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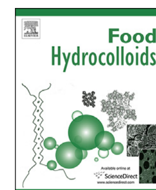

Bacterial cellulose-lactoferrin as an antimicrobial edible packaging

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A B S T R A C T

Bacterial cellulose (BC) films from two distinct sources (obtained by static culture with *Gluconacetobacter xylinus* ATCC 53582 (BC1) and from a commercial source (BC2)) were modified by bovine lactoferrin (bLF) adsorption. The functionalized films (BC + bLF) were assessed as edible antimicrobial packaging, for use in direct contact with highly perishable foods, specifically fresh sausage as a model of meat products. BC + bLF films and sausage casings were characterized regarding their water vapour permeability (WVP), mechanical properties, and bactericidal efficiency against two food pathogens, *Escherichia coli* and *Staphylococcus aureus*. Considering their edibility, an *in vitro* gastrointestinal tract model was used to study the changes occurring in the BC films during passage through the gastrointestinal tract. Moreover, the cytotoxicity of the BC films against 3T3 mouse embryo fibroblasts was evaluated.

BC1 and BC2 showed equivalent density, WVP and maximum tensile strength. The percentage of bactericidal efficiency of BC1 and BC2 with adsorbed bLF (BC1 + bLF and BC2 + bLF, respectively) in the standalone films and in inoculated fresh sausages, was similar against *E. coli* (mean reduction 69% in the films *per se* versus 94% in the sausages) and *S. aureus* (mean reduction 97% in the films *per se* versus 36% in the case sausages). Moreover, the BC1 + bLF and BC2 + bLF films significantly hindered the specific growth rate of both bacteria. Finally, no relevant cytotoxicity against 3T3 fibroblasts was found for the films before and after the simulated digestion. BC films with adsorbed bLF may constitute an approach in the development of bio-based edible antimicrobial packaging systems.

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1. Introduction

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. Consumer standards towards food freshness and safety have also 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 (Coma, 2008; Pereira de Abreu, Cruz, & Paseiro Losada, 2011). Moreover, the implementation of renewable and biodegradable materials must be adopted by the food industry in order to meet environmental standards (Cutter, 2006). Realini and Marcos (Realini & Marcos, 2014) reviewed the different categories of active and intelligent packaging systems, listing also available commercial applications, the latest packaging research trends and innovations. Antimicrobial packaging, as a form of active packaging, aims at preventing foodborne illness by eradicating or inhibiting the existing or contaminating food microflora, thus increasing the product safety and promoting the extension of its shelf-life (Pereira de Abreu et al., 2011; Quintavalla & Vicini, 2002). Irkin and Esmer (Irkin & Esmer, 2015) reviewed the most important knowledge on the application of natural antimicrobial packaging with model food systems and their antimicrobial effects on food products.

Natural polymers exhibit several advantages as coatings and films such as edibility, biodegradability, biocompatibility and barrier properties (Pérez-Pérez, Regalado-González, Barbosa-Rodríguez, Rodríguez-Rodríguez, & Villaseñor-Ortega, 2006;

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Siracusa, Rocculi, Romani, & Rosa, 2008). Nanostructured bio-based materials are further endowed with higher surface area-to-volume ratio, as compared to their micro-sized counterparts (Azeredo, 2013; Duncan, 2011). Bacterial cellulose (BC) is an outstanding polymer extruded by *Gluconacetobacter xylinus* to yield a 3D nanofibrillar pure cellulosic network. BC exhibits high tensile strength, *in situ* moldability, water holding capacity, biocompatibility and biodegradability. These unique properties allowed exploring its potential mostly in the biomedical field, where temporary skin substitutes and artificial blood vessels appear as patented products. In Asian countries, as obtained by "traditional" fermentation methods, BC is produced and sold as "nata de coco", a low-calorie sweetened dessert and high-fiber food (Andrade, Pertile, Dourado, & Gama, 2010; Shi, Zhang, Phillips, & Yang, 2014). The use of BC as an active and/or intelligent food packaging is virtually unexploited. Nguyen et al. (Nguyen, Gidley, & Dykes, 2008) published a proof-of-concept study on the use of BC films containing adsorbed nisin, to control *Listeria monocytogenes* and other bacteria, on the surface of vacuum-packaged frankfurters, as models for higher value meat products. Zhu et al. (Zhu et al., 2010) showed that ε-polylysine (ε-PL) embedded onto BC, had no significant loss of antimicrobial activity after autoclaving at 121 °C for 30 min. Also, sausages packaged with a BC + ε-PL composite showed a longer shelf-life than controls (without ε-PL). Additionally, Almeida et al. (Almeida, Prestes, Woiciechowski, & Wosiacki, 2011) evaluated the potential of BC for the conservation of minimally processed fruits. Furthermore, Maneesri et al. (Maneesri, Masniyom, & Pongpiriyadacha, 2012) studied the use of BC films containing Sarapee (*Mammea siamensis*) flower extract, against *Salmonella typhimurium*. To further potentiate the antibacterial activity of BC, ε-PL was cross-linked onto the surface of BC nanofibers, using procyanidins (Gao et al., 2014). Composites from BC + ε-PL exhibited enhanced antibacterial activity against both *Escherichia coli* (Gram-negative bacteria) and *Staphylococcus aureus* (Gram-positive bacteria).

The existing literature on the use of BC as an active food packaging has been confined to demonstrating its *in vitro* antimicrobial effect towards some strains of pathogens. This work describes the surface functionalization of BC from distinct sources with bovine lactoferrin (bLF) and the assessment of its potential as an edible antimicrobial film, for use in direct contact with highly perishable foods, specifically meat products. The properties of BC (e.g. mechanical, porosity, crystallinity, fibre length and diameter) are known to vary according to the type of strain, composition of the culture media (nutrients), fermentation conditions (static versus agitated culture) and post-processing (purification) (Sani & Dahman, 2010; Tang, Jia, Jia, & Yang, 2009). Two distinct BC sources (as obtained by static culture with *G. xylinus* ATCC 53582 and from a commercial source) were thus functionalized with bLF, to evaluate their antimicrobial properties against *E. coli* and *S. aureus*, which represent two major microbial contaminants of meat food. Fresh sausage from a local butcher shop was used in the antimicrobial tests. BC + bLF films and the sausage casings, composed of pig small intestine, were characterized regarding their water vapour permeability (WVP), mechanical properties and bactericidal efficiency. BC films were herein tested, for the first time with BC, using an *in vitro* gastrointestinal tract model, to simulate the digestion of substrates. Finally, the cytotoxicity of the edible BC films against 3T3 mouse embryo fibroblasts was evaluated.

BLF is a glycoprotein with approximately 80 kDa that has been found to possess a wide range of activities, such as immunoregulatory, anti-cancer and antimicrobial. It is considered a safe ingredient. Industrial scale production ranges between 80 and 100 tonnes a year (EFSA, 2012; Wakabayashi, Yamauchi, & Takase, 2006). Moreover, bLF antimicrobial action against foodborne pathogens such as *S. aureus* (Bhimani, Vendrov, & Furmanski, 1999), *E. coli* (Håkansson et al., 2001; Håversen et al., 2000) and *Clostridium* spp (Teraguchi et al., 1995) have been reported.

2. Materials and methods

BC from two different origins were used in this study: i) "BC1", produced in the laboratory by static culture of *G. xylinus* ATCC 53582, ii) "BC2", purchased commercial grade 2 raw *nata de coco*, from HTK Food CO. Ltd. (Vietnam). bLF protein was purchased from DMV International (USA) with a 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 with adsorbed bLF were named BC1 + bLF and BC2 + bLF, for the laboratory produced and the commercially acquired BC, respectively. Fresh sausages, bought at a local butcher shop (and produced in the northwest region of Portugal), were used as a model food product to evaluate the antimicrobial activity of the films. 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". The casing, composed of pig small intestine, was used as control in the assessment of the water vapour permeability (WVP), mechanical properties, and in the bactericidal efficiency, as described below.

2.1. Culture media

G. xylinus (ATCC 53582) was maintained in Hestrin-Schramm culture medium (HS) (Hestrin & Schramm, 1954) in solid state with 2% (w/v) agar (Himedia). BC1 fermentation occurred in static culture conditions using 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% glucose (Sigma–Aldrich), 0.5% peptone (Sigma–Aldrich), 0.5% yeast extract (Himedia), 0.34% $Na_2HPO_4 \cdot 2H_2O$ (Sigma–Aldrich), 0.15% citric acid (Pronolab). 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. These microorganisms were incubated overnight, prior to any experiment to produce the inoculum. The liquid culture media used for the antibacterial assays was Nutrient Broth (NB) (Himedia), and all liquid media pre-inoculum and experiments were carried out using a 1/5 culture medium/flask volume ratio in an incubator at 37 °C, under shaking at 120 rpm (Heidoph). HS (solid and liquid), MH agar and NB were autoclaved for 20 min, at 120 °C and 1 bar (AJC 88) before use.

