of covalent bounds between BC and bLF. Additionally, the FTIR-ATR spectra demonstrated that the processing methodology does not alter the supramolecular structure of either BC or bLF.

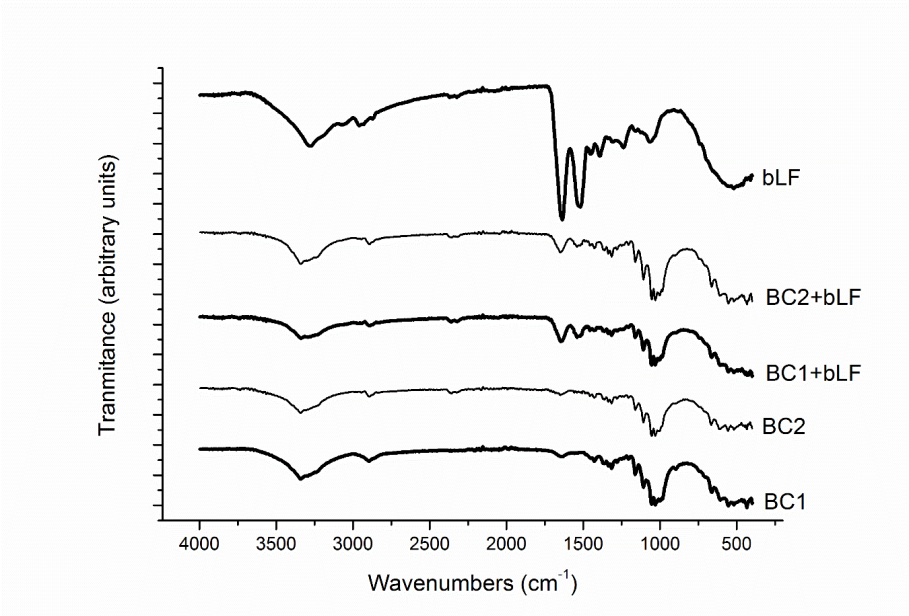


**Figure 4.3.5.** Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) of: "BC1" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC53582 with absorbed bovine lactoferrin, "BC2+bLF" processed commercial bacterial cellulose with absorbed bovine lactoferrin and "bLF" bovine lactoferrin.

#### 4.3.1.6. Swelling behaviour

BC high water binding capacity may confer advantages to the encased food, such as the prevention of dripping and the reduction of water condensation in the outer case, thus improving product presentation and concomitantly, the consumer acceptance [63]. Moreover, it prevents the loss of moister, hence reducing the loss of commercial weight value. The direct contact with a food product that has high water content such as meat will induce the swelling of the BC films during the uptake of water molecules. Therefore, the estimation of the processes films rate of swelling is crucial if considering its use as a packaging system. The drying process eliminates the plasticizing effect of the water molecules, increasing the proximity of neighbour BC fibrils. This promotes the formation of additional hydrogen bounds [111, 340]. The water holding capacity of the dried BC films will never be equivalent to never dried BC membranes. Thus, rehydration is inhibited by a more aggregated BC nanofibrillar structure. All the processed BC films achieved a swelling value ranging between approximately 215% and 380% after just 90 seconds of immersion in PBS (Figure 4.3.6.). These values are significantly higher than the ones reported by Rambo and collaborators [118]. These authors obtained approximately 80% of swelling in BC produced by *G. xylinus* ATCC 23769. BC1, BC2 and BC1+bLF share the same swelling profile, although BC1+bLF showed a higher swelling profile. BC2+bLF has a quite distinct profile, characterized by a second increase phase of

roughly 205% $h^{-1}$, subsequent to the 90 seconds, that subsists until 18 hours of incubation. This may be due to the higher load of bLF in the BC2+bLF that probably prevents the formation of hydrogen bonds between BC fibrils. Consequently, this provides more freedom for water molecules to rehydrate the BC matrix, and ultimately leads to a higher swelling profile.

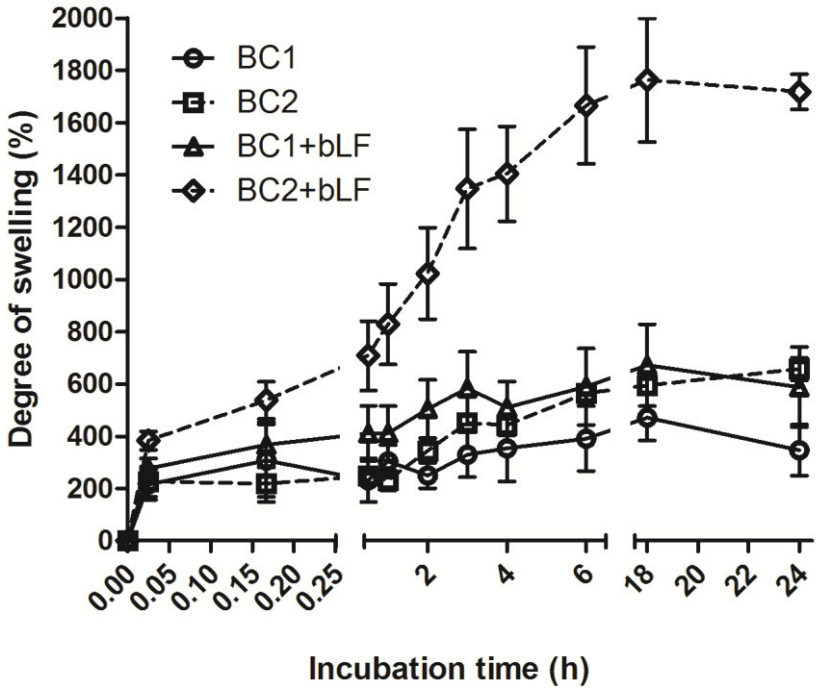


**Figure 4.3.6.** Profile of the degree of swelling: "-●-" processed bacterial cellulose produced by *Gluconacetobacter xylinus* ATCC 53582 (BC1), "-■-"processed commercial bacterial cellulose (BC2), "-▲-" processed bacterial cellulose synthesized by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and "-◆-"processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF). The values are presented using mean ± standard error, obtained from three independent assays (n = 3).

#### 4.3.1.7. Dry weight and thickness

BC membranes thickness values increased after bLF absorption, and this increment was found to be more pronounced in the BC1+bLF (Figure4.3.7. a)). However, the density of BC did not showed same tendency (Figure4.3.7. b)). Interestingly, the density values of BC1 and BC1+bLF are near the 0.99 mg $mm^{-3}$ density value reported by Yamanka and co-workers [108].

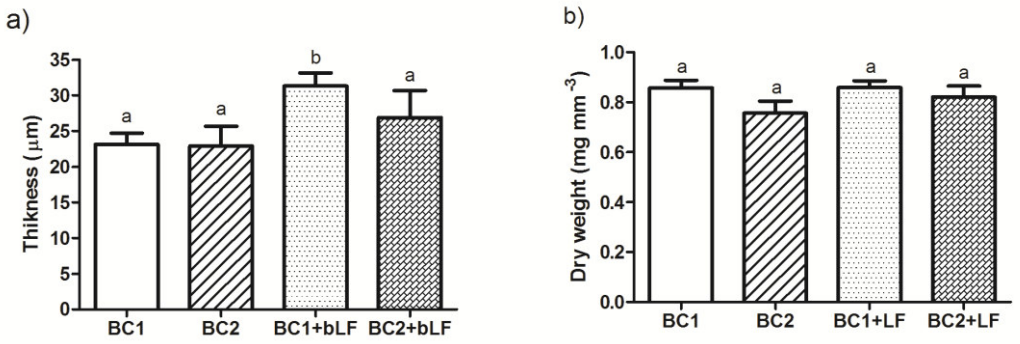


**Figure 4.3.7.** The values of: **a)** thickness and **b)** dry weight per volume of the processed BC membranes: "▭" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), "▨" processed commercial bacterial cellulose (BC2), "▭" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and "▨" processed commercial bacterial cellulose with absorbed lactoferrin (BC2+bLF). Distinct letters between different columns denote statistical different values Kruskal-Wallis with Dunn's multiple comparison post-hoc test. The results are displayed using mean ± standard error, obtained **a)** from thirty five independent assays (n =35), and **b)** from seventy independent assays (n = 70).

#### 4.3.1.8. Water vapour permeability

One essential feature of the edible films is their permeability to water vapour. In order to avoid the loss of moisture and consequently the weight of several types of meats and sausages, casings with low permeability to water vapour are used in the food industry. Other meat commodities, such as chorizo and fresh sausage, require a high permeability to gases. Fresh sausage is usually produced at ambient temperature, using fresh meat mixed with spices, remaining uncooked throughout all manufacturing process. For instance, Portuguese chorizo characteristic rich flavour and chewy texture derive from the bacterial fermentation, that generates moisture and $CO_2$ that must be able to permeate through the casing, otherwise the gas accumulation may compromise the casing physical integrity [341]. Due to their characteristic hydrophilic nature, polysaccharides and proteins usually offer poor water vapour permeability [342], thus making the processed films good candidates for this type of chorizo. Since fresh sausages are stored embedded in storing liquid, the WVP is usually not a concern. However, during cooking higher WVP may permit the permeation of water vapour, and thus avoid the casing disruption.

WVP values herein determined were found to be similar for BC1, BC2 and BC2+bLF, although BC1+bLF possess a higher value (Figure 4.3.8.). BC1 films showed a higher hydrophilicity when compared with BC2, although this is not perceptible in the WVP values. BC1+bLF presented also exhibited a higher thickness after the drying process, despite the relatively lower bLF load. This may be due to the formation of a less compact fibril network, and thus resulting in less tortuous matrix, offering less resistance to the transfer of water molecules. The WVP values are

approximately three fold lower than other bio-based edible films such as chitosan [73, 343], κ-Carrageenan mixed with locust bean gum [329], 7 fold when compared to whey protein films [344] [344] and cassava starch [345], but it is similar to high molecular weight methylcellulose edible films [346-348]. This higher value of permeability may represent a technical advantage when used in fresh sausage and other uncooked meats.

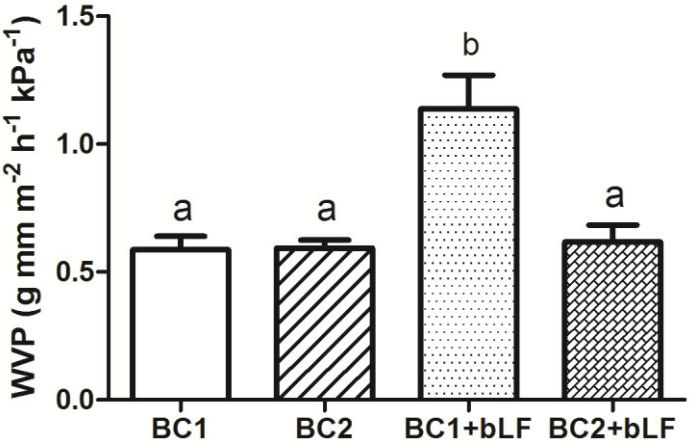


**Figure 4.3.8.** Water vapour permeability (WVP) values of: " ▭ " processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), " ▨ " processed commercial bacterial cellulose (BC2), " ▭ " processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and " ▨ " processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF). The results are displayed using mean ± standard error, obtained from five independent assays (n = 5). Different letters between distinct columns denote significant differences using two-way ANOVA with Tukey post-hoc test ($p < 0.05$).

#### 4.3.1.9. Mechanical properties

The casing must be able to endure the sausage filling process without bulging, thus the evaluation of the mechanical properties denote a key characterization step. The food product used as case study in this thesis possesses high moisture content, additionally it is stored in a liquid filled package; therefore the mechanical properties assessment was performed under water saturating conditions. The mechanical properties and the typical strain profiles are represented in Table 4.3.3. and Figure 4.3.9., respectively. All processed BC films have a $\sigma_{max}$ within the range described by the literature [118]. BC1 and BC2 behaviour towards tensile traction is quite distinct. BC1 has an elastic modulus ($E$) approximately 4 fold higher than that of BC2. Also, BC2 elongation at break was found to be over three times higher, comparing to BC1. bLF absorption into BC matrixes influenced in the same extent the performance of both types of BC, which is in accordance with other studies reported in the literature. For instance, Zhu and co-workers [60] showed a similar influence on the mechanical properties of embedding BC films with ε- polylysine.

**Table 4.3.3.** The mechanical properties: "*E*" elastic modulus, "$\sigma_{max}$" maximum stress and elongation at break "$\varepsilon_{break}$" of: "control" fresh sausage's porcine small intestine casing, "BC1" processed bacterial cellulose produced by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed bacterial processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin and "BC2+bLF" processed commercial bacterial cellulose with absorbed lactoferrin.

| | ***E* (MPa)** | **$\sigma_{max}$ (MPa)** | **$\varepsilon_{break}$ (%)** |
|---|---|---|---|
| Control | 46.1 ± 9.7 | 55.7 ± 4.2 | 42.5 ± 6.5 |
| BC1 | 643.3 ± 35.9 | 54.9 ± 8.4 | 8.1 ± 0.5 |
| BC2 | 153.4 ± 44.7 | 36.7 ± 4.4 | 27.8 ± 8.8 |
| BC1+bLF | 433.0 ± 100 | 41.7 ± 4.6 | 7.6 ± 0.3 |
| BC2+bLF | 114.5 ± 24.5 | 25.6 ± 3.6 | 24.7 ± 1.1 |

For control samples, the fresh sausages casing (pig's small intestine) was used. The casing was removed from the sausages immediately before conducting the mechanical assays. These cases consist of the small intestine and showed a considerable lower elasticity modulus, fourteen fold lower when compared to BC1. These differences were less evident when compared to BC1+bLF and BC2+bLF, although it still was 9 fold and 2.5 fold lower, respectively. The control maximum tensile strength is similar to BC1, and roughly 2 fold higher than BC2 +bLF. These values are in accordance to the maximum tensile values reported for pig intestine (43.3 MPa) and collagen (34.8 MPa) casings [349].

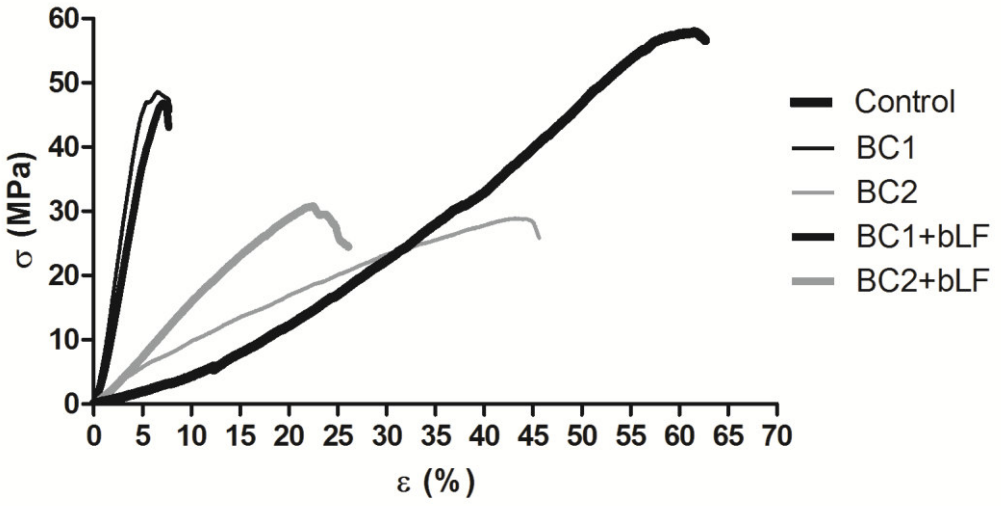


**Figure 4.3.9.** Typical tensile tests profiles of: "▬" pig small intestine fresh sausage casing (control) "▬" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), "▬" processed commercial bacterial cellulose (BC2), "▬" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and "▬" processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF).

## 4.3.2. Bactericidal characterization

### 4.3.2.1. Specific growth rate impact analysis

*E. coli* is a Gram-negative Bacillus major member of the normal intestinal microflora, but at the same time is one of the most frequent causative agent of intestinal infections [350] and an important foodborne pathogen, whereas *S. aureus* is a Gram-positive coccus and is a recurrent agent of food poisoning [204]. The bLF MIC against the tested microorganisms is described in the chapter 5. The non-specific bactericidal properties of bLF have been widely described. Briefly, some of the proposed antibacterial actions include (i) the bLF iron binding capacity may deprive the environmental $Fe^{3+}$, concomitantly impede bacterial carbohydrate metabolism [207]; (ii) the $Ca^{2+}$ chelating by the sialic acid present on the glycan moiety leading to the disruption of LPS, since this ion is an essential element in the stability of Gram-negative outer layer (this action is comparable to ethylenediaminetetraacetic acid (EDTA) and polymixin B [219]); (iii) the specific binding to the lipid A component of the LPS; (iv) the bLF high cationic net charge, it is electrostatically attracted to negatively charged bacteria surface, where it may destabilize the membrane [351].

The influence on the specific growth rate (μ) of *E. coli* and *S. aureus* in NB culture medium of the different bLF concentrations is illustrated in the Figure 4.3.10. bLF concentrations of 5 mg $mL^{-1}$ and 10 mg $mL^{-1}$ were found to significantly impair the growth of *E. coli*, whereas all the tested concentrations exerted a significant reduction of the *S. aureus* specific growth rate.

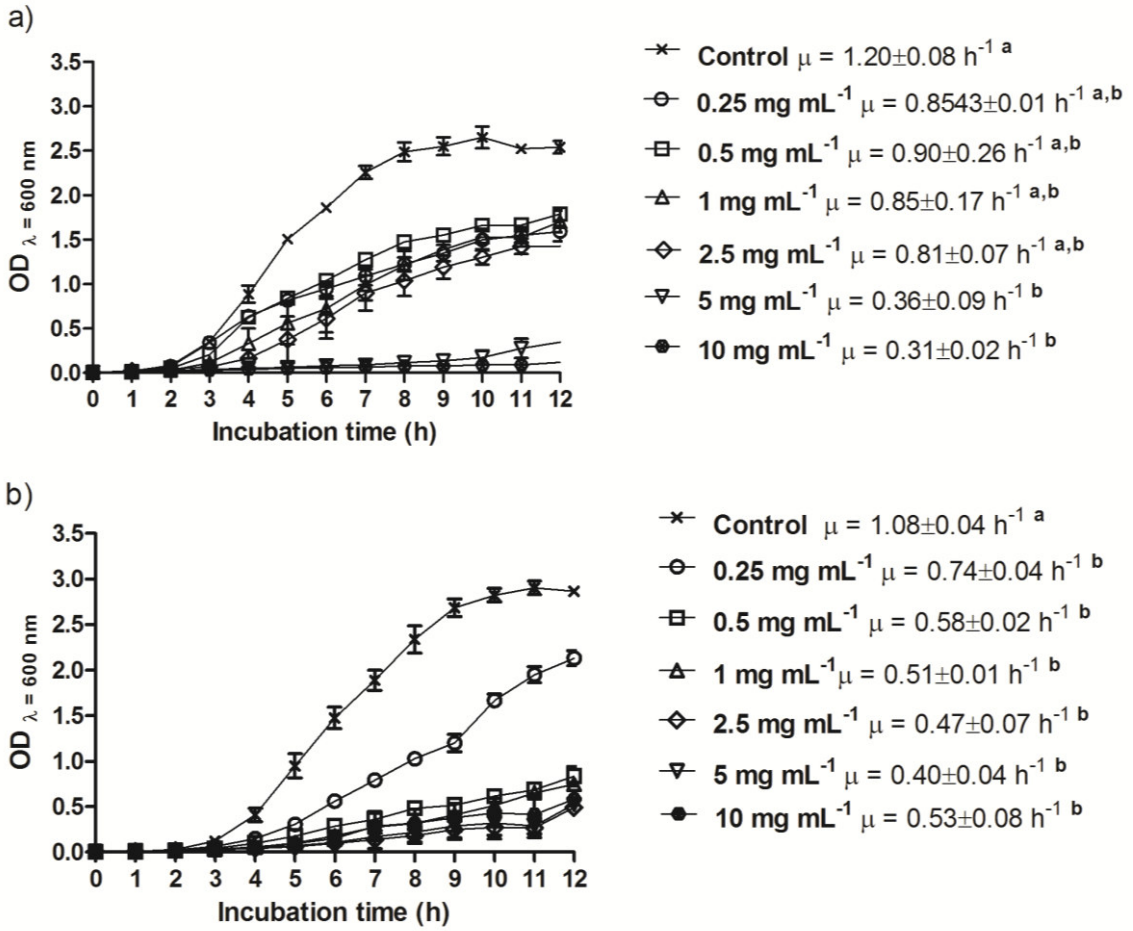


**Figure 4.3.10.** Growth profiles of: **a)** *Escherichia coli* and **b)** *Staphylococcus aureus*, when exposed to different concentrations of bovine lactoferrin (bLF): 0 mg mL$^{-1}$ (Control), 0.25 mg mL$^{-1}$, 0.5 mg mL$^{-1}$, 1 mg mL$^{-1}$, 2.5 mg mL$^{-1}$, 5 mg mL$^{-1}$ and 10 mg mL$^{-1}$. The specific growth rate (μ) is present in the legend, and different letters denote significant differences analysed using one-way ANOVA and Tukey post-hoc test ($p < 0.05$). All results are shown using mean ± standard error, obtained from three independent assays (n = 3). The cultures were performed in nutrient broth (NB), at 37°C with an orbital agitation of 120 rpm.

To evaluate if the processed films could also affect the specific growth rate of microorganisms, film patches were incubated in NB media and the growth profiles were analysed (0.6 circular processed films with 22 mm of diameter per mL of culture medium). The quantity of processed films used was defined to ensure the excess of culture media throughout the experiment, bearing in mind the volume loss due to sampling and absorption via film swelling. As shown in Figure 4.3.11., the BC1 and BC2 processed films did not affected the specific growth rate of none of the studied microorganisms. Interestingly, the BC2 led to higher specific growth rate profiles for both microorganisms as compared to the control. Also, BC1 showed a higher specific growth rate in comparison to the control when cultured with *E. coli* most likely due to the increase of the aeration of the culture media, since the presence of the circular patches may have increased the culture

turbulence during agitation [352]. Overall, processed films without absorbed bLF did not present any significant differences compared to the control. Considering the release models displayed in Figure 4.3.3. and the films dry weight (Figure 4.3.7.), the estimated bLF released into the culture media was approximately 0.8 mg $mL^{-1}$ in bothBC1+bLF and BC2+bLF. Therefore, the expected impact on the specific growth rate should be similar to the obtained in the flasks with 1 mg $mL^{-1}$ of bLF which was 0.85 $h^{-1}$ for *E. coli* and 0.51 $h^{-1}$ for *S. aureus*. BC1+bLF showed these expected growth rate when cultured with *E. coli* (0.86 $h^{-1}$), although it did not displayed a significant difference compared with the control. *E. coli* cultured with BC2+bLF presented a growth rate (0.48 $h^{-1}$) lower than expected, although with a high degree of variability within replicas. In the case of *S. aureus* cultures, the growth rate values are lower than the ones expected in the case of BC1+bLF (0.39 $h^{-1}$) and higher for the BC2+bLF (0.70 $h^{-1}$). All the results associated with the processed membranes showed a considerably higher variability as compared to untreated BC samples. This inconsistent bias may be related with the effect of BC in the turbulence of the culture medium, but also due to the swelling experienced by the BC edible films. Microorganisms are usually prone to adhere to surfaces. In the case of bLF-modified BC, it is possible that the adhering bacteria became exposed to higher bLF concentrations (during swelling of BC and leaching of bLF), thus leading to a greater reduction of the growth rate [353]. However, this effect may have not occurred in a reproducible manner in all experiments using modified BC samples. Moreover, it is important to notice that the optical density measurements of biomass has some limitations, namely an increase in the optical density gives an indication of bacterial growth but not of bacterial viability [354].

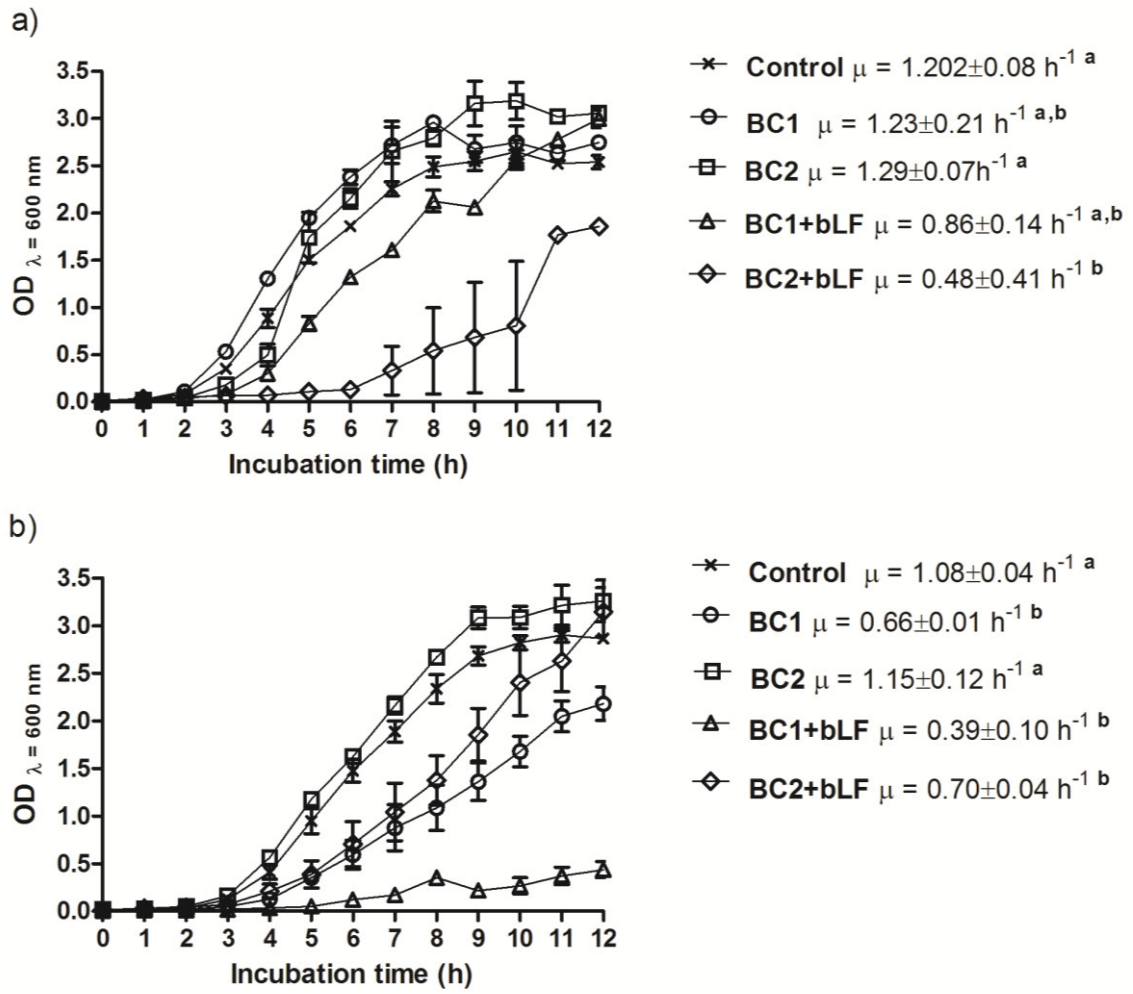


**Figure 4.3.11.** Growth profile of **a)** *Escherichia coli* and **b)** *Staphylococcus aureus* in contact with processed bacterial cellulose films: "Control" without any processed bacterial cellulose films, "BC1" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin and "BC2+bLF" processed commercial bacterial cellulose with absorbed bovine lactoferrin. The specific growth rate (μ) is present in the legend, and different letters between distinct columns denote significant differences analyzed using two-way ANOVA with Tukey post-hoc test ($p < 0.05$). The values displayed are represented using mean ± standard error, obtained from three independent assays (n = 3).

#### 4.3.2.2. Live/dead viability and scanning electron microscopy (SEM) analysis

The live/dead assessment test (Figure 4.3.12.) showed a relative higher killing ratio (red cells) for the processed BC films with absorbed bLF as compared to BC processed films.

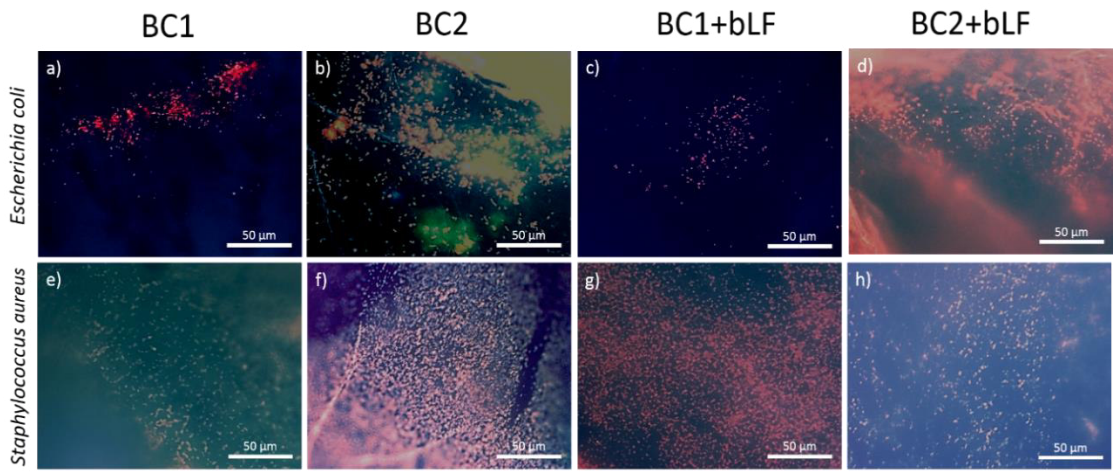


**Figure 4.3.12.** Live/dead assessment images of the bacteria at the surface of the processed films after 4 hours of incubation in nutrient broth at 37 °C with 120 rpm of orbital agitation: in contact with *Escherichia coli*: **a)** processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC53582, **b)** processed commercial bacterial cellulose, **c)** processed bacterial cellulose produced by *G. xylinus* ATCC53582 with absorbed bovine lactoferrin, **d)** processed commercial cellulose with absorbed bovine lactoferrin; incubated with *Staphylococcus aureus*: **e)** processed bacterial cellulose synthesized by *G. xylinus* ATCC53582, **f)** processed commercial bacterial cellulose, **g)** processed bacterial cellulose produced by *G. xylinus* ATCC53582 with absorbed bovine lactoferrin, **h)** processed commercial cellulose with absorbed bovine lactoferrin.

SEM of the processed BC samples cultured with either *E. coli* or *S. aureus*, revealed a clear difference in the bacteria morphology present on the surface of the processed films (Figure 4.3.13.). Clearly, the BC1+bLF and BC2+bLF surfaces are covered with *E.coli* (Figure 4.3.13. c), d)) and *S. aureus* (Figure 4.3.13. g), h)) unviable bacteria debris, thus confirming the bactericidal effect of bLF. Some *S. aureus* death was also visualized in the BC2 (Figure 4.3.13. f)).