2.2. Bacterial cellulose processing

The same purification procedure was undertaken in both BC1 and BC2 membranes: once the BC1 production was terminated, or 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. After cooling, the membranes were rinsed with tap water and submerged into 4% (w/v) NaOH (Fisher) for 24 h to remove any unwanted residues from the BC matrix. Afterwards, BC membranes were abundantly rinsed with distilled water until the pH equalled that of the distilled water. Finally, the never-dried BC membranes were cut into 1 mm thick slices, autoclaved in distilled water and stored in aseptic conditions until further use.

2.3. Adsorption of bLF onto BC

Adsorption of bLF onto BC nanofibres was achieved under sterile conditions, by immersing never-dried BC films in phosphate buffer saline (PBS) (pH 7.4) (control samples) and in PBS with 10 mg mL^{-1} of bLF (BC + bLF samples), for 24 h, with mild stirring at room temperature (RT = 25 °C), using a ratio of 4.5 mg of BC per mL of solution. Afterwards, the films were vacuum dried at RT for 48 h and stored at in a desiccator aseptically, no longer than a week before use. PBS was autoclaved for 20 min, at 120 °C and 1 bar (AJC 88) before use. bLF solutions were prepared immediately before use in PBS and were filter-sterilized (0.22 μm). PBS composition (w/v) was 0.8% NaCl (Fisher), 0.02% KCl (Fischer), 0.14% Na_2HPO_4 (Sigma), 0.03% KH_2PO_4 (Flücka).

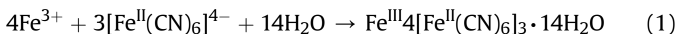
Prussian blue staining and the adsorption-desorption studies described below, were performed using never-dried BC membranes. All the other assays were done using vacuum dried films.

2.4. BC edible films physicochemical characterization

2.4.1. Prussian blue staining of BC + bLF films

BC1 and BC2 disks (0.5 mm in diameter) were separately immersed into PBS or bLF solutions for 24 h at RT under mild stirring. Afterwards, the disks were collected and the excess of solution was removed using Whatman 1 filter paper. BC disks were immediately immersed in a Prussian blue solution for 30 min at RT. 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 (Buser, Schwarzenbach, Petter, & Ludi, 1977; Goncalves et al., 2013). The disks were then rinsed with distilled water, transversely cut using plastic scissors and placed on a microscopy slide. Samples were analysed in a stereo microscope (Olympus).

$[Fe(CN)_6]^{4-}$ reaction with Fe^{3+} (Equation (1)) originates an intense blue coloration (Tafesse, 2003; Verdaguer, Galvez, Grade, & Desplanches, 2002).



$$4Fe^{3+} + 3[Fe^{II}(CN)_6]^{4-} + 14H_2O \rightarrow Fe^{III}4[Fe^{II}(CN)_6]_3 \cdot 14H_2O \quad (1)$$

2.4.2. Ultraviolet–visible spectroscopy and Fourier transform infrared spectroscopy with attenuated total reflectance

Ultraviolet–visible (UV) spectroscopy allows the identification of the characteristic absorbance profiles of proteins (Belitz & Grosch, 1999). Absorbance profiles were registered within the wavelengths ranging from 200 nm to 700 nm (Jasco).

BC and BC + bLF films were analysed by 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 to 400 cm^{-1}.

2.4.3. bLF adsorption-desorption profile onto and from BC

To assess the bLF loading capacity of BC, 25 × 170 mm films, obtained after purification, were immersed in 10 mg mL^{-1} bLF solutions under mild agitation at RT for 24 h. Afterwards, the films were vacuum-dried at RT for 48 h in a desiccator. Then, to determine the bLF release rate from the BC films, these were again immersed in a PBS solution at 25 °C with mild agitation. In both assays (adsorption and desorption), 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 = 280 nm) (Knauer). Three film replicates of each type of BC were used in this assay. Each sample retrieved was measured three times. The bLF release profile was adjusted to a modified Gompertz Equation (Equation (2)) (Zwietering, Jongenburger, Rombouts, & van 't Riet, 1990) as follows:

$$X(t) = X_{max}\, exp\left[-\,exp\left[\frac{R_r e}{X_{max}}(\psi + t) + 1\right]\right] \quad (2)$$

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

2.4.4. Swelling behaviour

A gravimetric method based on the methodology described by Sangsanoh and Supaphol was used to determine the degree of swelling in BC samples (Sangsanoh & Supaphol, 2006). Circular BC films with 5 mm diameter were used in these assays. Samples were immersed in PBS solutions at RT 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 the following Equation (3):

$$Degree\ of\ Swelling\ (\%) = \frac{(m_s - m_d)}{m_d} \times 100 \quad (3)$$

where, "m_s" represents the mass of the membrane after it is submersed in the PBS solution (mg) and "m_d" the mass of the sample in is dried state (mg).

2.4.5. Water vapour permeability

The water vapour transmission of all produced films was assessed gravimetrically, using an adaptation of the methodology described in the ASTM E96-95 (ASTM, 1980). BC films were placed on top of glass vials containing 1/3 of ultrapure water and fixed using parafilm. Samples were equilibrated overnight in a desiccator containing silica gel at RT. Afterwards, the vials were placed in a desiccator with silica gel and fans in its interior to maintain homogenous conditions, at 25 °C as described by Cerqueira et al. (Miguel A Cerqueira, Costa, Fuciños, Pastrana, & Vicente, 2014). The relative humidity gradient (RH) was 0/100 (RH outside/RH inside). The glass vials were weighed within a 10-h period. The WVP was calculated according to Equation (4):

$$WVP = \frac{WVT \times x}{A \times P_0 \times (RH_1 - RH_2)} \quad (4)$$

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.

2.4.6. Mechanical properties

The macroscopic physical characteristics of the BC + bLF films were calculated by determining the dry mass of 50 samples of each film (Adam Equipment), and by recording the thickness of 30 replicas of each film using a digital micrometre (Mitutoyo). The ultimate goal for the BC + bLF films is to encase meat products, which inherently possess high water activity (Borch, Kant-Muermans, & Blixt, 1996). Therefore, the mechanical properties were evaluated using re-hydrated BC samples, using a Shimadzu Universal Testing Machine (AG-IS - load cell of 50 N). Immediately prior to the stress strain tests, the films were immersed in distilled water for no less than 5 min. The defined strain rate was 0.5 mm/min at RT. The maximum stress (σ_{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 (5)) in 5 samples of each film (Menard, 2008).

$$\sigma = E\varepsilon \tag{5}$$

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

2.5. Bactericidal activity characterization

The antibacterial properties of the BC + bLF edible films were assessed against *E. coli* and *S. aureus* kindly provided by the Faculty of Pharmacy of University of Porto (Porto, Portugal).

2.5.1. Specific growth rate impact analysis

In order to evaluate the influence of bLF in the specific growth rate of the model microorganisms, culture flasks containing NB with 0, 0.25, 0.5, 1, 2.5, 5 and 10 mg/mL of bLF were inoculated with approximately 1.5×10^6 CFU/mL of either *E. coli* or *S. aureus*, and were subsequently incubated at 37 °C with 120 rpm of orbital shaking. Additionally, BC + bLF disks (and controls consisting of BC without bLF) with 22 mm diameter were aseptically introduced in culture flasks containing NB medium (4.5 mg of BC disks per mL of culture medium) and these 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.