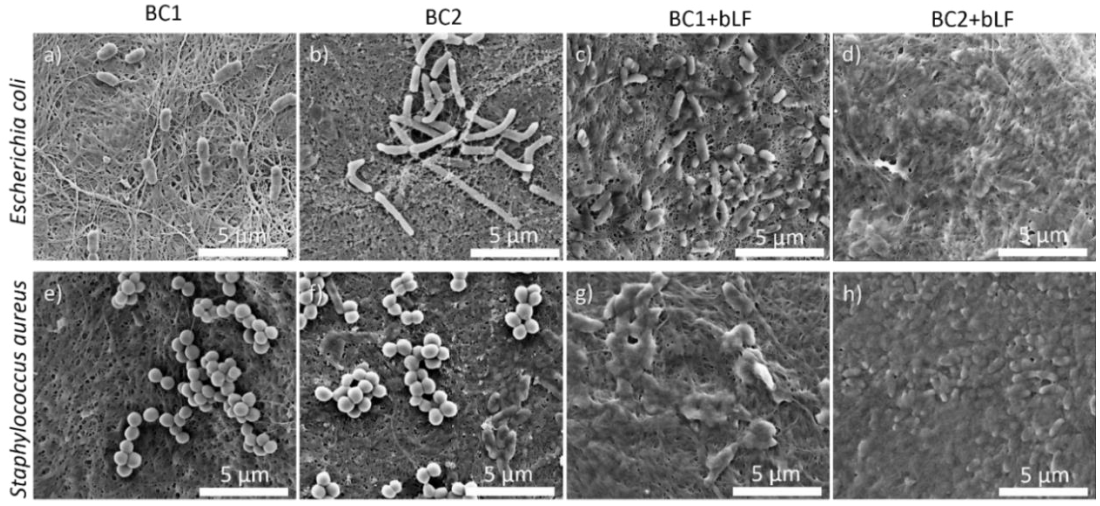


**Figure 4.3.13.** Scanning electron microscope (SEM) images of the bacteria in the surface of the processed films after 4 hours of incubation in nutrient broth at 37 °C with 120 rpm of orbital agitation: in contact with *Escherichia coli*: **a)** processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC53582, **b)** processed commercial bacterial cellulose, **c)** processed bacterial cellulose produced by *G. xylinus* ATCC53582 with absorbed bovine lactoferrin, **d)** processed commercial cellulose with absorbed bovine lactoferrin; incubated with *Staphylococcus aureus*: **e)** processed bacterial cellulose synthesized by *G. xylinus* ATCC53582, **f)** processed commercial bacterial cellulose, **g)** processed bacterial cellulose produced by *G. xylinus* ATCC53582 with absorbed bovine lactoferrin, **h)** processed commercial cellulose with absorbed bovine lactoferrin.

#### 4.3.2.3. Evaluation of contact bactericidal effectiveness (contact killing)

The processed film bactericidal efficiency was quantified. Films containing bLF demonstrated the tendency for a higher effectiveness in the percentage of CFU reduction (Figure 4.3.14.). BC1+bLF and BC2+bLF showed a significant *E. coli* CFU reduction when compared to the reduction displayed by BC1 and BC2. BC1 and BC2 presented a low percentage of reduction (below 10%), whereas the BC1+bLF and BC2+bLF showed a considerably higher reduction, of approximately 65% and 73%, respectively. Even though BC1+bLF and BC2+bLF films promoted a higher reduction against *S. aureus*, this reduction was not significant when compared to the reduction exerted by BC1 and BC2. The processed films without bLF showed an unusual high reduction of *S. aureus* CFU of approximately 65% for both film types. This is probably due to the fact that *S. aureus* is more sensitive to the conditions imposed by the BC surfaces, which may also have led to the higher CFU reduction experienced by the contact with the processed films bearing bLF. Both BC1+bLF and BC2+bLF reduced approximately 96% of *S. aureus* viable bacteria, both displaying a 1 log reduction. Interestingly, albeit the significantly higher bLF load present in BC2+bLF, no differences in the reduction of the viability of either *E. coli* or *S. aureus* when compared to BC1+bLF were detected, most likely due to similar amounts of bLF available at the surface of the films.

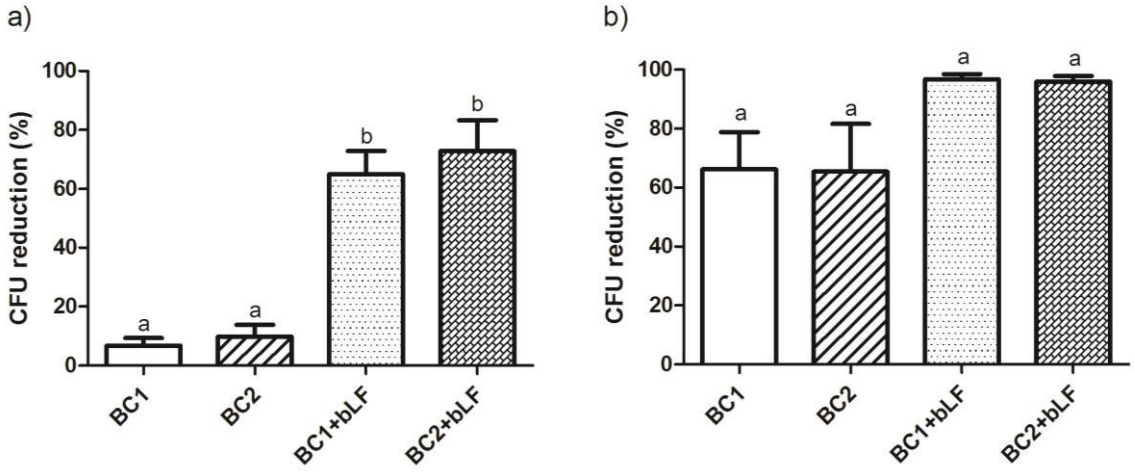


**Figure 4.3.14.** Percentage of colony forming units (CFU) reduction of **a)** *Escherichia coli* and **b)** *Staphylococcus aureus* by: " ▭ " processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), " ▨ " processed commercial bacterial cellulose (BC2), " ▭ " processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with absorbed bovine lactoferrin (BC1+bLF) and " ▨ " processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF). During a period of 2 hours at 37 °C. Results are displayed using mean ± standard error, obtained from three independent assays (n = 3). Distinct letters between different columns denote significant differences analysed using **a)** two-way ANOVA with Tukey post-hoc test, and **b)** Kruskal-Wallis with Dunn's post-hoc test ($p < 0.05$).

BC films were also applied to fresh sausage and their antibacterial efficiency was analysed through contact killing. Absence of nutrient shortage and good humidity conditions were assumed, since the microorganisms were in direct contact with fresh sausages, so the exposure time was increased to 24 hours (Figure 4.3.15.). Even though the assay occurred under optimal temperature conditions for microbial growth, a tendency to reduce viable *E. coli* bacteria could be easily identifiable (Figure 4.3.15.a)). When in contact with *E. coli*, BC1, BC1+bLF and BC2+bLF films demonstrated a similar reduction in the CFU percentage. BC1+bLF and BC2+bLF showed a higher reduction in the viability of bacteria, which reached 93.6% and 95.9%, respectively. On the other hand, *S. aureus* displayed growth in BC1 and BC2, while BC1+bLF and BC2+bLF displayed a slight inhibition (approximately 32.9% and 39.7%, respectively) (Figure 4.3.15.b).

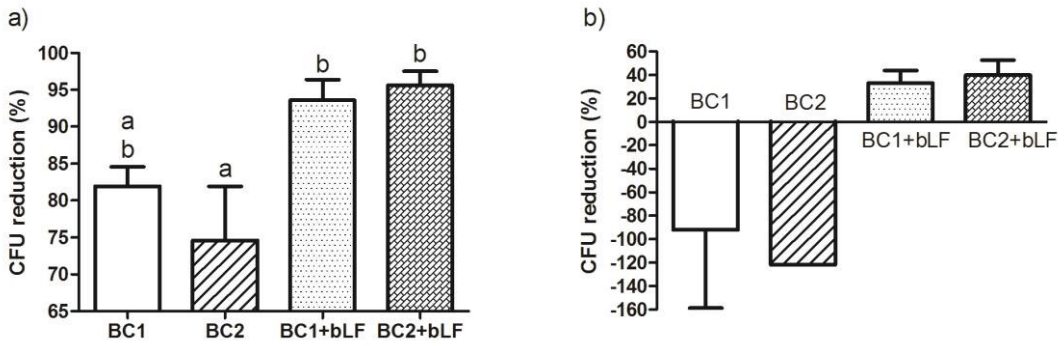


**Figure 4.3.15.** Percentage of reduction of colony forming units (CFU) of **a)** *Escherichia coli* and **b)** *Staphylococcus aureus* by: " ▭ " processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582 (BC1), " ▨ " processed commercial bacterial cellulose (BC2), " ▭ " processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with adsorbed bovine lactoferrin (BC1+bLF) and " ▨ " processed commercial bacterial cellulose with absorbed bovine lactoferrin (BC2+bLF). During a period of 24 hours at 37°C. Results are displayed using mean ± standard error, obtained from three independent assays (n = 3). Distinct letters between different columns denote significant differences analysed using **a)** two-way ANOVA with Tukey post-hoc test, and **b)** the statistical treatment was not valid.

## 4.3.3. Cytotoxicity

### 4.3.3.1. Cell viability assessment

An essential assumption for a film to be considered edible is its absence of cytotoxicity. Therefore, the new BC-based materials were evaluated regarding its effect in the 3T3 cell proliferation and viability, both resulting from direct contact and after simulating a dynamic gastrointestinal digestion in a TNO system. Direct contact with 3T3 cells showed a slight impairment of the cell proliferation after 24 hours of exposure, thus showing low cell adhesion and low proliferation as compared to the control (polystyrene surface). This is in accordance with the results previously obtained in our group and it was mainly due to the high hydrophilic nature offered by the processed BCs (Figure

4.3.16.a)) [135, 136]. Among processed BC films the cell proliferation is identical, with similar values of total cell count in films with and without bLF. Cell viability did not suffered any significant impact, namely for the cells seeded on BC1. BC2, BC1+bLF and BC2+bLF showed a lower percentage of viability mainly at 48 hours demonstrating approximately 20% of viability loss at this time point (Figure 4.3.16.b)). No cytotoxicity was expected since 10 mg $mL^{-1}$ of bLF has been reported to be non-toxic to CACO-2 cells [222].

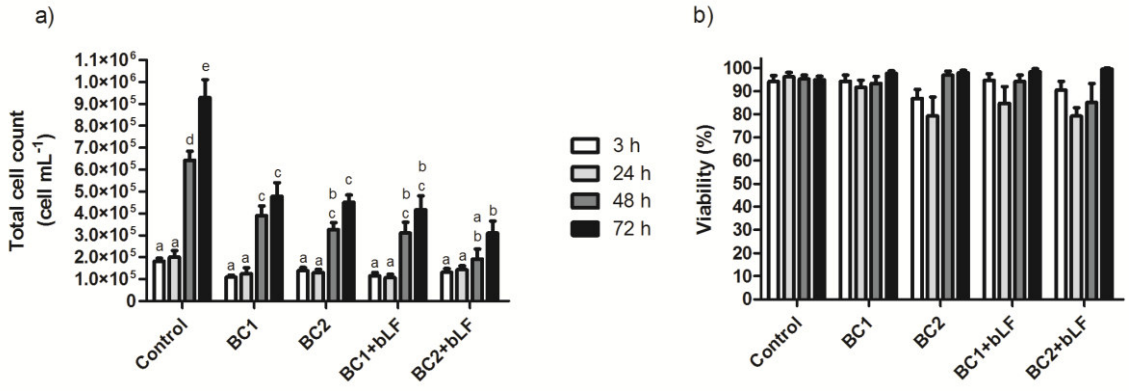


**Figure 4.3.16.** Cytotoxicity analysis using mammalian mouse embryo fibroblasts 3T3 (ATCC CCL-164) at "▭" 3 hours, "▭" 24 hours, "▭"48 and "▭" 72 hours of incubation at 37°, 5% $CO_2$ humidified atmosphere: **a)** total cell count, **b)** percentage of viability. The fibroblast cells were directly seeded over: "Control" culture plate polystyrene surface, "BC1" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with adsorbed bovine lactoferrin and "BC2+bLF" processed commercial bacterial cellulose with absorbed bovine lactoferrin. Results are expressed in mean ± standard error, obtained from six independent assays (n = 6). Statistical analysis: **a)** two-way ANOVA with Bonferroni post-hoc test, different letters between different columns denote significant differences, **b)** Kruskal-Wallis and Dunn's multiple comparison test exhibited no statistical significant differences between samples ($p < 0.05$).

### 4.3.3.2. Mimetic and dynamic in-vitro gastro-intestinal model

After digestion in the mimetic dynamic gastrointestinal system, the 3T3 cells were challenged with the several digestion products (Figure 4.3.17.). The resulting products of the digestion were diluted (1/5), otherwise the cell viability was equal to 0%. Higher dilution values (1/10) led to similar results as compared to the control (*data not shown*). Significant differences in the total cell count were only visible after 72 hours of incubation (Figure 4.3.17. a)). The cell number of BC1 remained identical throughout the experiment, although the minimum percentage of viability was 80%. BC2+bLF samples presented the higher percentage of viability and demonstrated a significant increase of the cell number after 72 hours of incubation. SIES samples were non-cytotoxic to the cells. However, these lack several of the gastrointestinal components. The production of an accurate control would imply a gastrointestinal digestion without the injection of any processed

films, which was not feasible. The ilium's main fluid is SIES, and therefore it was used as the control sample. BC1+bLF and BC2+bLF could have exerted growth stimulation, since bLF has been reported to act as an growth factor in cell culture, however only a modest tendency could be visualized in  when compared to BC2 [355].

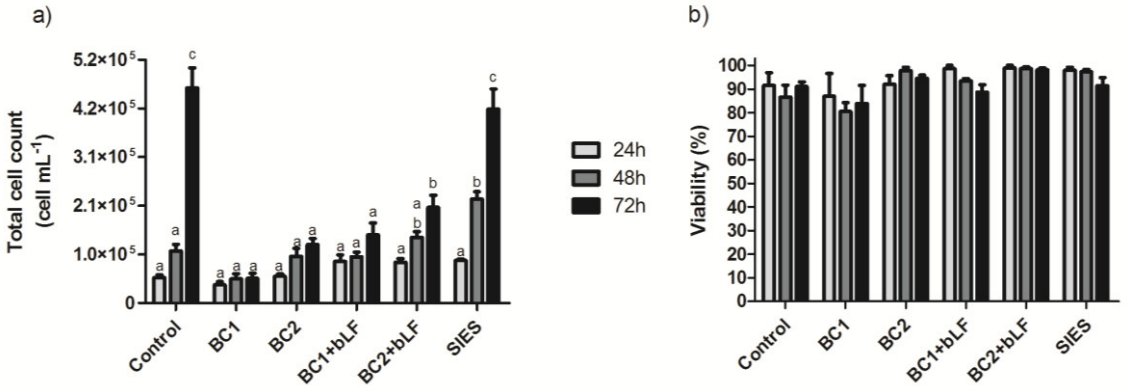


**Figure 4.3.17.** Cytotoxicity analysis using mammalian mouse embryo fibroblasts 3T3 (ATCC CCL-164) at "▭" 3 hours, "▭" 24 hours, "▭"48 and "▭" 72 hours of incubation at 37°, 5% $CO_2$ humidified atmosphere: **a)** total cell count, **b)** percentage of viability. The fibroblast cells were directly seeded over: "Control" culture plate polystyrene surface, "BC1" processed bacterial cellulose synthesized by *Gluconacetobacter xylinus* ATCC 53582, "BC2" processed commercial bacterial cellulose, "BC1+bLF" processed bacterial cellulose produced by *G. xylinus* ATCC 53582 with adsorbed bovine lactoferrin, "BC2 + bLF" processed commercial bacterial cellulose with absorbed lactoferrin and "SIES" small intestinal electrolytic solution. Results are expressed in mean ± standard error, obtained from six independent assays (n = 6). Statistical analysis: **a)** two-way ANOVA with Bonferroni post-hoc test, different letters between different columns denote significant differences, **b)** Kruskal-Wallis and Dunn's multiple comparison test exhibited no statistical significant differences between samples ($p < 0.05$).

## 4.4. Conclusions

Two different BC were evaluated as a structural material for active edible films. BC1 was produced under controlled laboratory conditions using *G. xylinus* ATCC 53582, whereas BC2 was commercially acquired. Even though BC is composed by highly pure cellulose nanofibrils as a part of *G. xylinus* carbon metabolism of, it may present different physical properties due to distinct fibril network architecture, that may be obtained due to the variability of culture conditions, such as different oxygen tension and culture components, along with differing strains [83, 108]. Thus, both BC studied showed distinct properties and interactions with the antimicrobial multifunctional protein, bLF. Prussian blue formation analysis showed that bLF is evenly absorbed through all BC nanofibril matrixes, although BC2 presented a significant higher loading capacity. The maximum bLF loading was achieved after 4 hours of incubation in either BC1 (approximately 30 mg $mL^{-1}_{bLF}$ $g^{-1}_{BC}$) and BC2 (approximately 40 mg $mL^{-1}_{bLF}$ $g^{-1}_{BC}$). The amount of bLF released is also significantly higher for BC2+bLF (~8 mg $mL^{-1}$ bLF $g^{-1}$ BC) than for BC1+bLF (~5 mg $mL^{-1}$ bLF $g^{-1}$ BC), achieved

after 40 minutes of incubation in PBS. Surface free energy shows that the BC1 surface is more hydrophilic that BC2, moreover BC2+bLF is more hydrophilic than BC1+bLF, may indicate a higher bLF release. Spectrometry corroborated the higher bLF loading capacity of BC2 but it also demonstrated that the processing of BC films did not affect the structure of both BC and bLF. Interestingly, BC1 demonstrated a significant increase in the thickness after bLF absorption, whereas BC2 showed a more modest increase. Dry weight per volume of BC also showed an increase after bLF absorption, possibly due a higher bLF loading capacity of the BC2. WVP values may prove useful when applied in some specific meat applications. Pig small intestine casing elastic modulus was found to be considerably lower than the processed films. Although its maximum stress value is comparable to BC1 and BC1+bLF, the elongation at break is considerably higher. A slight impairment of the mechanical properties of the BC films was registered as a consequence of bLF addition. Even though synthesized by Gram-negative bacteria, the purification process used in both BC revealed a residual and highly acceptable level of endotoxin. *S. aureus* specific growth rate was affected in a significant manner by all the tested bLF concentrations, whereas the *E. coli* specific growth rate was only significant impaired by 5 mg $mL^{-1}$, and 10 mg $mL^{-1}$ of bLF. BC1+bLF and BC2+bLF were able to reduce the CFU viability of *E. coli* and *S. aureus*. The specific growth rate of microorganisms was also compromised by the contact with BC1+bLF and BC+bLF. These results were corroborated by live/dead assays, SEM and contact killing assays. The performance of the edible films containing bLF also showed superior capacity to reduce viable bacteria, once compared to the fresh sausage natural casing. All processed BC edible films were found to be non-toxic.

# Chapter 5

## Assessment of the properties of fish gelatin and bovine lactoferrin-fish gelatine electrospun nanocomposites

# 5. Assessment of the properties of fish gelatine and bovine lactoferrin-fish gelatine electrospun nanocomposites


## Abstract

The use of active packaging with antimicrobial properties may present several advantages, such as the spanning the food shelf-life, and increasing food safety, even when the optimal storage conditions are not met. This chapter describes the production and characterization of a novel fully protein-based electrospun fibrous nanocomposite holding antimicrobial properties. Its composition is exclusively comprised of proteins, with fish gelatine (FG) as structural matrix material and bovine lactoferrin (bLF) as the active antimicrobial agent. Electrospun FG with random and aligned orientations were crosslinked with glutaraldehyde vapour in order to improve stability in aqueous solutions. Moreover, these materials were found to be non-toxic against 3T3 fibroblasts.

Two different methods were used to incorporate bLF into the FG nanofibres, namely (i) using bLF as filler in the electrospinning formulation with different concentrations (2, 5 and 10%wt); and (ii) through adsorption, immersing FG electrospun nanofibres into a solution with 40 mg $mL^{-1}$ of bLF. The nanocomposite materials comprising FG and bLF were crosslinked with glutaraldehyde vapour after electrospinning process. On the other hand solely the electrospun FG fibres were submitted to a crosslinking step, prior to its immersion into bLF solution.

Fourier transform infrared spectroscopy analysis with attenuated total reflectance showed that the structure of both FG, and FG+bLF nanocomposites, remained intact through the ectrospinning blending and crosslinking procedure. bLF concentrations of 10 mg $mL^{-1}$ and over 40 mg $mL^{-1}$ were found to be bLF minimum inhibitory concentration for *Escherichia coli* and *Staphylococcus aureus* respectively.

The samples containing the higher concentration of bLF as filler exhibited a remakable reduction of a 6 log reduction (99.9999% reduction) in the number of CFUs of *E. coli* and *S. aureus*.


## 5.1. Introduction

Innovative bio-based materials holding biological activities are being designed for the development of better commodities. The use of protein-based polymers such as gelatine, casein, soy, whey for

the development of new bio-based materials is widely established; yet their properties may be further enhanced through the use of advanced technologies such as electrospinning. Electrospinning is a versatile technique that allows the fabrication of fibres ranging from the micro to the nanometer size [356]. A usual electrospinning setup includes a syringe pump that injects a polymer solution through a blunt stainless steel needle that is connected to a high voltage source, and a grounded collector. Briefly, the high voltage applied in the needle induces free charges in the polymer solution, once the charged solution reaches the tip of the needle it encounters strong electrostatic forces that propel a polymer jet from the needle to the grounded collector [357, 358]. As the jet travels through the atmosphere between the needle tip to a ground collector, the solvent evaporates and solid polymer fibres are deposited on the collector [356]. Gelatine is one of the most widely used protein sources, due to its gelling properties, transparency, oxygen impermeability, and resilience [142]. Gelatine is obtained from the partial hydrolysis of collagen, usually through acid or alkali treatments giving rise to gelatine type A and type B, respectively [144, 148]. Different denaturation process as well as diverse collagen sources result into gelatines with distinct physicochemical properties [144, 147]. Fish gelatine (FG) is a source of gelatine with high potential to be exploited since it may be extracted from the inedible components of the process catch which roughly accounted for 50 million tonnes in 2013 [154]. Moreover, FG consumption is free of religious restrictions, but most importantly it is absent of bovine spongiform encephalopathy, and other prions [143, 145].

Cationic proteins and peptides with antimicrobial properties represent a promising response to the emerging threat of antimicrobial resistance since they may synergistically act with, or even substitute antibiotics, that are becoming alarmingly ineffective [7, 359].

Bovine lactoferrin (bLF), a glycoprotein usually purified from non-pasteurized milk and cheese whey, is an important member of the innate immune system [175]. The impressive number of roles attributed to bLF includes, among others, a wide spectrum antimicrobial activity against bacteria, virus, yeast and parasitic protozoa [172, 180]. The mechanism for bLF antibacterial activity is related to its potent iron chelating capacity, specific and unspecific binding to components of bacteria envelope (proteins, lipotheichoic acid, porins, phopholipids), causing the disruption of lipopolysaccharide (LPS) or the destabilization of the membranes polarity [184, 208, 209].

This chapter describes the production and characterization of FG electrospun fibres, followed by the synthesis of FG electrospun membranes containing different amounts of bLF, as a novel edible antimicrobial material.

## 5.2. Materials and methods

Bovine lactoferrin (bLF) was obtained from DMV International (USA) containing (% dry weight) 96% protein, 0.5% ash and 3.5% moisture, and an iron content of approximately 120 ppm. FG and formic acid (FA) were purchased from Sigma-Aldrich and N,N-dimethylformamide (DMF) (Merck). Polymer solution was prepared by dissolving fish gelatine in a mixture of 40% FA and 60% DMF (v/v) at 50 °C to achieve a final gelatine concentration of 30 wt%. A film of gelatine was processed form the abovementioned solutions by adding 1 % of glutaraldehyde to the solution that was solvent casted in to a Teflon dish. For the preparation of gelatine/bLF composites with different amounts of bLF (0, 2, 5 and 10 wt%), the filler was dispersed in the FA/DMF solution with the help of an ultrasound bath (Bandelin, Sonorex Super RK 106) for 4 hours at room temperature. Gelatine was then added to the solution and placed under agitation with the help of magnetic stirrer at 50 °C.

### 5.2.1. Electrospun membranes preparation and characterization

#### 5.2.1.1. Electrospinning

The protein solution was placed in a commercial plastic syringe (10 mL) fitted with a steel needle with 500 μm of inner diameter. Electrospinning was conducted at 1.25 kV $cm^{-1}$ (distance to collector of 20 cm) with a high voltage power supply from *Glassman* (model PS/FC30P04). A syringe pump (*Syringepump*) was used to feed the solutions into the needle tip at a rate of 0.2 mL $h^{-1}$. The generated electrospun mats were collected in grounded metal collecting plates (random fibres) and in a drum collector at 750 rpm (aligned fibres). After processing, the membranes were stored at room temperature in a desiccator with silica gel, protected from light, until further use.

#### 5.2.1.2. Crosslinking

Electrospun fibre mats were placed in a vapour chamber (vacuum-temp, JPSelecta) with 20 mL of glutaraldehyde (GA, 50 % PS, Panreac) placed at the bottom of the chamber. The fibre mats were exposed to GA-saturated air for 2, 4, 6, 24 and 48 hours at room temperature (25 °C). Unreacted GA aldehyde groups were neutralized by immersion in 100 mmol glycine aqueous solution for 30 minutes at pH 7 [360] and thoroughly washed with sterilized deionized water. Finally, the membranes were placed in a vacuum freeze-drying equipment (Scanvac CoolSafe VP100) for at least 24 h. To obtain electrospun mats with adsorbed bLF, pristine gelatine fibre membranes were cut into 9 mm diameter disks and immersed in 40 $mg.mL^{-1}$ bLF aqueous solution for 24 °C under

mild orbital agitation. The disks were then retrieved from the solution and freeze-dried (Scanvac CoolSafe VP100) for at least 24 h and used for subsequent assays.

## 5.2.1. Characterization of electrospun fibre mats

### 5.2.1.1. Crosslinking degree

The crosslinking degree of fish gelatine electrospun fibres with and without bLF was determined by the ninhydrin assay (NHN). Ninhydrin reacts with free amino groups to produce the purple coloured ninhydrin-amino complex, which optical density is proportional to the number of free amino groups [361-363]. Ninhydrin solution was prepared as follows: Solution A: 1.05 g of citric acid, 10 mL (1.0 M) NaOH and SNCl2.2H2O were mixed and the volume filled to 25 mL with deionized water; Solution B: 1.00 g ninhydrin was added to 25 mL of ethylene glycol monomethyl ether and stirred at room temperature until complete dissolution. The two solutions, A and B, were mixed and stirred at room temperature for 45 minutes and stored in a dark bottle. The crosslinking degree was assessed in 1.5 mg of lyophilized electrospun gelatine membranes with and without bLF. The fibre mats were incubated with 1 mL of ninhydrin solution and heated to 100 °C in a water bath for 20 minutes. The solution was then cooled down to room temperature, diluted with 5 mL of 50% isopropanol, and the optical absorbance was measured at 570 nm with a spectrophotometer (UV, 2501 PC Shimadzu). The amount of free NH2 groups in the sample was determined by comparison to a standard curve of glycine vs absorbance. The crosslinking degree ($X_{CL}$) is determined according to Equation 5.2.1.:

$$X_{CL} = \frac{[(NHN\ reactive\ amine)_{free} - (NHN\ reactive\ amine)_{fixed}]}{(NHN\ reactive\ amine)_{fresh}} \times 100 \qquad \text{Equation 5.1.1.}$$

where “free” is the mole fraction of free NH2 in the non-crosslinked samples and “fixed” is the mole fraction of free NH2 remaining in the crosslinked samples. Three different regions of each type of electrospun samples were evaluated and the results were averaged.

### 5.2.1.1. Scanning electron microscopy

To evaluate fibber size and architecture electrospun fibres were coated with a thin gold layer using a sputter coater (Polaron, model SC502) and their morphology was analysed using a scanning electron microscope (SEM, Cambridge Leica) with an accelerating voltage of 15 kV. Nanofibre average diameter and the distribution were calculated over approximately 40 fibres using SEM

images (5000x magnification) and Image J software [307]. Cryo-SEM images were obtained using a cryogenic fixture within JEOL JSM5410 equipment. Fully swelled samples were transferred to a cryogenic chamber, frozen in liquid nitrogen, and frost sublimated. The frozen samples where then coated with gold *in-situ*.

#### 5.2.1.2. Hydrolytic degradation

*In vitro* degradation of crosslinked gelatine electrospun membranes was carried out in a phosphate buffer saline (Phosphate buffer saline (PBS) pH 7.4 (w/v), 0.8% NaCl (Fisher), 0.02% KCl (Fischer), 0.14% $Na_2HPO_4$ (Sigma), 0.03% $KH_2PO_4$ (Flücka)) solution. The membrane samples were cut into squares of 25 x 25 $mm^2$ (triplicate samples were used for statistical purposes), immersed in 15 mL of PBS and incubated in an air circulation oven (HERAEUS Vacuotherm) at 37 °C for 15 days. The pH of the PBS solution was measured periodically and PBS was renewed every 72 h. After specific periods of time, a membrane was removed from the PBS, washed with ultrapure water and dried in a vacuum oven (JP SELECTA Vacuotherm) at room temperature until constant mass was reached. Samples were weighed before and after degradation in an electronic quartz microbalance (M5P) from Sartorius) with a resolution ≤0.001 mg. The extent of hydrolytic degradation ($W_L$) was calculated by Equation 5.2.2.:

$$W_L = \left(1 - \frac{m_s}{m_0}\right) \qquad \textbf{Equation 5.1.2.}$$

where, $m_s$ is the sample mass after an incubation period and $m_0$ is the initial sample mass.

#### 5.2.1.3. Thermal degradation analysis and calorimetric analysis

The behaviour of the electrospun materials was analysed through differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC measurements were performed in a Perkin–Elmer Pyris-1 apparatus at a heating rate of 10 °C $min^{-1}$. Samples for the DSC studies were cut into small pieces from the middle region of the electrospun membranes and placed in 40 mL aluminum pans. All experiments were performed under a nitrogen purge. The thermal degradation kinetics of the samples was characterized by thermogravimetric analysis in a Perkin–Elmer Pyris-1 TGA apparatus at heating rate scans from 10°C $min^{-1}$ up to 40 °C $min^{-1}$ performed under a nitrogen atmosphere.

5.2.1.4. Fourier transform infrared spectroscopy with attenuated total reflectance

To assess the possible chemical alterations during sample processing an cross linking, Fourier transform infrared (FTIR) spectra were acquired at room temperature with an Alpha FTIR (Bruker) in attenuated total reflectance (ATR) mode from 4000 to 600 $cm^{-1}$. FTIR spectra were collected after 32 scans with a resolution of 4 $cm^{-1}$.

5.2.1.5. Swelling behaviour

Swelling was measured in circular samples of ~5 mm diameter by the gravimetric method. Each sample, after submersion in phosphate buffer saline solution (PBS) (pH = 7.4, at room temperature) for different time intervals (between 0 and 60 minutes), was withdrawn and placed between two pieces of tissue paper to remove excess of buffer [327]. The degree of swelling (%) was calculated according to Equation 5.2.3.:

$$Degree\ of\ Swelling\ (\%) = \frac{(W_s - W_d)}{W_d} \times 100 \qquad \textbf{Equation 5.1.3.}$$

Where, "$W_s$" weight of the sample in its wet state after it is submersed in the PBS solution (mg) and "$W_d$" weight of the sample in is dried state (mg).The value corresponds to the average of 3 independent assays.