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

NB inoculated with approximately 1.5×10^6 CFU/mL of either *E. coli* or *S. aureus*, was poured on top of BC + bLF disks, with 5 mm and 22 mm diameter, inside 96 and 24 well plates respectively, and these were afterwards incubated at 37 °C and 150 rpm for 4 h. The 5 mm disks were used in a Live/Dead viability assessment. Before each of the following step, the films were gently rinsed with PBS as follows: the culture medium was removed and then the disks were immersed for 10 min in propidium iodide (PI) (0.33% (w/v) in PBS). Subsequently, the disks were submerged in a formaldehyde solution (3.67% (v/v)) for 10 min at RT to fixate the cells. Next, the disks were immersed in a PBS solution with 4′,6-diamidino-2-phenylindole (DAPI) (0.01% (w/v) in PBS) for 10 min and subsequently rinsed twice prior to observation on the fluorescence microscope (Olympus BX51). Images were obtained with different fluorescence filters and were further merged using ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

Scanning electron microscopy (SEM) was performed on the 22 mm BC disks, after cell fixation according to the procedure described by Andrade et al. (Andrade et al., 2011). Briefly, the culture media was removed and 1 mL of 2.5% (v/v) glutaraldehyde in PBS was added to each sample. The disks were maintained for 1 h at RT to promote the fixation of the bacterial cells onto CB. Immediately after, the films were gently rinsed with distilled water and submitted to a dehydration process using serial ethanol dilutions, with increasing percentages of ethanol (55, 70, 80, 90, 95, 100% (v/v)). Samples were submerged in each solution for a period of 30 min at RT. Finally, the remaining ethanol in the films was evaporated at RT. The BC disks were coated with gold-sputtered (Polaron) and were observed by SEM (LEICA) with a magnification of 20,000 × using an electron accelerating voltage of 5 kV.

2.5.3. Evaluation of direct contact bactericidal effect

The American Association of Textile Chemists and Colourists (AATCC) test method 100-TM100 (AATCC, 2012) was herein adapted to evaluate the contact bactericidal efficiency of the BC + bLF films, so-called contact killing. Briefly, BC disks with 9 mm diameter were placed in a 48 well plate and inoculated with either 9.2×10^5 CFU/mL of *E. coli* or 9.4×10^5 CFU/mL of *S. aureus*. After 2 h of incubation at 37 °C, the samples were covered with 500 μL of PBS, and carefully stirred for no less than 1 min, to homogenise the bacterial population. Afterwards, the CFU/mL was determined in MH agar plates incubated for 24 h at 37 °C. The percentage of CFU reduction was determined as follows (Equation (6)):

$$R = \frac{A - B}{A} \times 100 \tag{6}$$

where, "R" is the percentage of CFU reduction, "A" the CFU/mL of the inoculum, and "B" the CFU/mL obtained after 2 h of contact with the films at 37 °C. Three disks replicas were used for each bacteria.

2.5.4. Evaluation of BC + bLF films bactericidal effect

An adaptation of the AATCC test method 100-TM100 (AATCC, 2012) was used for the bactericidal assays of BC + bLF films using fresh sausage samples. BC disks (with and without bLF) with 22 mm diameter were individually placed at the bottom of sterilized flasks, under aseptic conditions, and were further inoculated with 9.5×10^5 CFU/mL of *E. coli* and 1.0×10^6 CFU/mL of *S. aureus*. Immediately after inoculation, a fresh sausage piece with 12 mm diameter and 1.0 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 min to minimize the hypothetical endogenous contamination. As a control, the same procedure was used for a 22 mm disk of the casing as removed from the fresh sausage. After 24 h of incubation at 37 °C, 5 mL of PBS were poured into the flasks and submitted to a vigorous agitation. The obtained CFU/mL was determined and the percentage of reduction was calculated using the following equation:

$$R(\%) = \frac{A - C}{A} \times 100 \tag{7}$$

where, "R" is the percentage of CFU reduction, "A" is the CFU/mL obtained immediately after inoculation, and "C" is the CFU/mL achieved after 24 h of incubation at 37 °C.

2.6. Endotoxin level determination

G. xylinus is a Gram-negative bacterium. To assure the efficiency of the BC purification procedure described in section 2.2, as applied to BC samples from different sources, the levels of endotoxin were quantified. Endotoxin determination was performed using the limulus amebocyte lysate test Pierce® LAL Chromogenic Endotoxin Quantitation kit (Thermo Scientific). The optical density was measured at λ = 405 nm, with a linear range set between 0.1 and 1.0 endotoxin units (EU) mL^{-1}.

2.7. In vitro dynamic gastrointestinal model

The BC films (with and without bLF) were submitted to a dynamic *in vitro* gastrointestinal system similar to the intestinal tract model (TIM) 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 this system was to determine if the edible BC films would generate cytotoxic elements during the gastrointestinal digestion. Electrolyte gastric solution was composed of 0.48% (w/v) NaCl,

0.22% (w/v) KCl, 0.02% (w/v) $CaCl_2$, 0.15% (w/v) $NaKHCO_3$), pepsin 600 U mL^{-1} and lipase 40 U mL^{-1}, whereas the SIES (small intestine electrolyte solution) was composed of 0.5% (w/v) NaCl, 0.06% (w/v) KCl and 0.025% (w/v) $CaCl_2$. In all gastrointestinal compartments the pH was adjusted with HCl (37% (w/v)) or $NaHCO_3$ (10.6% (w/v)).

To simulate mouth digestion, the films were submitted to a protocol described by Sessa et al. (Sessa, Tsao, Liu, Ferrari, & Donsì, 2011). Briefly, 10 mL of human volunteer's saliva was collected. Edible BC films with 3 × 8 cm (with and without bLF) were shredded with scissors, immersed in the saliva solution and incubated at 37 °C for 10 min. Afterwards, 30 mL of PBS were added to the mixture and all contents shredded using a blender. Finally, the mixture was inserted in the gastrointestinal model. The digestion conditions were described by Reis et al. (Reis et al., 2008). with some modifications. In the stomach compartment, the sample contacted with the gastric solution added at a flow rate of 0.33 mL/min. The pH was gradually dropped from the initial 7.4, to 4.5 at 5 min, 4.2 at 20 min, 2.8 at 40 min, 2.1 at 60 min, 1.8 at 90 min, and finally kept 1.7 after 120 min of digestion, to mimic the process occurring after food ingestion. Then, the stomach compartment was gradually emptied. In the duodenum compartment, the samples were exposed to SIES enriched with 7% (w/v) pancreatin and 4% (v/v) bile at a flow rate of 0.6 mL/min. The pH was maintained at 6.5 and the duodenum was gradually emptied. When the sample reached the jejunum, it was mixed with SIES with 10% bile (v/v) added at a flow rate of 2.13 mL/min, and the pH was maintained at 6.8. The jejunum compartment was gradually emptied. In the ileum, the added solution consisted solely of SIES, added at 2.0 mL/min and the pH value was maintained at 7.2. After 3 h, all the volume present in the ileum was collected, immediately frozen at −80 °C, and stored until further use.

2.8. Cell viability assessment

The potential cytotoxicity of the BC and BC + bLF films before and following passage through an *in vitro* gastrointestinal system was evaluated with 3T3 mouse embryo fibroblasts (ATCC CCL-164). To test the BC and BC + bLF films *per se* cells were seeded at a density of 1.3 × 10^5 cells per well, on top of each BC sample previously placed on the bottom of 24 well plates. Fibroblasts were incubated in Dulbecco's Modified Eagle's medium (DMEM) (Biochrom), with 10% (v/v) fetal bovine serum (FBS) (Invitrogen) and 1% (v/v) of penicillin/streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells grown directly on the polystyrene surface were used as control. The cell growth was followed for 72 h. At this time, BC films were washed with PBS to remove non-adhered cells, and were further incubated with trypsin to recover the adhered cells. The total number of fibroblasts adhering onto the BC samples was quantified in a Neubauer chamber, using trypan blue (ratio 1:1) (Flücka) to distinguish viable from non-viable (blue) cells.

Additionally, the trypan blue exclusion assay was used to test the digestion fluids that resulted from the simulated gastrointestinal digestion of the BC films (with and without bLF). The frozen samples previously collected from the ilium were thawed and filter sterilized using a 0.2 μm filter. 96 well plates were used to seed 3T3 cells at a density of 1.3 × 10^5 cells/well. Cells were left to adhere in the polystyrene surface for 24 h and afterwards were exposed to the diluted (1/5) degradation products (section 2.7) in culture media for 72 h. Control samples cells were exposed to a diluted (1/5) PBS and small intestinal electrolyte solution (SIES). Afterwards, cells were washed with PBS and trypsinized. Cells suspension was then added at a 1:1 ratio to trypan blue and viable/non-viable cells were determined. All experiments were conducted in triplicate.

2.9. Statistical analysis

All data are presented as the mean ± standard error of the mean. Statistical analysis included t-test, one-way ANOVA and two-way ANOVA with Tukey post-hoc tests whenever the analysed results displayed a parametric distribution, otherwise Kurskal-Wallis with a Dunn's post hoc test was used. All statistical analysis was performed using Statistica 8.0 software (Statsoft, Inc.) using an alpha value of 0.05.