5.2.1.6. Water vapour permeability

Water vapour permeability (WVP) of the gelatine films and membranes was determined in triplicate according to the ASTM E96-95 method [364]. The cells (glass cup with outer inner diameter of 25 mm and an inner diameter of 23 mm) were filled with silica gel, fixed with a rubber O-ring and placed in desiccators containing saturated $MgNO_3$ solution at 50 ± 3% relative humidity (RH) which were maintained in temperature-controlled incubators (JPSelecta) at 25 °C. The RH gradient was 50:0 (RHoutside: RHinside). The cells were weighted in a Mettler Toledo AG AE200 precision balance (± 0.0001g) during 10 h. The WVP was calculated according to Equation 5.2.4.:

$$WVP = \frac{WVT \times x}{A \times P_0 \times (RH_1 - RH_2)} \qquad \textbf{Equation 5.1.4.}$$

where *WVT* is the mass change (flux, $g.h^{-1}$); *x* is the film thickness (mm); *A* is the membrane area exposed to the permeant (m2); $P_0$ the vapour pressure of pure water (1.359 kPa); *(RH1 – RH2)* is the relative humidity gradient at 25 °C. Samples were allowed to equilibrate for 1 h before the cells were initially weighed.

## 5.2.2. Eukaryotic cell assays

### 5.2.2.1. Cell culture

Mouse embryo fibroblast 3T3 cells were routinely grown in Dulbecco's Modified Eagle's medium (DMEM) (Biochrom) supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen) and 1% of a penicillin/streptomycin solution, and maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. Culture medium was changed every 2–3 days and cells were sub-cultured once they reached 70–80% confluence by treatment with a 0.25 % trypsin/EDTA solution for 5 minutes. For cell culture, circular gelatine membranes with 13 mm of diameter were sterilized by immersion in 70% ethanol for 30 minutes. Membranes were then thoroughly washed with sterile PBS to eliminate any residual ethanol.

### 5.2.2.2. Cell viability analysis

For cell viability studies, 3T3 fibroblasts at a cell density of $1.2 \times 10^4$ cells $well^{-1}$ were seeded on the gelatine membranes previously placed in 24-well plates. Cells were also seeded in wells not coated with gelatine to be used as controls. The number of total cells adhering to the gelatine membranes was quantified at 3, 24, 48, and 72 hours of incubation using a Neubauer chamber; viable cells were distinguished from nonviable ones using the trypan blue exclusion dye, as trypan blue will only label cells with the damaged membrane.

### 5.2.2.3. Cell Imaging

SEM was used in order to evaluate the morphology of the fibroblast cells incubated on the gelatine membranes. Samples were prepared according to the protocol described by Andrade et al. [332]. Briefly, after 72 hours of incubation, the culture medium was discarded of the 24-well plates, the membranes were rinsed twice with PBS, and cells were fixated using with 0.7 mL of 2.5% (v/v) of glutaraldehyde in PBS for 1 hour. Then, membranes were rinsed with distilled water and dehydrated using ethanol solutions with sequentially higher concentrations (55, 70, 80, 90, 95, and 100% v/v ethanol in distilled water), for 30 minutes each, and then allowed to evaporate overnight at room temperature.

## 5.2.3. Antibacterial activity

### 5.2.3.1. Minimal inhibitory concentration (MIC) of bovine lactoferrin

The antimicrobial performance of bLF against clinical samples of *E. coli* and *Staphylococcus aureus* (kindly provided by the Faculty of Pharmacy, University of Porto, Portugal) was determined using broth microdilution tests. Bacterial pre-cultures were prepared in 20 mL of Nutrient Broth (NB, Himedia) with a volume ratio of 1:5 and incubated overnight at 37 °C and 120 rpm. Bacterial cell suspensions with a concentration of $1.5x10^6$ CFU.$mL^{-1}$ (determined from growth curve) and diluted in PBS were inoculated in 200 µL of NB with 0.0, 0.1, 0.3, 0.6, 1.2, 2.5, 5.0, 10.0, 20.0, 40.0 mg.$mL^{-1}$ of bLF content. The optical density ($\lambda$ = 600 nm) was measured after 24 hours of incubation at 37 °C and 120 rpm. The MIC assay was performed with at least 6 replicas of each bacteria and concentration.

### 5.2.3.1. Evaluation of contact bactericidal effectiveness (contact killing)

An adaptation of the American Association of Textile Chemists and Colourists (AATCC) Test Method 100-2012 [333] was employed to access the contact bactericidal efficiency of the electrospun fibre mats. Briefly, a pre-culture of each bacteria was prepared in 20 mL of NB with a volume ratio of 1:5 and incubated overnight at 37 °C and 120 rpm. Electrospun samples with 9 mm diameter were inoculated with 10 µl of cell suspension (diluted in PBS) with a concentration of $9.6x10^5$ CFU $mL^{-1}$ for *E. coli* and $1.1x10^6$ CFU $mL^{-1}$ for *S. aureus*, followed by incubation at 37 °C for 2 hours. After incubation, 500 µL of PBS were added and carefully orbital stirred for at least 1 minute. Finally, the samples were submitted to successive serial dilutions and plated in Mueller Hinton (MH, Himedia) agar plates, and incubated for 24 h at 37 °C. The contact killing assays were performed using at least 5 replicas for each bacteria.

## 5.2.4. Statistical analysis

Statistical differences were evaluated using the one-way ANOVA and post-hoc Tuckey´s multiple comparison as post-test ($p < 0.05$) The statistical results were obtained using GraphPad Prism software (version 5.0) with an $\alpha$ value of significance equal to 0.05.

## 5.3. Results and discussion

Electrospinning is a versatile process for fibre production. However, despite the apparent simplicity of the process, the morphology/structure of the spun fibres is highly affected by several experimental parameters such as the applied voltage, the collector procedure nature, as well as by a wide range of solution properties namely concentration, conductivity, viscosity, surface tension, and solvent volatility [365].

### 5.3.1. Fish gelatine electrospun fibre mats

#### 5.3.1.1. Fish gelatine electrospun mats fibre characterization

Random (Figure 5.3.1. a) and b)) and oriented (Figure 5.3.1. c) and d)) gelatine electrospun fibres were obtained from dissolution in DMF/FA solvent mixture. Uncrosslinked fibres (Figure 5.3.1. a) and c)) showed a smooth fibre surface without bead defects and with a mean diameter of 240 ± 58 nm (Figure 5.3.1. a)) and 223 ± 47 nm (Figure 5.3.1. b)) for the random and aligned fibres, respectively. The difference in the average diameter between aligned and random fibres may due to the extra mechanical stretch given by the rotating drum [365].

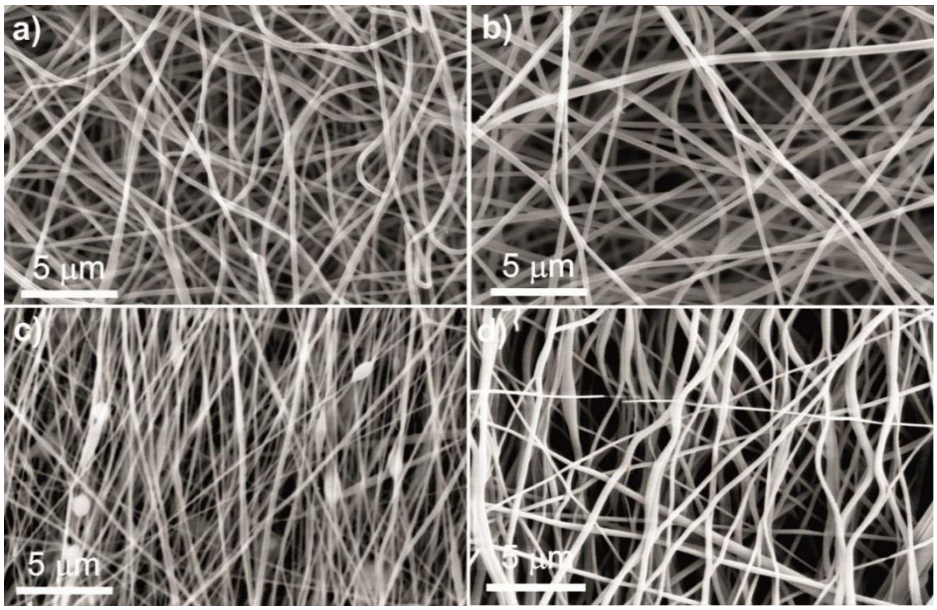


**Figure 5.3.1.** Scanning electron microscopy (SEM) images showing the morphology of as-spun fish gelatin membranes: **a)** and **c)** as-spun randomly aligned fibres, before and after glutaraldehyde crosslinking, respectively and **b)** and **d)** as-spun aligned fibres at 750 rpm, before and after GA crosslinking, respectively. Samples were collected with an applied electric field of 1.25 $kV.cm^{-1}$, a feed rate of 0.2 $mL.h^{-1}$ and a needle inner diameter of 0.5 mm.

Fish gelatine fibre mats were then exposed to glutaraldehyde vapour phase and the effect of the crosslink agent in the fibre morphology was characterized by SEM (Figure 5.3.1. b) and 5.3.1. d)).

It was found that as-spun gelatine does not suffer significant changes in the fibre diameter (Figure 5.3.2.) after exposure to glutaraldehyde, independently of the fibre orientation and exposure time. After crosslinking the fibre mats changed their colour from white to pale yellow and slightly shrank slightly when compared to their original dimensions. These colour changes (along with the shrinkage effect) have been attributed to the formation of aldimine linkages (-CH=N-) between the free amino acids of lysine or hydroxylysine amino acids residues of the protein, and the aldehyde groups of glutaraldehyde [366, 367].

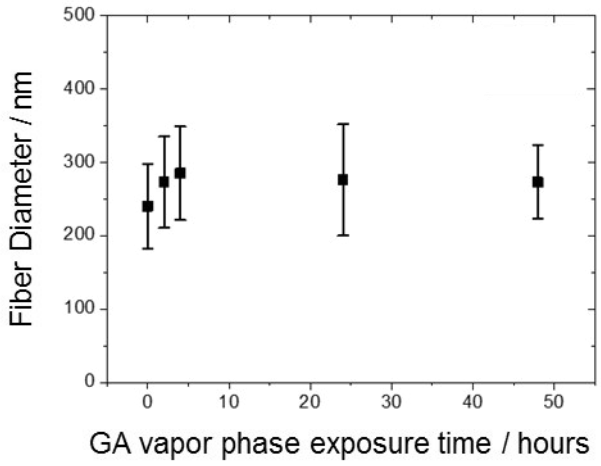


**Figure 5.3.2.** Evolution of the mean fibre diameter with glutaraldehyde vapour phase exposure time.

### 5.3.1.2. Fish gelatine electrospun mats swelling degree

Crosslinked gelatine fibre mats exposed for 24 hours to glutaraldehyde vapour were immersed in PBS at room temperature, and the swelling behaviour was measured over time (Figure 5.3.3. a)). For comparison purposes, same procedure was performed to crosslinked gelatine films obtained by solvent casting (Figure 5.3.3. a)). It was found that the electrospun gelatine sample can absorb as much as 1400% water after 5 minutes immersed in PBS, while gelatine films can absorb around 260% after 10 minutes of swelling (Figure 5.3.3. a)), and remaining almost constant for longer swelling time (Figure 5.3.3. a)). As shown in Figure 5.3.3. b), the microstructure of the electrospun gelatine sample after swelling changed from the typical fibrous structure of electrospun samples to an almost spherical porous microstructure [368], which is attributed to the confluence of fibres. Gelatine crosslinking mediated by GA is explained through the reaction of the aldehyde functional groups with free non-protonated ε-amino groups ($-NH_2$) of lysine or hydroxyl-lysine through a nucleophilic addition-type reaction [369], resulting in a more organized amorphous structures with more free space between the polymer networks. At pH around 7, the ionization of amine groups in

crosslinked gelatine fibre mats increases, as well as the concentration of ionic groups in the polymer network, thus resulting in an appreciable increase in the water absorbency [370].

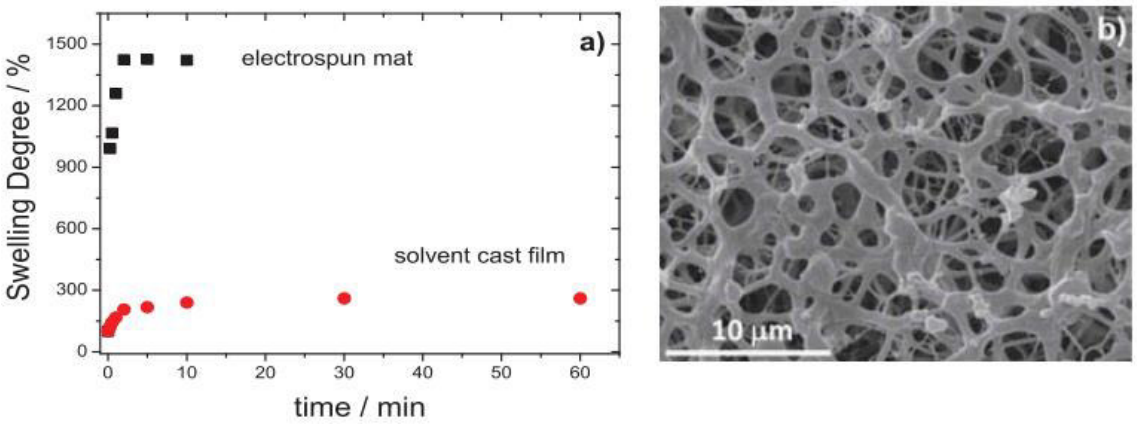


**Figure 5.3.3. a)** Swelling degree as a function of time of as-spun fish gelatine fibre mats and solvent-cast films after immersion in phosphate buffer saline (PBS) at room temperature; **b)** Fibre microstructure of the electrospun mats after 1 hour immersed in PBS.

### 5.3.1.3. Fish gelatine electrospun mats fibres water vapour permeability

Hydrophilic materials such as gelatine polymer, exhibit positive slope relationships between the amounts of water vapour that cross the material as a function of time, resulting from variations in the water vapour partial pressure at the underside of films during testing (Figure 5.3.4.). Water vapour permeability (WVP) was performed for gelatine electrospun mats at room temperature (Figure 5.3.4.). Applying Equation 5.2.4. it was found a WVP value of 1.37 ± 0.02 g mm $m^{-2} h^{-1} kPa^{-1}$ and 0.13 ± 0.10 g mm $m^{-2} h^{-1} kPa^{-1}$ for the as-spun crosslinked membrane, and for the gelatine films, respectively. As expected, the differences obtained between electrospun membranes and polymeric films are due to the higher porosity of the first ones. The electrospun fibre mats present a WVP much higher than the gelatine films, being the WVP value herein obtained similar to the ones reported in the literature [33].