3. Results and discussion

In this work, BC from two distinct sources was studied for its bLF adsorption ability and saturation limit. Its functionality (antimicrobial activity) and cytotoxicity was also evaluated.

3.1. BC edible films physicochemical characterization

3.1.1. Prussian blue staining of BC + bLF films

A simple methodology to functionalize the BC films consists on the adsorption of bLF onto the BC nanofibrillar network. Determining the concentration of bLF in the supernatant, although allowing to quantitatively inferring as to the amount of adsorbed protein, it does not provide information regarding the distribution profile of the protein within the 3D nanofibrillar BC matrix. Therefore, a Prussian blue formation analysis was performed on both BC1 and BC2 films to qualitatively ascertain such distribution of the adsorbed protein within the 3D matrix. The bLF used possesses 120 ppm of Fe^{3+} in its iron binding clefts, thus its presence should lead to Prussian blue formation (according to Equation (1)). The transversal cuts analysed by stereo microscopy showed that BC films solely exposed to PBS did not display the characteristic coloration originated by the Prussian blue (Fig. 1Aa) and b)), whereas BC films with adsorbed bLF, were heavily stained throughout all the BC matrices (Fig. 1Ac) and d)).

3.1.2. Ultraviolet–visible spectroscopy and Fourier transform infrared spectroscopy with attenuated total reflectance

The presence of bLF was further analysed by UV–vis and FTIR spectrometry. 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 (Fig. 1B) (Aisen & Leibman, 1972; Belitz & Grosch, 1999). The high absorption peaks visible between 230 and 200 nm in the BC films with absorbed bLF also correspond to histidine, cysteine, methionine and aromatic amino acid residues (Belitz & Grosch, 1999). In BC samples, the sharp and steep band from FTIR-ATR spectra (Fig. 1C), at approximately 1060 cm^{-1}, corresponds to the C–O–C stretching. The low intensity band found near the 2890 cm^{-1} corresponds to the aliphatic C–H stretching, and finally, the broad band of O–H stretching vibration is present at approximately 3350 cm^{-1} (Müller et al., 2012). BC1 + LF and BC2 + LF exhibit two characteristic amide adsorption bands at around 1530 cm^{-1} and 1640 cm^{-1}, corresponding 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^- could not be identified in the bLF/BC films. Moreover, the Amide A (3280 cm^{-1}) band is probably masked by the cellulose O–H stretching (Xavier, Chaudhari, Verma, Pal, & Pradeep, 2010).

3.1.3. bLF adsorption–desorption profile onto and from BC

Analysis on the adsorption/desorption profile allowed estimating the saturation limit of bLF for both types of BC samples. It's noteworthy to mention that in previous experiments, following protein adsorption in never-dried BC films, we observed no

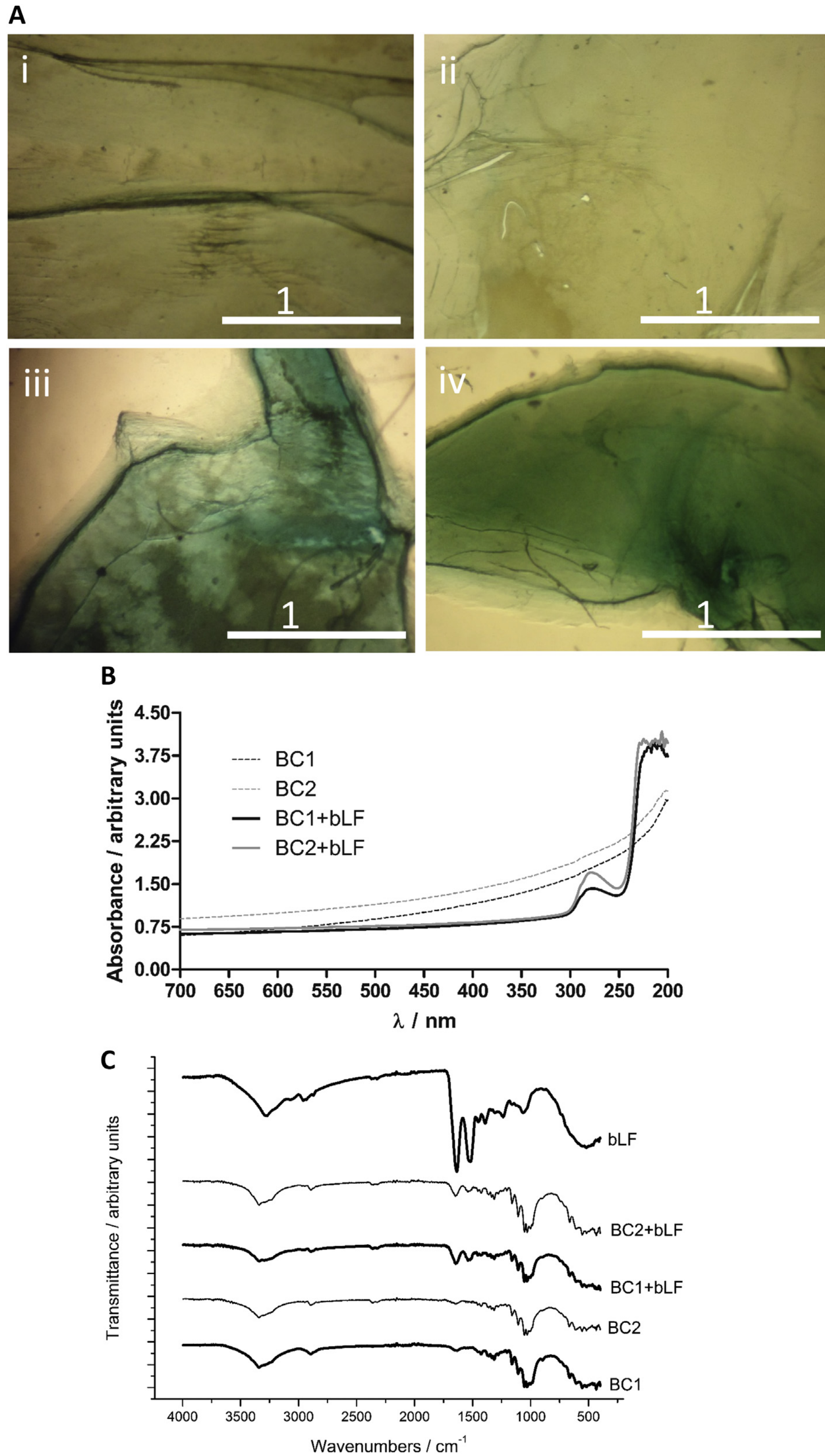


Fig. 1. A – Analysis of Prussian blue formation in bacterial cellulose (BC) films. i) bacterial cellulose from ATCC 53582 (BC1), ii) commercial bacterial cellulose (BC2), iii) BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and iv) BC2 with adsorbed bovine lactoferrin (BC2 + bLF). The images were obtained using a stereo microscope (Olympus). B – Ultraviolet–visible spectra of BC films: "- - -" BC1, "- - -"BC2, "▬" BC1 + bLF and "▬"BC2 + bLF. C – Fourier transform infrared spectroscopy with attenuated total reflectance (FTIR-ATR) of bovine lactoferrin powder (bLF), "BC1", "BC2", "BC1 + bLF"and "BC2 + bLF".

significant differences (t test, $p < 0.05$) in the protein desorption profile, between the dried and never-dried samples, thus suggesting the rehydration of the dried films was enough to promote a protein desorption comparable to the never-dried films. Therefore, we herein present the adsorption/desorption profiles only with the never-dried samples (further discussion on the swelling behaviour of the BC samples is given below). Fig. 2A shows that both BC films present a similar cumulative adsorption profile, divided in 3 phases, namely an initial adsorption phase, of up to 1 h of incubation for BC1 and up to 0.33 h for BC2; this was followed by a step increase (second phase) until the highest concentration plateau is achieved (third phase), after approximately 4 h incubation, reached simultaneously by both types of BC films. The sharp bLF adsorption rate was roughly estimated as 4.4 and 6.4 mg/mL.h for BC1 and BC2, respectively. After 24 h of incubation, the adsorbed bLF per gram of BC1 was 31.8 mg/mL; while BC2 adsorbed 40.6 mg/mL. This difference between the two types of BC was found to be statistically significant (t test, $p < 0.05$).

The *in vitro* release profiles obtained were adjusted using a modified Gompertz model (Equation (2)) (Fig. 2B), due to its' adequate fitness (Dash, Murthy, Nath, & Chowdhury, 2010). The fitting values for the release profile are presented on Table 1. A significant difference is registered in the cumulative release of bLF at 20 min, as well as at 1440 min, with the highest value corresponding to BC2 (t test, $p < 0.001$). BC2 seems to uptake a higher amount bLF and also to release a higher concentration of bLF. However, both BC films released almost the same relative amount of absorbed bLF, 18.5% and 17.3% for BC1 and BC2, respectively. The release rate of BC2 is however, slightly higher than BC1.

Table 1
Bovine lactoferrin (bLF) release values estimated using a modified Gompertz model (Equation (2)), "X_{max}" maximum bLF cumulative release, "R_r" rate of bLF release and "ψ" lag period prior to the bLF release.

	BC1 + bLF	BC2 + bLF
X_{max} (mg/mL$_{bLF}$·g$_{BC}$)	5.2	8.1
R_r (mg/mL$_{bLF}$·g$_{BC}$·min)	0.4	0.7
ψ (min)	0.0	0.8

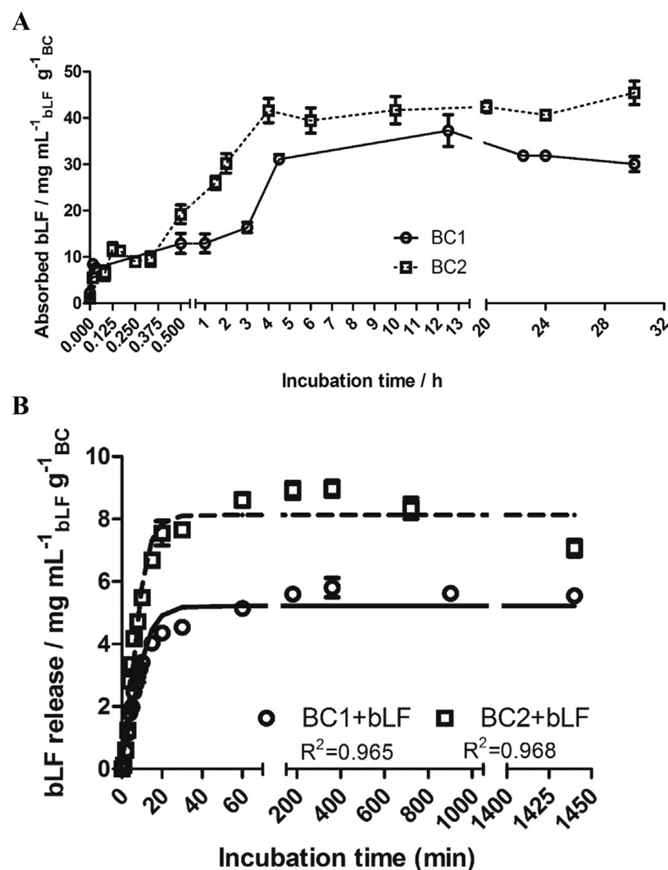


Fig. 2. A – Adsorption profiles of bovine lactoferrin (bLF) by: "-o-"bacterial cellulose membranes from ATCC 53582 (BC1), and "-□-"commercial bacterial cellulose BC2. B – Cumulative release profiles of bLF from BC samples: "o" bLF cumulative release from BC1 films (BC1 + bLF), "□" cumulative release of bLF from BC2 films (BC2 + bLF), "—" bLF release adjusted by Equation (2) for BC1 and "- -" bLF release adjusted by Equation (2) for BC2. All values correspond to the mean ± standard error of nine independent assays (n = 9).

3.1.4. 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 casing, thus improving product presentation and concomitantly, the consumer acceptance (Gennadios, Hanna, & Kurth, 1997). Moreover, it prevents the loss of moisture, 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 BC films during the uptake of water molecules. Therefore, the estimation of the rate of swelling from BC films is crucial if considering their use as a packaging system. However, with BC, the drying process may reduce the plasticizing effect of the water molecules, by increasing the proximity of neighbour BC fibrils and promoting the strong formation of additional hydrogen bounds (Clasen, Sultanova, Wilhelms, Heisig, & Kulicke, 2006; Lin, Hsu, Chen, & Chen, 2009). Consequently, rehydration may be inhibited by a more aggregated BC nanofibrillar structure. Thus, on one hand, the water holding capacity of the dried BC films will never be equivalent to the never-dried BC membranes; on the other hand, this could affect bLF desorption. In this work, all BC films showed a swelling value ranging between approximately 215% and 380% just after 90 s of immersion in PBS (Fig. 3). For BC without protein, these values are actually significantly higher than the approximately 80% reported by Rambo et al. for BC from *G. xylinus* ATCC 23769 (Rambo et al., 2008). BC1, BC2

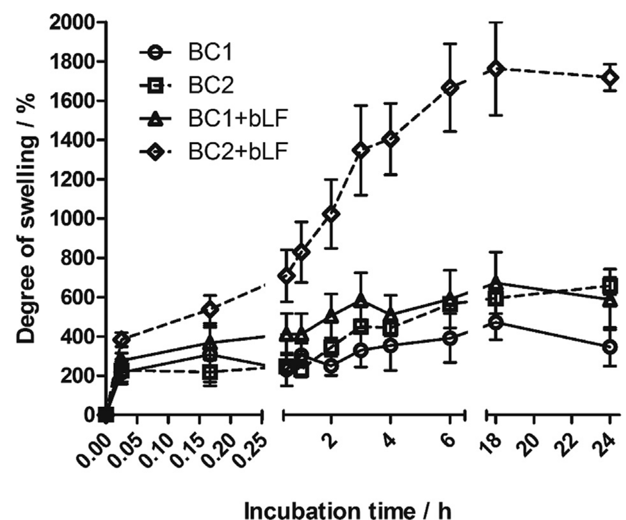


Fig. 3. Profile of the degree of swelling: "-o-"bacterial cellulose from ATCC 53582 (BC1), "-□-"commercial bacterial cellulose (BC2), "-▲-" BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and "-◇-" BC2 with adsorbed bovine lactoferrin (BC2 + bLF). The values represent the mean ± standard error of three independent assays (n = 3).

and BC1 + bLF share the same swelling profile. BC2 + bLF in particular has a quite distinct profile, characterized by a second increase phase of approximately 205% h^{-1}, subsequent to the 90 s, that subsists up to 24 h of immersion. This may be due to the higher protein loading capacity exhibited by BC2 + bLF that, by further coating the surface of BC fibres, the adsorbed protein probably prevents the formation of hydrogen bonds between BC fibrils, during dehydration. Consequently, this provides more freedom for water molecules to rehydrate the BC matrix and ultimately leads to a higher swelling profile.

3.1.5. Water vapour permeability

One essential feature of edible films is their permeability to water vapour. In order to avoid the loss of moisture and consequently the weight loss of several types of meats and sausages, casings with low permeability to water vapour are used in the food industry. Fresh sausage is usually produced at ambient temperature, using fresh meat mixed with spices, remaining uncooked throughout all manufacturing process (Cruz, 2010). Due to their characteristic hydrophilic nature, polysaccharides and proteins usually offer poor water vapour permeability (Lacroix, 2009). The WVP values exhibited by the BC films were approximately 5 fold lower than those of the pig's small intestine casing (Fig. 4). The WVP values herein determined were found to be similar for BC1, BC2 and BC2 + bLF. BC1 + bLF possess a higher value; however, as observed in section 3.1.4, BC2 + LF exhibit a higher swelling behaviour.

The WVP values of BC are approximately three fold lower than those of other bio-based edible films, such as chitosan (Cerqueira, Souza, Teixeira, & Vicente, 2012; Martins, Cerqueira, & Vicente, 2012), κ-carrageenan mixed with locust bean gum (Cerqueira et al., 2014), 7 fold higher when compared to whey protein films (Fang, Tung, Britt, Yada, & Dalgleish, 2002) and cassava starch (Henrique, Teófilo, Sabino, Ferreira, & Cereda, 2007), but it is similar to high molecular weight methylcellulose edible films (Chambi & Grosso, 2011; Nazan Turhan & Şahbaz, 2004; Park, Weller, Vergano, & Testin, 1993). These results suggest that BC may present a technical advantage in comparison to pig small intestine casing, by its improved maintenance of the products moisture content, while possibly allowing the release of water during the cooking process, thus maintaining the integrity of the casing.

3.1.6. Mechanical properties

The pig's small intestine casing (control) was approximately 3.5 fold thicker than the BC substrates, while no pronounced differences were observed among the BC membranes thickness (Fig. 5A). The volumetric mass density of all BC substrates was similar (Fig. 5B). The density values of BC and BC with bLF were similar to the 0.99 mg/mm^3 density value reported by Yamanka and co-workers (Yamanaka et al., 1989).