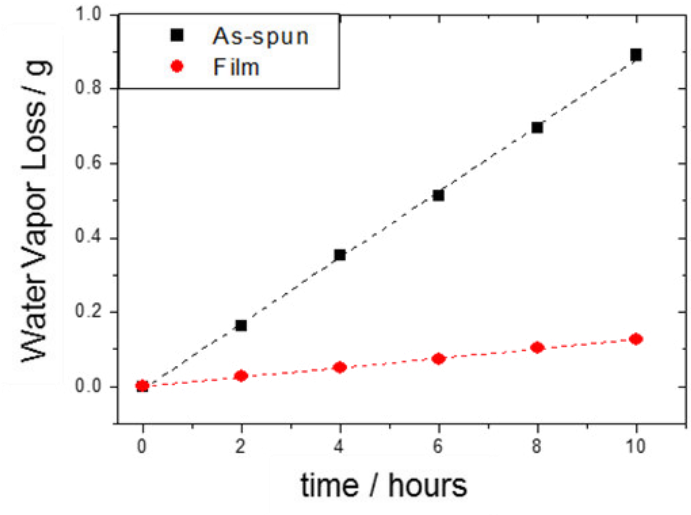


**Figure 5.3.4.** Water vapour loss for the gelatine films and electrospun crosslinked fibre mats.

### 5.3.1.4. Random fibre fish gelatine electrospun mats hydrolytic degradation of electrospun

Rosellini *et al.* [371] showed that the *in vitro* degradation behaviour at 37 °C of gelatine crosslinked with GA films in PBS is quite similar to that obtained in PBS collagenase solution. Electrospun crosslinked samples were immersed in PBS solution and the extent of hydrolytic degradation was determined (Figure 5.3.5.). It was found that crosslinked electrospun gelatine fibres took less than 5 minutes to reach the equilibrium swelling state (data not shown) and that can uptake as much as 14 times of its initial mass[372]. Gelatine weight loss occurred rapidly during the first week of incubation, losing around 50% of the sample initial mass (Figure 5.3.5. a)) after approximately 9 days. After 15 days, a residual mass of 32% remained. Zhou *et al.* [373] suggested that gelatine mass loss during the *in vitro* and *in vivo* degradation occurs primarily due to solvation and depolymerisation of polymeric chains. In the current work it was also observed that electrospun crosslinked membranes still showed integrity after a week under *in vitro* degradation conditions (Figure 5.3.5. b) and c)).

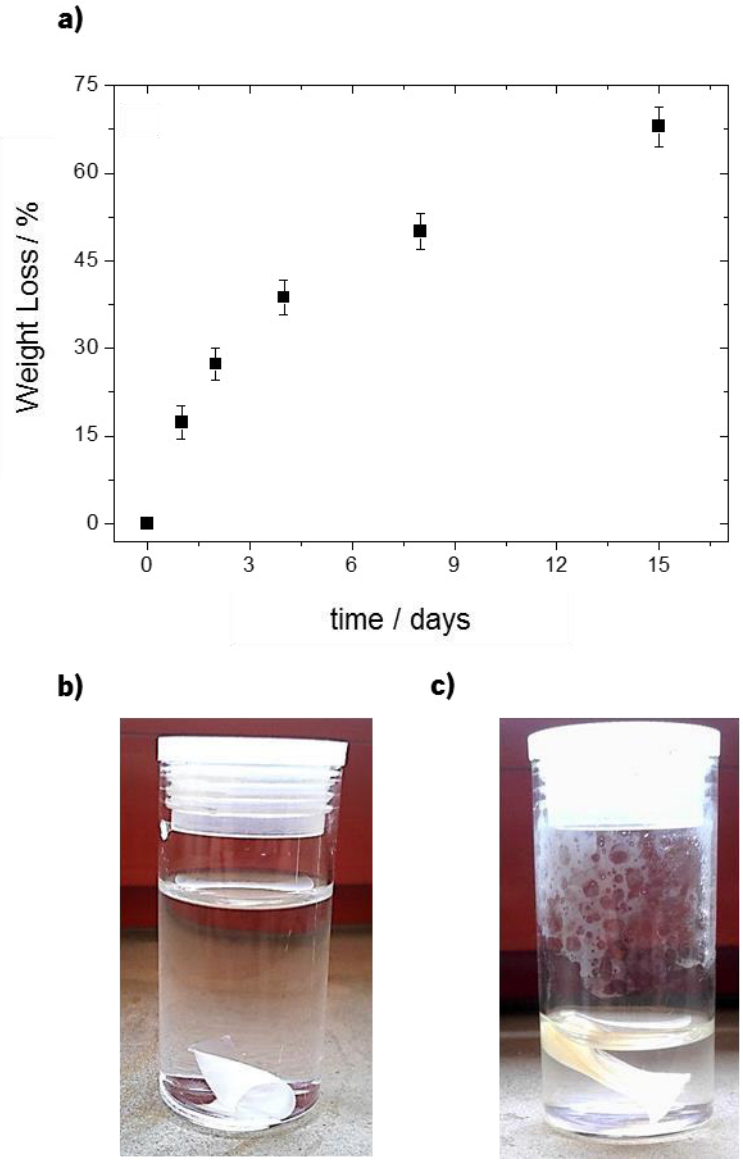


**Figure 5.3.5. a)** Hydrolytic degradation kinetics for the electrospun gelatin, **b)** gelatine membranes immersed in phosphate buffer saline (PBS) for 1 hour at 37 °C and **c)** gelatine membranes immersed in PBS for 7 days at 37 °C.

### 5.3.1.5. Fish gelatine electrospun fibre mats thermal degradation and calorimetry

Thermal degradation of gelatine occurs in three different stages (Figure 5.3.6.). The first stage occurs at temperatures from room temperature up to 200 °C with a mass loss of around 12%. The first stage of thermal degradation is related to the loss of adsorbed water due to the handling of the sample at room conditions without any special care and, consequently, these data were not considered for the determination of the kinetic parameters [374, 375]. Water bound to protein may be divided into three types according to its state: a) water bound by high-energy sorption centres that occurs inside collagen triple helix and which plays a major role in its stabilization by intramolecular bonds, the amount of water depending on the degree of helicity of the macromolecules; b) water absorbed by polar groups of gelatine and collagen macromolecules,

bound to proteins by hydrogen bonds, located outside the helical fragments and also contributing to the stabilization of the collagen helical structure. The amount of this water in gelatine probably corresponds to the so-called mono-molecular layer and can be considered as structural water; c) water absorbed by proteins to give polymolecular layers, consisting of the total amount of water bound in gelatine and the amount of structural water [376].

The second degradation stage occurs between 200 and 400 °C and is associated with protein degradation [374], Finally, the third stage occurs between 400 and 600 °C and corresponds to the thermal decomposition of the gelatine networks (Figure 5.3.6.) [377]. In general, the thermal stability of the gelatine electrospun membranes is similar to that of the original powder (Figure 5.3.6. b)).

The kinetics of the mass loss was studied through the analysis of experiments performed at different heating rates. As expected, increasing heating rates shift the onset temperature of the degradation processes to higher temperatures, not affecting any of the main characteristics of the process itself. A model for the decomposition kinetics has the following general form:

$$\frac{\partial \alpha(t)}{\partial t} = k(T) f[\alpha(t)] \qquad \textbf{Equation 5.3.1.}$$

where, $\alpha$ represents the degree of conversion of the sample under degradation, defined by Equation 5.3.72.:

$$\alpha = {(w_0 - w_t)}\Big/{(w_0 - w_\infty)} \qquad \textbf{Equation 5.3.2.}$$

where $w_0$, $w_t$ and $w_\infty$ are the sample weights before degradation, at a given time $t$ and after complete degradation, respectively. The rate constant $k(t)$ changes with the absolute temperature according to the Arrhenius equation (equation 18). $f(\alpha)$ represents the net result of elementary steps, as the polymer degradation is often a chain reaction. For the reaction model $f(\alpha) = (1-\alpha)^n$, where $n$ is the reaction order assumed to remain constant during the degradation process.

The Ozawa-Flynn-Wall (OFW) [378, 379] method assumes that the conversion function $f(\alpha)$ does not change with the heating rate for all values of the degree of conversion $\alpha$. It involves measuring the temperatures corresponding to fixed values of $\alpha$ from the experiments performed at different heating rates, $\beta$ . In this theory (Equation 5.3.3.):

$$\ln(\beta) = \frac{\ln(AE_{act})}{R} - \ln[f(\alpha)] - \frac{E_{act}}{RT}$$ **Equation 5.3.3.**

where $A$ is a pre-exponential factor ($min^{-1}$), R is the gas constant (8.31 $J.mol^{-1}.K^{-1}$), and $E_{act}$ is the activation energy of the degradation process. By plotting $\ln(\beta)$ *vs* $^{1}/_{T}$, the activation energy can be obtained, regardless of the reaction order of the system (Figure34 c) and d)). The validity of this model is based in the assumption that the conversion is constant for different heating rates [378, 379].

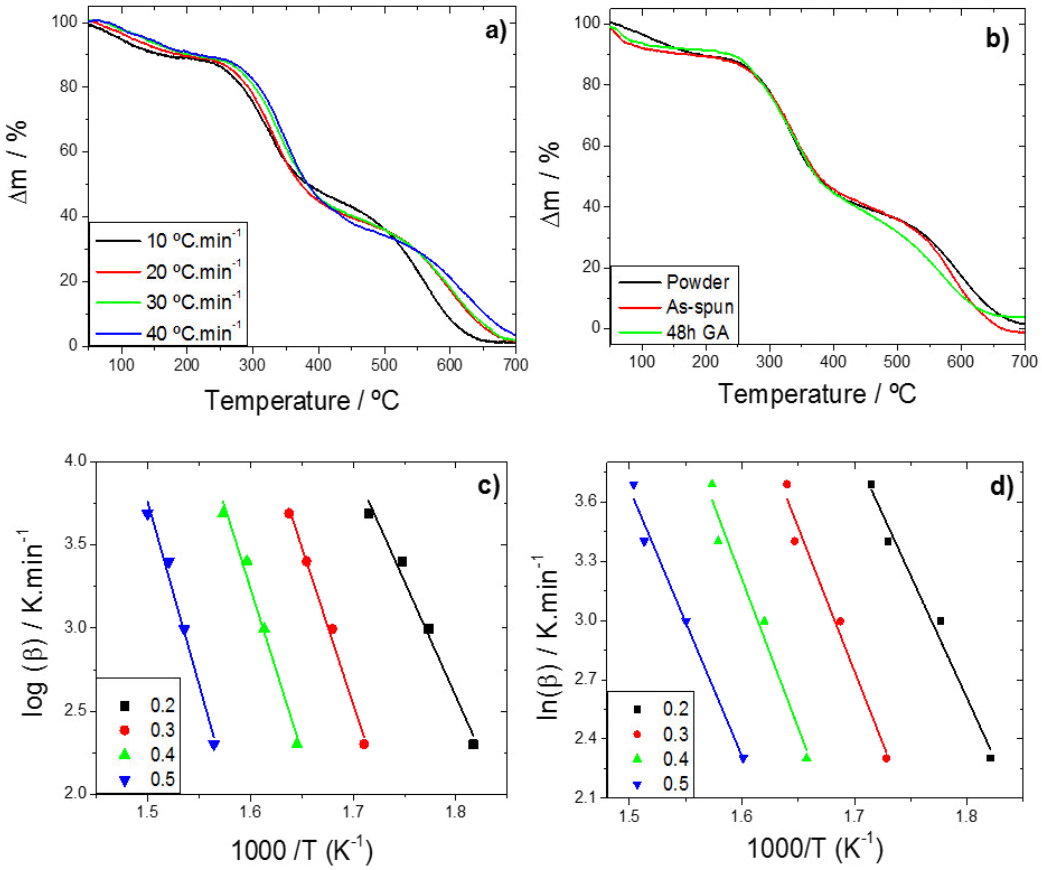


**Figure 5.3.6**. Thermogravimetric analysis (TGA) thermograms for **a)** gelatin powder, obtained at different heating rates; **b)** electrospun membranes before and after different crosslinking periods, collected at a rate of 20 °C $min^{-1}$; **c)** Ozawa-Flynn-Wall (OFW) fitting for fish gelatin powder and **d)** OFW fitting obtained for the gelatin fibre membranes crosslinked with glutaraldehyde during 48 hours for different conversions.

The activation energy was calculated for the raw material and for the as-spun membranes before and after crosslinking with glutaraldehyde (Table 5.3.1.). The values show that dissolution and processing conditions does not affect the thermal degradation of the material. It was previously reported that the [374] thermal activation energy for gelatine is between 175 and 275 $kJ.mol^{-1}$, the difference with the present investigation being attributed to the gelatine source, *i.e.* animal or fish

gelatine, and molecular weight. After crosslinking, a decrease of $E_{act}$ was observed in both transition stages of the electrospun mats (Table 5.3.1.), which was probably due to random scission of polymer chains [374]. Crosslinking is associated with more rigid molecules with less ordered structures. In this work protein-protein interaction is, therefore, apparently affected by chemical crosslinking, and is involved in the decrease of thermal stability of gelatine electrospun mats. During heating, the initially ordered structure of the samples is gradually destroyed after breaking of inter and intra-molecular hydrogen bonds, which are responsible for the maintenance of the polymeric chain [374, 380].

**Table 5.3.1.** Evaluation of $E_{act}$ for the fish gelatine powder and electrospun materials before and after glutaraldehyde crosslinking.

| Sample | $E_{act}$ (kJ.mol$^{-1}$) | |
|---|---|---|
| | First-Step | Second-Step |
| Powder | 175 ± 27 | 177 ± 28 |
| As-spun | 175 ± 35 | 189 ± 35 |
| 48h-GA | 110 ± 13 | 110 ± 15 |

Thermal properties of gelatine raw materials and electrospun mats were also analysed by differential scanning calorimetry (DSC). DSC curves obtained for the different gelatine membranes showed similar trends, indicating that electrospinning and crosslinking with glutaraldehyde processes do not influence gelatine thermal properties. A broad endothermic peak was observed in the range of temperatures between 30 and 180 °C, being associated with the evaporation of bound water that was previously absorbed by the polymer membrane when in contact with air. TGA experiments showed that almost 12% of water can be absorbed by gelatine (Figure 5.3.7.) when in contact with air. Further, at 190 °C an endothermic shoulder was observed, related to the helix-coil transition, i.e., the electrospinning process does not alter the formation of the characteristic helical structure between the gelatine chains [381].

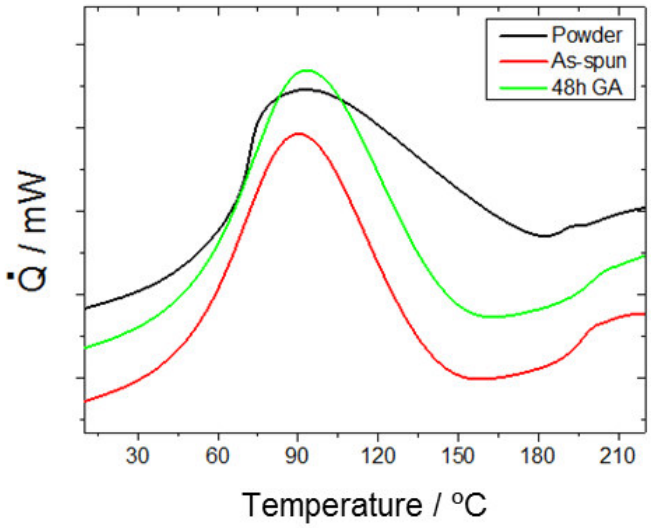


**Figure 5.3.7**. Differential scanning calorimetry (DSC) curves of gelatin raw material, as-spun and crosslinked with glutaraldehyde for 48 hours.

### 5.3.2. Eukaryotic cell assessment in random and aligned fibre fish gelatine electrospun fibre mats

Cell culture experiments were carried out on electrospun random and aligned fibre mats crosslinked with glutaraldehyde. The typical overall morphology of the mouse embryo fibroblast 3T3 cells seeded in both gelatine random and aligned fibres is illustrated in Figures 5.3.8. a) and b), respectively. Cell viability was quantified by the trypan blue dye exclusion test.

The initial cell adhesion (3 hours) was found to be low with a slight percentage of non-viable cells, especially for the aligned fibre mats (Figure 5.3.8. c)). After 3 days it was possible to observe that the fibrous structure of the scaffold remained intact (Figure 5.3.8. b)), and the cells growing on top the scaffolds reached confluence. These results suggest that both scaffolds are suitable for cell growth.

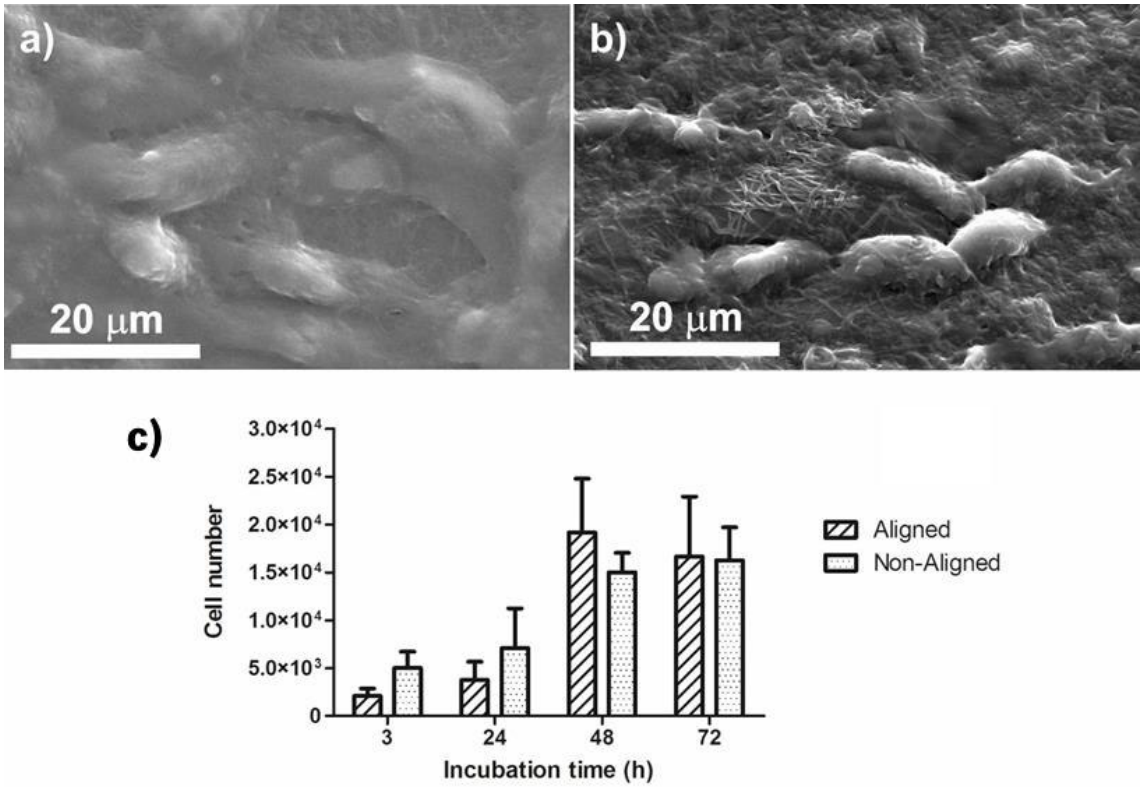


**Figure 5.3.8.** Scanning electron microscopy (SEM) images of mouse embryo fibroblast 3T3 cells in glutaraldehyde crosslinked gelatine mats after 72 hours culture: **a)** random fibres; **b)** oriented fibres; **c)** viable cell count. The results are expressed in mean ± standard error, obtained from four independent assays (n = 4).

## 5.3.3. Active fish gelatine and bovine lactoferrin electrospun composite fibre mats

### 5.3.3.1. Electrospun fish gelatine and bovine lactoferrin nanocomposite fibre characterization

The morphology and size distribution of crosslinked electrospun fibres showed to be greatly affected by the bLF content. Indeed, while the fish gelatine fibres showed to be non-defective with a smooth surface, increasing the concentration of bLF in the spinning solution led to the production of defective fibres (Figure 5.3.9.). With a concentration of 2 wt%, the electrospun fibres showed a morphology very similar to neat gelatine however, at increased concentrations of the filler (5 wt% and 10 wt%) beaded fibres were formed (Figure 5.3.9. c)), the presence of such defects being more pronounced at the highest bLF content (Figure 5.3.9. d)). Under the experimental conditions, the randomly oriented fish gelatine membranes presented an average fibre diameter of 196 ± 54 nm, whereas a reduction was observed on average fibre diameter for the sample with 10 wt% bLF (91 ± 30 nm) (Figure 5.3.10.). The slight decrease on the average fibre diameter when bLF is present

may suggest that the critical electrical field needed for stable Taylor cone formation and the subsequent polymer jet, is influenced by the presence of bLF in the solution.

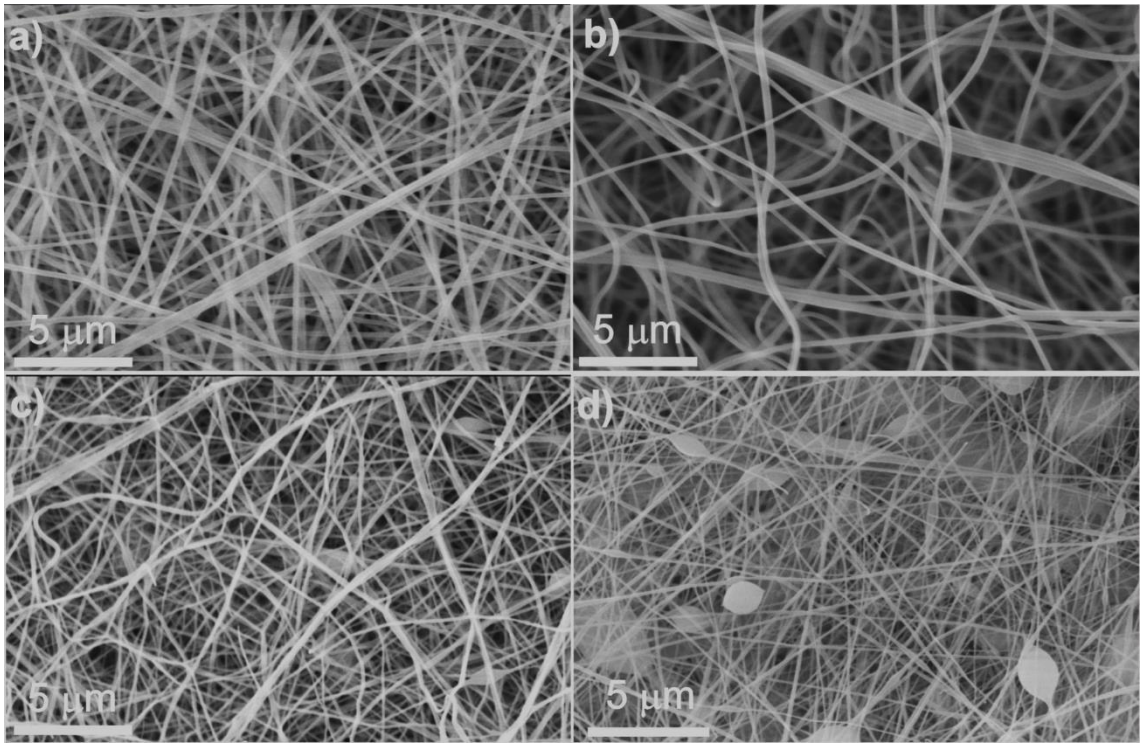


**Figure 5.3.9.** Scanning electron microscopy (SEM) images showing the morphology of fish gelatine electrospun composite membranes with: **a)** 0 wt%, **b)** 2 wt%, **c)** 5 wt% and **d)** 10 wt% bovine lactoferrin (bLF), after crosslinking with glutaraldehyde in a vapour chamber during 48 hours. Samples were collected in a static grounded collector with an applied electric field of 1.25 kV $cm^{-1}$, a feed rate of 0.2 mL $h^{-1}$ and a needle inner diameter of 0.5 mm.

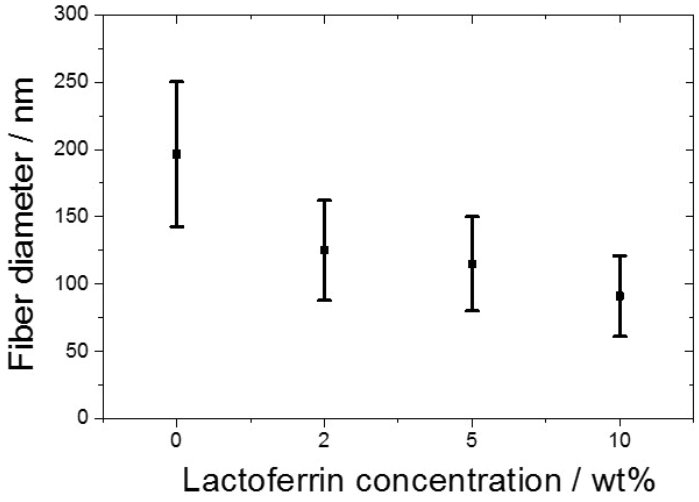


**Figure 5.3.10.** Influence of bovine lactoferrin (bLF) on the average fibre diameter after crosslinking with glutaraldehyde in a vapour chamber during 48 hours. The results are expressed in mean ± standard error.

5.3.3.1. Electrospun fish gelatine and bovine lactoferrin nanocomposite crosslinking degree

Gelatine is a water soluble material and consequently, as-spun fibre membranes partially or totally dissolve when in contact with an aqueous medium or high moisture environments [382]. In order to increase the sample stability to aqueous environments, gelatine fibre mats were exposed to a saturated atmosphere of glutaraldehyde thus promoting chemical crosslinking of the material. It was found that this chemical treatment did not influence the fibre average diameter for an exposure time up to 48 hours [372] and the crosslinked fibres showed the same visual appearance as the as-spun ones. The crosslinking degree of gelatine electrospun membranes, with and without bLF, submitted to 48 hours glutaraldehyde vapour was calculated by ninhydrin assay, showing a crosslinking degree ranging from 78 to 84% (Figure 5.3.11.). These results demonstrate that glutaraldehyde is a favourable crosslinking reagent for gelatine electrospun membranes, as it can efficiently crosslink protein amino groups. Chemical crosslinking of gelatine with glutaraldehyde involves the reaction of ε-amino groups of lysine or arginine of the polypeptide chain with aldehyde groups of the crosslinking agent by a Schiff base reaction[383]. Moreover, crosslinking is typically associated to a colour change from white to pale yellow [382]. These colour changes have been attributed to the formation of aldimine linkages (-CH=N-) between the free amino acids of lysine or hydroxylysine amino acids residues of the protein, and the aldehyde groups of GA [366, 367].

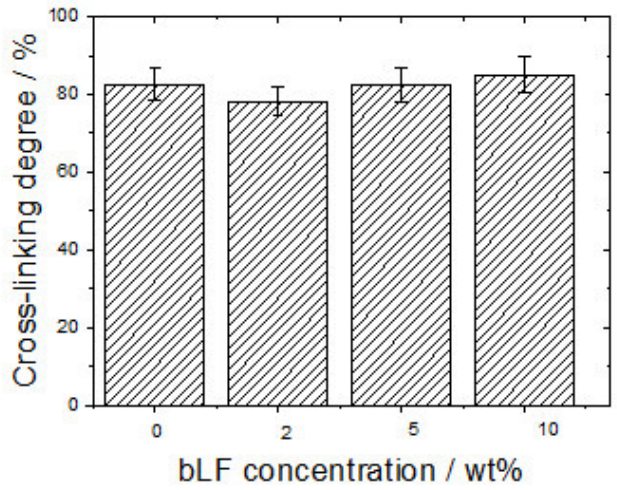


**Figure 5.3.11.** Crosslinking degree determined by ninhydrin assay for gelatine and bLF-gelatine electrospun membranes after exposure to glutaraldehyde in a vapour chamber for 48 hours. The results are expressed in mean ± standard error, obtained from five independent assays (n = 5).

#### 5.3.3.1. Electrospun fish gelatine and bovine lactoferrin nanocomposite fibre FTIR-ATR analysis

The infrared spectrum of lyophilized and electrospun pristine gelatine, as well as nanocomposite fibre mats and commercial pure lactoferrin, are shown in Figure 5.3.12.. All samples show the characteristic amide absorption bands of the peptide group namely, amide I at 1636 $cm^{-1}$ (C=O stretching vibration), amide II at 1514 $cm^{-1}$ (N-H bending with contribution of C-N stretching vibrations) and amide III at 1234 $cm^{-1}$ (assigned to N-H bending vibration) [384]. The broad absorption band observed between 3500 – 3100 $cm^{-1}$ was attributed to contributions from amide A (N-H stretching vibration) [384, 385] and free water present in the material due to storage at room conditions that can correspond to a 12% of total sample weight [367]. The absorption bands at 2850 and 2920 $cm^{-1}$ are attributed to C-H stretching vibration, corresponding to the symmetric and asymmetric stretching of alkyls chains, respectively, with a minor contribution of symmetric and asymmetric stretching vibration in the $CH_3$ groups at 2873 $cm^{-1}$ and 2955 $cm^{-1}$, respectively [386].

The absence of any shift in peak positions after addition of lactoferrin to gelatine fibres suggests that both the filler and the matrix did not suffer significant structural changes during polypeptide dissolution and subsequent processing by electrospinning (Figure 5.3.12.). Similarly, no differences were found between the spectra of pristine electrospun gelatine membranes and electrospun fibre mats with adsorbed bovine lactoferrin. As both gelatine and lactoferrin are proteinaceous materials, the infrared spectra is dominated by the peaks characteristic of the peptide bound.

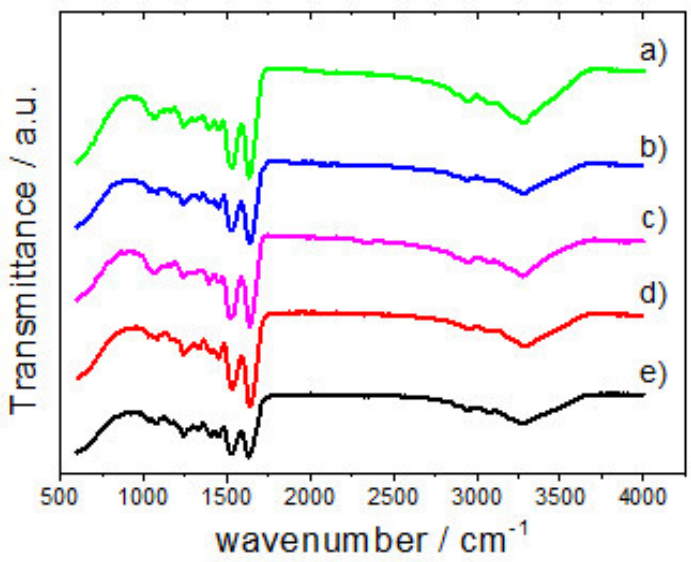


**Figure 5.3.12.** Experimentally determined Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) spectra for **a)** gelatine fibre mats with adsorbed bLF, **b)** electrospun fish gelatine-bovine lactoferrin 10 wt% composite, **c)** pure bovine lactoferrin, **d)** electrospun fish gelatine, and **e)** raw fish gelatine.

## 5.3.4. Antibacterial activity

### 5.3.4.1. Minimal inhibitory concentration (MIC) of bLF:

The antibacterial performance of bLF evaluated against the *E. coli* and *S. aureus*. The Gram-negative *E. coli* is a facultative anaerobe, widely known to be one of the major gastrointestinal microflora entities, but also a resourceful pathogenic [387]. The Gram-positive rod shape *S. aureus* is a commensal organism present in the human skin and mucosal, and is prolific in virulence factors and antimicrobial resistance [388].

The major constituents of bacteria cytoplasmic envelope are the anionic phosphatidylglycerol, and zwitterion phosphatidylethanolanine, thus the overall bacteria cytosol membrane charge is negative [8, 211, 212]. bLF high positive net charge, mainly due its N-terminal domain and in inter-lobe linker and promotes interaction with the negatively charged components of the bacteria surface, such as teichoic acids and phospholipids (namely phosphatidylglycerol) [212, 213, 215]. Once in close proximity with the bacterial surface, several antimicrobial events may occur. bLF antimicrobial motifs, especially in its N-terminal, may exert the destabilization of the outer or inner membrane by their interaction with bLF hydrophobic residues [180]. In the case of the Gram-negative bacteria, the LPS envelope may be additionally be disrupted by the action of the bLF LPS binding loop [218], and/or by the $Ca^{2+}$ chelation by the sialic acid groups present in the bLF glycans, since $Ca^{2+}$ is essential for the stabilization of the inner core of LPS [219].