A food casing must be able to endure the sausage filling process without bulging. Thus, the evaluation of the mechanical properties constitutes a key characterization step. In this work, the food product used has high moisture content. Additionally, it is stored in a liquid filled package. Therefore, the assessment of the mechanical properties was performed under water saturating conditions. The mechanical properties and the typical strain profiles are presented in Table 2 and Fig. 5C, respectively. All BC films have a σ_{max} within the range described by the literature (Rambo et al., 2008). BC1 and BC2's behaviour towards tensile traction is relatively 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 than that of BC1. bLF absorption into BC matrixes influenced in the same extent, the performance of both types of BC, due to the plasticizing effect of water, which increases the strain at break and is in accordance with other studies reported in the literature. For instance, Zhu and co-workers (Zhu et al., 2010) showed a similar influence on the mechanical properties, following functionalization of BC films with ε-PL.

The fresh sausages 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, 14 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's maximum tensile strength is similar to that of BC1, and roughly 2 fold higher than that of BC2 + bLF. These values are in accordance to the maximum tensile values reported for pig's intestine (43.3 MPa) and collagen (34.8 MPa) casings (Cagri, Ustunol, Osburn, & Ryser, 2003).

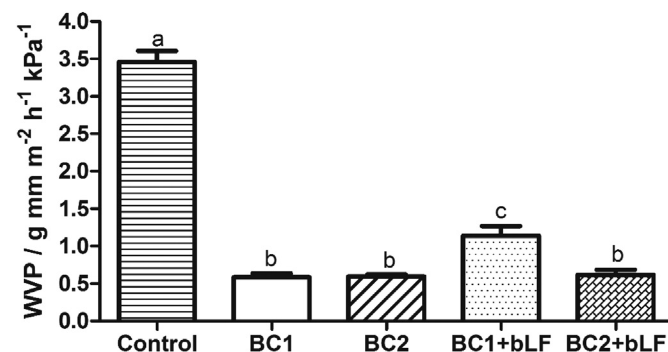


Fig. 4. Water vapour permeability (WVP) values of: "▤" pig small intestine casing (control), "▭"bacterial cellulose from ATCC 53582 (BC1), "▨"commercial bacterial cellulose (BC2), "▭" BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and "▩"BC2 with adsorbed bovine lactoferrin (BC2 + bLF). The results correspond to the mean ± standard error of five independent assays (n = 5). Different letters between distinct columns denote significant differences using two-way ANOVA with Tukey post-hoc test ($p < 0.05$).

3.2. Bactericidal characterization

3.2.1. Specific growth rate impact analysis

The non-specific bactericidal properties of bLF have been widely described in the literature (Rossi et al., 2002; Sanchez, Calvo, & Brock, 1992; Vorland et al., 1998). *E. coli*, Gram-negative bacilli, are a major representative of the normal intestinal microflora; also they are one of the most frequent causative agents of intestinal infections (Gadó et al., 1991) and thus considered important foodborne pathogen. *S. aureus*, Gram-positive cocci, are a recurrent agent of food poisoning (Bellamy et al., 1992).

The effect of bLF concentration on the specific growth rate (μ) of *E. coli* and *S. aureus* in NB culture medium is illustrated in Fig. 6A (for the sake of clarity, the right hand side of the figure contains the time-scale corresponding to the exponential growth phase, which allowed the determination of the μ values and their statistical comparison). As compared to the control, bLF concentrations of 5 mg/mL and 10 mg/mL were found to significantly impair the growth of *E. coli* whereas all the tested concentrations also significantly reduced the specific growth rate *of S. aureus*.

Unmodified BC disks were incubated in NB media and the growth profiles were analysed. As shown in Fig. 6B, overall, BC *per se* did not significantly affect the growth profile of both strains, as compared to the control. Considering the modulated release profile displayed in Fig. 2B and the films density (Fig. 5B), the expectable bLF release into the culture media would be of approximately 0.8 mg/mL in BC1 + bLF and BC2 + bLF. Therefore, the expectable impact on the specific growth rate should be similar to that of 1 mg/

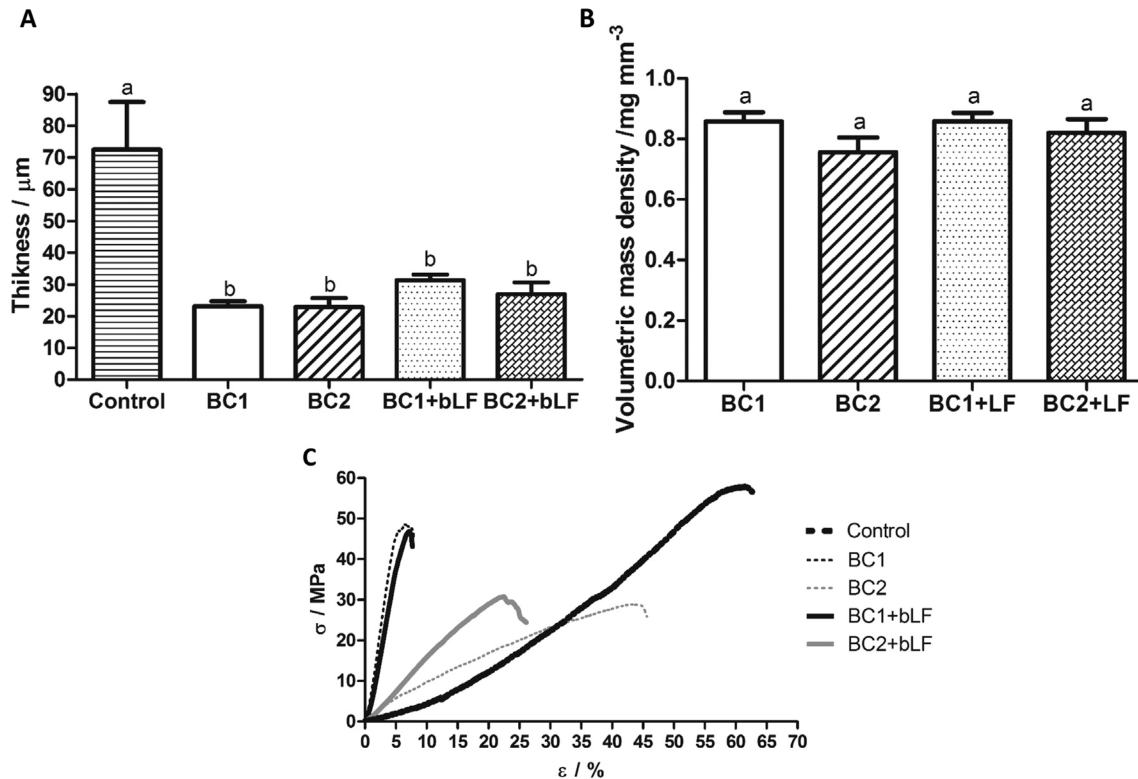


Fig. 5. The values of: A – thickness and B – volumetric mass density of: "▭" pig small intestine casing (control), "▭"bacterial cellulose from ATCC 53582 (BC1), "▨"commercial bacterial cellulose (BC2), "▭" BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and "▨" BC2 with adsorbed lactoferrin (BC2 + bLF). C – Typical tensile tests profiles of: "▬" control "- - -"BC1, "- - -"BC2, "▬" BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and "▬" BC2 with adsorbed bovine lactoferrin (BC2 + bLF). Distinct letters between different columns denote statistical different values as obtained from Kruskal–Wallis with Dunn's multiple comparison post-hoc test ($p < 0.05$). The results correspond to the mean ± standard error from (A) thirty five independent assays ($n = 35$) and (B) seventy independent assays ($n = 70$).

Table 2
"E" elastic modulus, "σ_{max}" maximum stress and elongation at break "ε_{break}" of: "control" fresh sausage's porcine small intestine casing, "BC1" bacterial cellulose from ATCC 53582, "BC2" commercial bacterial cellulose, "BC1 + bLF" BC1 with bLF and "BC2 + bLF" BC2 with bLF. The results correspond to the mean ± standard error from five independent assays ($n = 5$).

	E (MPa)	σ_{max} (MPa)	ε_{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

mL bLF, which was 0.85 h^{-1} for *E. coli* and 0.51 h^{-1} for *S. aureus*. *E. coli* showed this growth rate when cultured with BC1 + bLF although, comparing to the control, it did not display a significant difference. *E. coli* cultured with BC2 + bLF presented a growth rate of 0.48 h^{-1}, which was not statistically significant when compared to the control. In the case of *S. aureus*, the growth rate values are lower than the ones expected when grown in BC1 + bLF (0.39 h^{-1}) and higher for the BC2 + bLF (0.70 h^{-1}).

Microorganisms are usually prone to adhere to surfaces. In the case of BC + bLF films, 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 (Shimazaki et al., 1993). However, this effect may have not occurred in a reproducible manner in all experiments using BC + bLF films.

Optical density measurements of biomass provide an indication of the bacterial growth, however they do not allow obtaining information on the bacterial viability (Reller, Weinstein, Jorgensen, & Ferraro, 2009). Thus, an analysis of the bacteria viability was also done by conducting a live/dead assay as described below.

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

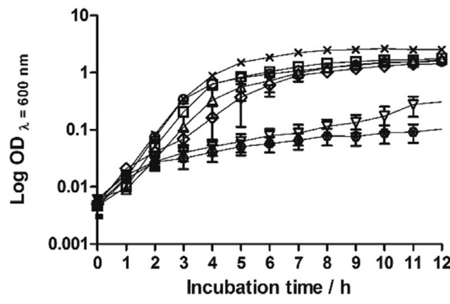
SEM images of the BC films cultured with either *E. coli* or *S. aureus*, revealed an evident difference in the bacteria's morphology present at their surfaces (Fig. 7A). Clearly, the BC1 + bLF and BC2 + bLF surfaces are covered with disrupted bacteria debris from *E. coli* (Fig. 7A iii), iv)) and *S. aureus* (Fig. 7A vii), viii)), thus confirming the bactericidal effect of bLF. Some *S. aureus* death could also be visualized in BC2 films (Fig. 7A vi)). The live/dead assessment test (Fig. 7B) showed a higher killing ratio (red cells) for BC with bLF as compared to BC without bLF.

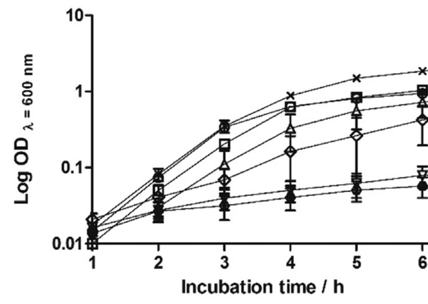
3.2.3. Bactericidal effect of BC films

The BC bactericidal efficiency was quantified using a high inoculum and optimal growth conditions for the tested bacteria. BC1 + bLF and BC2 + bLF showed a significant *E. coli* CFU reduction when compared to the reduction exhibited by BC1 and BC2 (Fig. 8A, i)). Indeed, BC films alone only killed less than 10% of bacteria, whereas the BC1 + bLF and BC2 + bLF showed a considerably higher contact killing in the range of 70% in average. Effectiveness against *S. aureus* from BC1 + bLF and BC2 + bLF films was (statistically) higher than that of BC1 and BC2 (Fig. 8A, ii)). However, BC films alone led to a reduction of *S. aureus* CFU by nearly 65%. Also, 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* as compared to BC1 + bLF were detected.

A

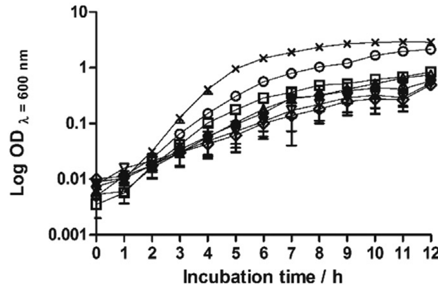
i)

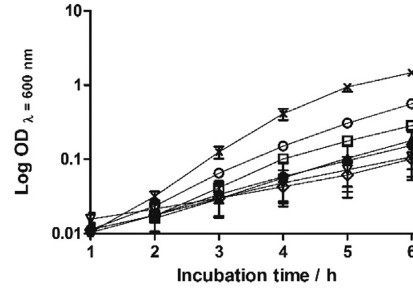




- **Control** μ = 1.20±0.08 h^{-1} [a]
- **0.25 mg mL^{-1}** μ = 0.8543±0.01 h^{-1} [a,b]
- **0.5 mg mL^{-1}** μ = 0.90±0.26 h^{-1} [a,b]
- **1 mg mL^{-1}** μ = 0.85±0.17 h^{-1} [a,b]
- **2.5 mg mL^{-1}** μ = 0.81±0.07 h^{-1} [a,b]
- **5 mg mL^{-1}** μ = 0.36±0.09 h^{-1} [b]
- **10 mg mL^{-1}** μ = 0.53±0.08 h^{-1} [b]

ii)

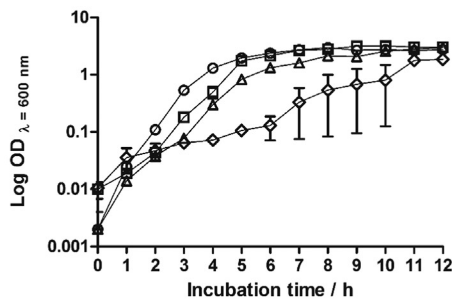




- **Control** μ = 1.08±0.04 h^{-1} [a]
- **0.25 mg mL^{-1}** μ = 0.74±0.04 h^{-1} [b]
- **0.5 mg mL^{-1}** μ = 0.58±0.02 h^{-1} [b]
- **1 mg mL^{-1}** μ = 0.51±0.01 h^{-1} [b]
- **2.5 mg mL^{-1}** μ = 0.47±0.07 h^{-1} [b]
- **5 mg mL^{-1}** μ = 0.40±0.04 h^{-1} [b]
- **10 mg mL^{-1}** μ = 0.53±0.08 h^{-1} [b]

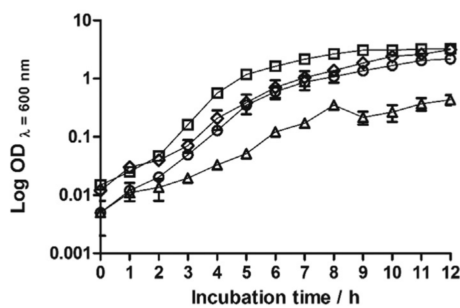
B

iii)



- **BC1** μ = 1.23±0.21 h^{-1} [a]
- **BC2** μ = 1.29±0.07 h^{-1} [a,b]
- **BC1+bLF** μ = 0.86±0.14 h^{-1} [a,b]
- **BC2+bLF** μ = 0.48±0.41 h^{-1} [b]

iv)



- **BC1** μ = 0.66±0.01 h^{-1} [a]
- **BC2** μ = 1.15±0.12 h^{-1} [b]
- **BC1+bLF** μ = 0.39±0.10 h^{-1} [c]
- **BC2+bLF** μ = 0.70±0.04 h^{-1} [a]