Moreover, bLF may exert an bacteriostatic activity due to its potent iron binding capacity, that may deprive the environment of this essential micronutrient, blocking several essential pathogen's biochemical reactions [207-210].

The MIC found in the literature for native bLF covers a wide range of values due to different experimental conditions such as, inoculum strength, pH, culture media composition, and strains used. Therefore, a key point of this research was to validate the bactericidal activity of the used bLF by calculating the MIC. The bLF MIC was found to be 10 mg $mL^{-1}$ for *E. coli* with approximately 100% of reduction, even though 5 mg $mL^{-1}$ resulted in 90% of reduction in the growth (Figure 5.3.13 a)). At lower concentrations, the effect of bLF in cell growth was minimal with a reduction of only 37% for 2.5 mg $mL^{-1}$, and negligible for concentrations below this value (Figure 5.3.13 a)). These results are in accordance the work of Nonnecke and co-workers [275]. As for *S. aureus*, only the concentration of 40 mg $mL^{-1}$ was found to be effective in inhibiting cell growth with a reduction of approximately 82% (Figure 5.3.13. b)). These bLF bactericidal profile for *E. coli* suggests an inverse correlation between concentration and growth inhibition, since higher concentrations lead to lower visible bacterial growth. Interestingly, this profile was completely different for *S. aureus* as it was not possible to establish a direct correlation between bLF concentration and inhibition of cell growth. For concentrations below 0.6 mg $mL^{-1}$ there was a negligible effect on bacterial cell growth, whereas a minimal inhibitory effect around 30% was found for concentrations in the range of 1.2 – 20 mg $mL^{-1}$. This non-linear tendency may result from the *S. aureus* ability to counter act the bactericidal polycationic effect of bLF by changing the nature of its teichoic acids, as well as by decreasing the number of negatively charged membrane phospholipids by replacing them with zwitterion phospholipids [181, 212].

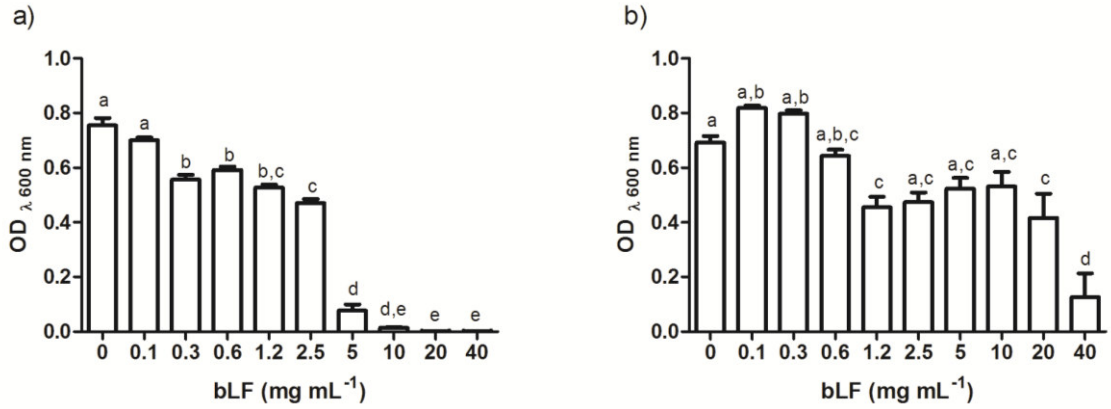


**Figure 5.3.13.** The visible growth (λ = 600 nm) of: **a)** *Escherichia coli* and **b)** *Staphylococcus aureus*, measured in culture medium NB with different concentrations of bovine lactoferrin (0, 0.1, 0.3, 0.6, 1.2, 2.5, 5, 10, 20 and 40 mg $mL^{-1}$), after 24h at 37 °C in a 96 well plate with 120 rpm of orbital rotation. For each bacteria, distinctive letters between different columns denote significant different values of One-way ANOVA post-hoc Tuckey´s multiple comparison test ($p < 0.05$). Results are expressed in mean ± standard error, obtained from six independent assays (n = 6).

### 5.3.4.2. Evaluation of contact bactericidal effectiveness (contact killing)

In the present work, fish gelatine with different amounts of bLF were prepared by electrospinning, followed by crosslinking with glutaraldehyde vapour, and their antibacterial efficiency was evaluated against *E. coli* and *S. aureus*, with an incubation time of 120 minutes. As shown in Figure 5.3.14., the electrospun fish gelatine mats by itself displayed a considerable reduction when compared with the inoculum. This can be explained by the swelling nature of the electrospun mats that do not provide the adequate conditions for bacterial cell growth. When compared with neat gelatine fibre mats, the membranes containing 2 wt% and 5 wt% of the filler showed only a slight decrease in the number of viable cells for both *E. coli* and *S. aureus*. However, the membranes with 10 wt% of bLF were highly effective in promoting a biocide effect for both *E. coli* and *S. aureus* showing a 6 log reduction (99.9999% reduction) in the number of CFUs.

To further understand the mechanism of bLF release and the subsequent antibacterial efficiency, the gelatine fibre mats were immersed in a solution of 40 mg $mL^{-1}$ of bLF for 24 hours. These samples (esFG/bLF) displayed an identical reduction as that observed for the neat fish gelatine electrospun mats (0 wt% of bLF). This can be explained by the low concentration of bLF adsorbed by the membranes which was estimated to be approximately $1.0x10^{-4}$ $mg.mm^{-2}$. Moreover, due to the weak binding between the filler and the surface of the matrix, the diffusion of bLF to the culture media occurs quite fast. On the other hand, the bLF present in the electrospun fibre mats are distributed randomly in the gelatine fibres, not only in the surface but also in the fibre bulk. This hampers the bLF diffusion into the culture media and increases the contact time of the antibacterial filler with bacteria improving its efficiency (Figure 5.3.14.).

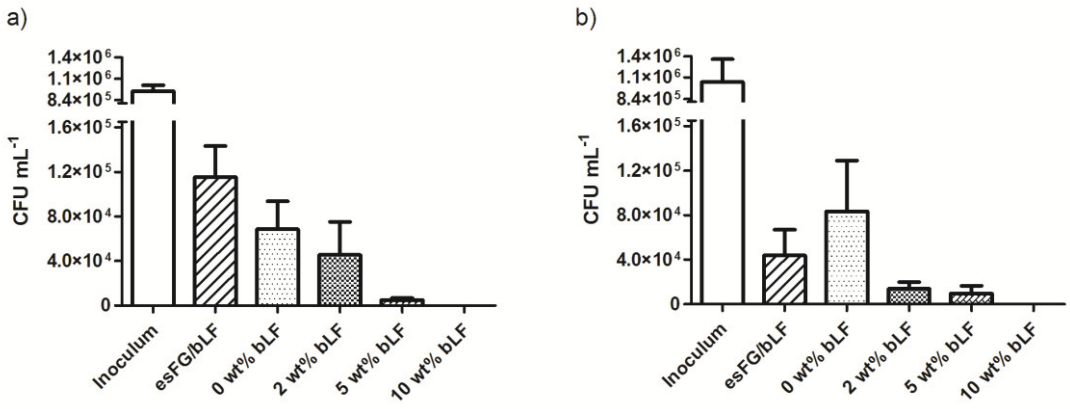


**Figure 5.3.14.** Colony forming units (CFU) per mL of **a)** *Escherichia coli* and **b)** *Staphylococcus aureus*, after 2 hours of contact at 37 °C with electrospun mats containing different concentrations of bLF (0, 2, 5 and 10 wt%) and bLF adsorbed to the surface of the fibres (esFG/bLF). Results are expressed in mean ± standard error of the mean, obtained from five independent experiments (n = 5).

## 5.4. Conclusions

The increasing demand of advanced materials with antimicrobial properties is justified by the urgent need to control the microbial population in a wide range of applications, such as food packaging, medical devices and technical textile [389]. This work exploits the potential of sub-products from fish (fish gelatine) and dairy (bovine lactoferrin) industries for the development of a novel antimicrobial fully protein-based nanocomposite. Fish gelatine was successfully processed into a structural matrix by electrospinning. The random and aligned fibres presented an average diameter between the 200 to 300 nm. The further crosslinking of gelatine membranes with glutaraldehyde conferred stability to the electrospun mats in aqueous solutions. However, the microstructure of the membranes changed from the characteristic fibrous structure to an almost spherical porous microstructure, which was attributed to the confluence of fibres during the water absorption process. These fibres were found to absorb more PBS under different exposure times than FG films.

No significant differences regarding the thermal degradation of both random fibres electrospun FG and FG powder were observed, however the electrospun process was found to lower the energy of activation. The electrospinning process did not influence the formation of the characteristic helical structure between the gelatine chains, while the cross linking lead to the formation of a more disordered structure and formation of polymer networks.

The water barrier properties of FG electrospun mats was evaluated and the WVP values found for the as-spun crosslinked membrane were around 10 times higher as compared to the FG films.

Preliminary cytotoxicity of these materials against 3T3 fibroblasts showed that both crosslinked random and aligned FG electrospun mats are suitable substrates for cell adhesion and proliferation. Indeed, neither the electrospinning nor the crosslinking were found to be harmful. Different bLF MIC were found for the bacteria herein studied, which can be explained by the different interactions that can be established between the protein and the bacterial membranes.

The electrospun nanocomposite generated using FG and bLF, and using FG electrospun samples with adsorbed bLF were compared regarding their contact killing efficiency. The FG electrospun mats with adsorbed LF showed a killing capacity equivalent to electrospun FG without bLF. The greater contact killing efficiency against *E. coli* and *S. aureus* was demonstrated by the electrospun mats bearing 10 wt% of bLF as a filler.

# Chapter 6

## General conclusions and future perspectives

# 6. General conclusions and future perspectives

Food industry and Governmental Institutions are struggling to significantly reduce the foodborne illness cases, which unfortunately are still apprehensively high [1]. The development and application of active packages that exert an antimicrobial action in the encased food product is an important strategy for the food product safety and to extend its shelf-life.

The use of natural antimicrobial agents allied to bio-based materials allows the formulation of superior antimicrobial packaging. Bio-based packaging implies petrochemical independence, edibility, and their inherent properties may be further improved by the use of nanotechnology methodologies.

The main goal of this thesis was the development of novel edible functionalized biomaterials with antimicrobial properties, to be used as active packaging for highly perishable food commodities, namely meat-based foodstuffs. The type of action intended for this packaging was bactericidal, in order to increase the food shelf-life by delaying bacterial growth, as well as to increase food safety (since bacteria are the major etiological agents of foodborne diseases).The bactericidal agent herein used was the glycoprotein bovine lactoferrin (bLF). Two distinct bio-based material sources, bacterial cellulose (BC) (polysaccharide) and fish gelatine (FG) (protein), were evaluated as support materials for the chosen antimicrobial agent. The functionalized films developed were fully bio-based and edible.

The use of *Gluconacetobacter xylinus* ATCC 53582 was chosen as the BC producing strain due to the higher BC yield in static culture conditions as compared with the ATCC 700178 strain.

Never dried BC membranes, pristine and with 25% of oxidation (by periodate) were aseptically embedded with bLF to evaluate their antimicrobial potential. The bLF uptake increased with the increasing concentration of bLF in the embedding solutions. The bactericidal activity against *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* was assessed. The oxidized BC showed higher uptake of bLF, but displayed a lower bactericidal activity in comparison to pristine BC. Based on these results, a different strategy was implemented that consisted in the bLF uptake onto the never dried BC membranes using bLF solutions at higher concentrations, followed by a vacuum drying process, which allowed obtaining significant bactericidal action towards tested microorganisms.

This same methodology was used to evaluate two different BC sources, one, as synthesised by *G. xylinus* ATCC53582 (BC1) and another, as commercially purchased "*nata de coco"* (BC2). BC2 presented a higher bLF absorption profile (approx. 45 mg $mL^{-1}$ $g^{-1}$) in comparison to BC1 (30 mg $mL^{-1}$ $g^{-1}$). Both BC's de-absorbed the same percentage of bLF (approx. 18%) The films thickness of both substrates was equivalent, approximately 25 μm, as well as their maximum tensile strength (approx. 42 MPa). The films bactericidal activity was tested in the standalone films, or in the fresh sausage case study. BC1+bLF and BC2+bLF displayed a higher contact bactericidal efficiency than BC1 and BC2, and also hampered the specific growth rate of *E. coli* and *S. aureus*. The mean reduction of standalone films BC1+bLF and BC2+bLF was of 69% and of 97% for *E. coli* and *S. aureus*, respectively. After the contamination of fresh sausage with the testing bacteria, the BC films with absorbed LF displayed a mean percentage of reduction of 94% for *E. coli* and 36% for *S. aureus*. Fibroblasts 3T3 cells, before and after digestion in an *in vitro* gastrointestinal tract model (TIM), were used to assess the toxicity of the standalone films and their subsequent digestion products. No relevant cytotoxicity was detected. Given the interesting mechanical properties, effective antibacterial activity and absence of cytotoxicity, it is possible to conclude that BC1+bLF and BC2+bLF may be considered effective antimicrobial edible active films.

The second bio-based material source used in this thesis was the protein FG. FG was successfully electrospun, originating a material with interesting properties after crosslinking with glutaraldehyde. The hydric and thermal degradation was determined using electrospun random fibres. FG random and aligned fibres were evaluated and showed no cytotoxicity when cultured with 3T3, and both architectures show similar cell division. Additionally the electrospun FG mats displayed a WVP value highly similar to BC1+bLF (1.37 and 1.13 g mm $m^{-2}$ $h^{-1}$ $kPa^{-1}$)

According to these promising results, the minimal inhibitory concentration (MIC) of bLF against *E. coli* and *S. aureus* was determined and bLF was used to functionalize the FG electrospun mats, as filler in the FG fibres and also through adsorption. The highest concentration of bLF used as filler (10%wt) led to over log 6 (99.9999 %) reduction of *E. coli and S. aureus* counts, thus displaying an outstanding antibacterial activity that should be further explored.

Future perspectives and recommendations resulting from this thesis include:

- Further improvement of the aqueous stability of the electrospun FG with bLF filler nanocomposites, in order to withstand moderate and higher temperatures;

- Characterization of the aqueous degradability of the electrospun FG with bLF filler nanocomposites;
- The assessment of the antioxidant potential of the BC and FG films containing bLF as food casings, through oxygen radical quantification using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), which is commonly used for accessing the antioxidant capacity of herbal products;
- The resulting solutions obtained after TIM digestion should be further analysed. The reported percentage of intact bLF after human gastrointestinal is 60% [171]. However, it is unknown the fraction that is actually converted into intact active peptides, namely lactoferricin B, lactoferrampin and peptide LF1-11. The putative beneficial effect of these peptides is still poorly understood. Thus, to analyse if the bLF structure is intact, immunoassays should be performed. To evaluate the products of its proteolysis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis could be used;
- Cation-exchange chromatography or magnetic affinity separation should be used to determine the bLF release profile from the nanocomposite electrospun materials;
- The cytotoxicity of the nanocomposite electrospun mats comprising FG and bLF (10%wt) should be assessed, using both 3T3 and epithelium CaCO-2 cells before and after TIM digestion.
- The resulting solutions form the TIM digestion of each of the mentioned films, should be screened for potential beneficial effects, which may be evaluated through *Bifidobacterium* cultures;
- Additional toxicity tests should be performed on the materials that displayed a higher potential to be used as active edible material, i.e. BC1+bLF, BC2+bLF, and the electrospun nanocomposite composed of FG and bLF (10%wt). The suggested future toxicity tests should be aligned with the "Guidelines of Scientific Committee on food for the presentation of an application for safety assessment of the substance to be used in food contact materials prior to its authorization" in order to prepare an active food packaging data dossier.

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