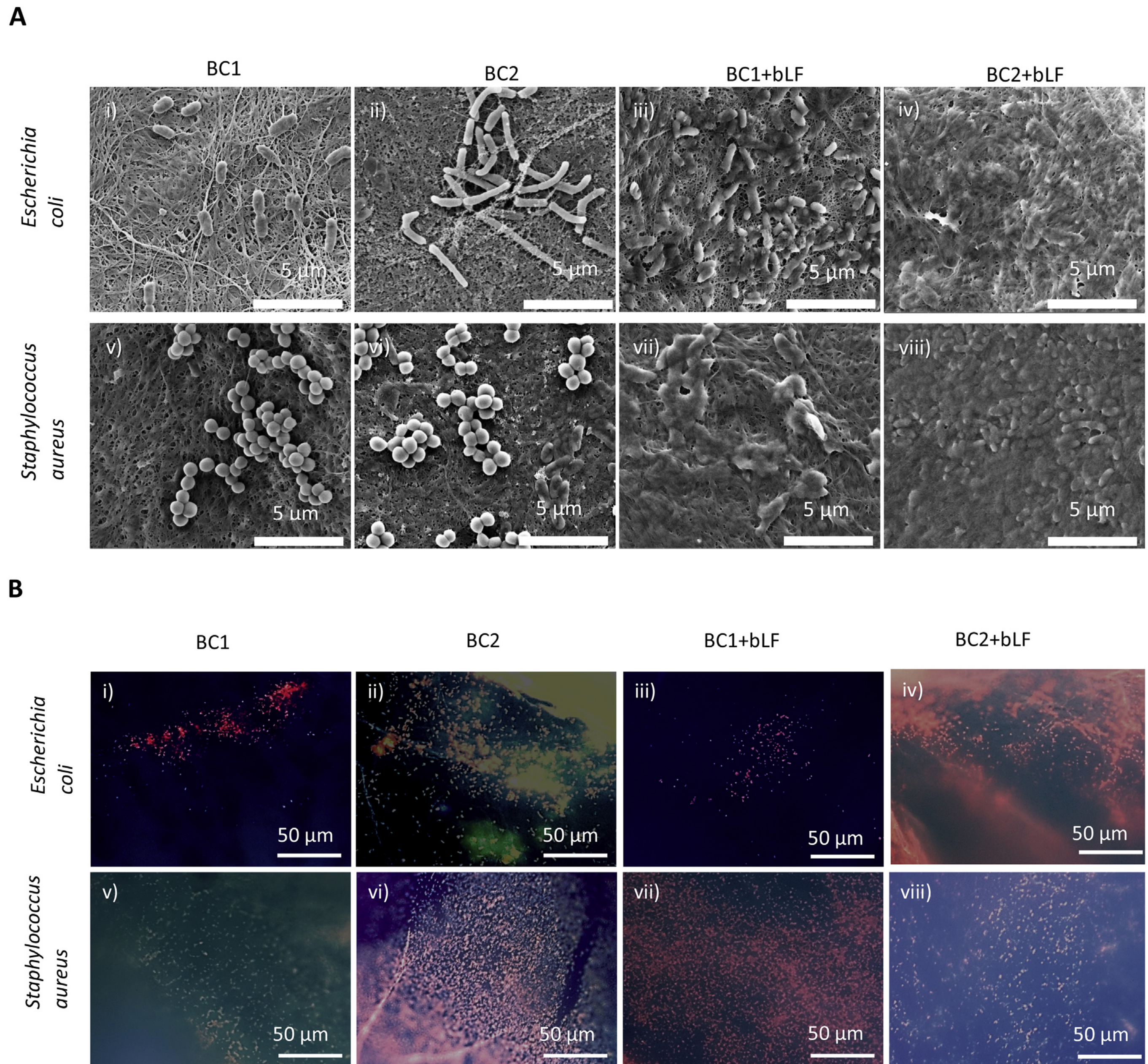


Fig. 7. A – Scanning electron microscope (SEM) images of the bacteria at the surface of the bacterial cellulose (BC) films after 4 h of incubation in nutrient broth at 37 °C, 120 rpm agitation: in contact with *Escherichia coli* and *Staphylococcus aureus*. B – Images from the live/dead assays with BC1, BC2, BC1 + bLF and BC2 + bLF.

BC films contaminated with bacteria were also used to wrap fresh sausage slices to further evaluate the films antibacterial efficiency through contact killing. This test aimed to evaluate the efficiency of the films under direct contact with a perishable food product. For these experiments, the contamination of the food product was induced (by adding 9.5×10^5 CFU/mL of *E. coli* and 1.0×10^6 CFU/mL of *S. aureus*). Nutrient and humidity conditions were considered adequate for microbial growth, since the microorganisms were in direct contact with the fresh sausage; also the exposure time was set to 24 h (Fig. 8B). Even though the assay occurred under optimal temperature conditions for microbial growth, a statistically significant reduction of viable *E. coli* was found (Fig. 8B iii)). When in contact with *E. coli*, BC1 and BC2 exhibited a similar reduction in the CFU percentage (approximately 78.5%). BC1 + bLF and BC2 + bLF showed a higher reduction in the bacteria viability, which reached 93.6% (corresponding to 1-log

Fig. 6. A – Growth profiles of: i) *Escherichia coli* and ii) *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}. B – Growth profile of iii) *E. coli* and iv) *S. aureus* in contact with bacterial cellulose (BC) films: "Control" without BC films, "BC1" BC from ATCC 53582, "BC2" commercial BC, "BC1 + bLF" BC1 with adsorbed bLF and "BC2 + bLF" BC2 with adsorbed bLF. The specific growth rate (μ) is provided in the legend, and different letters between distinct rates denote significant differences as analysed by one-way ANOVA (i. and ii.) and two-way ANOVA (iii. and iv.) with Tukey post-hoc test ($p < 0.05$). The values correspond to the mean ± standard error from three independent assays (n = 3). The cultures were performed in nutrient broth (NB), at 37 °C with an orbital shaking of 120 rpm.

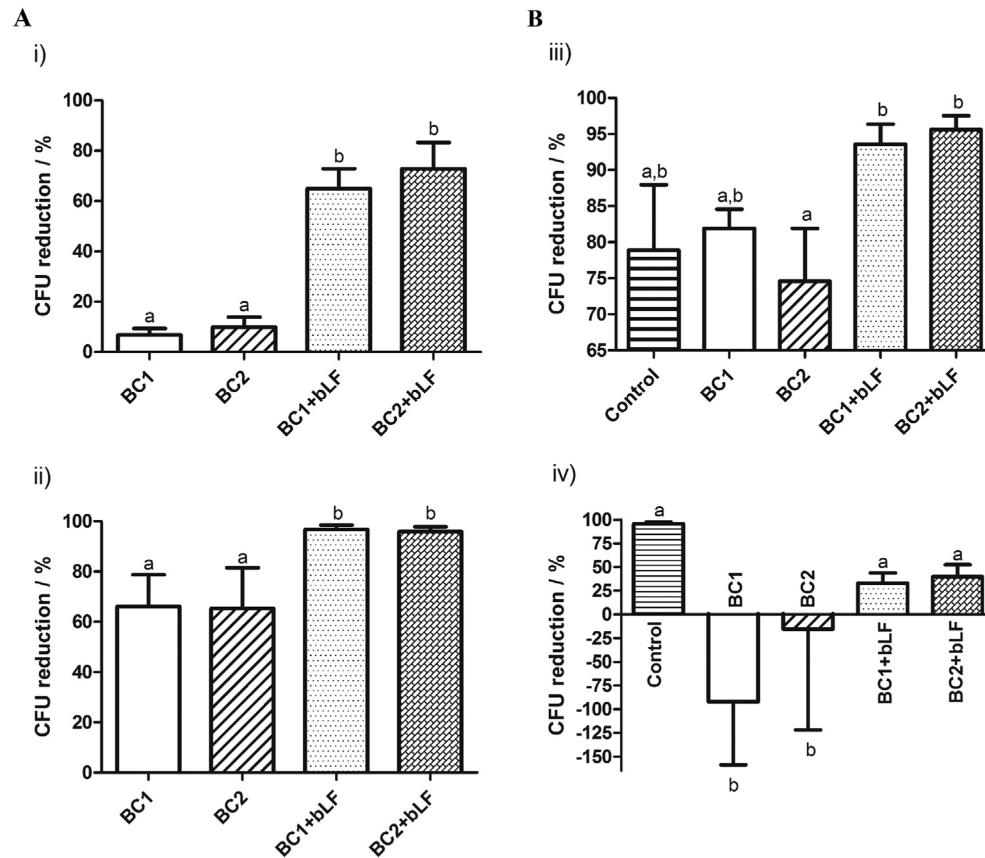


Fig. 8. A – Percentage of colony forming units (CFU) reduction of i) *Escherichia coli* and ii) *Staphylococcus aureus* by: "▭"bacterial cellulose from ATCC 53582 (BC1), "▨"commercial bacterial cellulose (BC2), "▭" BC1 with adsorbed bovine lactoferrin (BC1 + bLF) and "▨"BC2 with adsorbed bovine lactoferrin (BC2 + bLF), for an exposure period of 2 h at 37 °C. B –CFU percentage of reduction of iii) *E. coli* and iv) *S. aureus* in: "▤"pig small intestine casing (control), "▭"BC1, "▨"BC2, "▭"BC1 + bLF and "▨"BC2 + bLF, for an exposure period of 24 h at 37 °C. Distinct letters between different columns denote significant differences analysed using two-way ANOVA with Tukey post-hoc test ($p < 0.05$). All results correspond to the mean ± standard error from three independent assays ($n = 3$).

reduction) and 95.9% (corresponding to 1-log reduction), respectively. On the other hand, *S. aureus* actually grew in both BC1 and BC2, while with pig small intestine, BC1 + bLF and BC2 + bLF inhibition of growth was shown (approximately 52.5%, 32.9% and 39.7%, respectively) (Fig. 8B iv). It's noteworthy to mention that, the initial contamination level was indeed high, a condition most likely not encountered in a real case scenario (considering the mass of the fresh sausage used in the assays, the initial microbial load would correspond to 1.2×10^4 CFU/g product for *E. coli* and 1.3×10^4 CFU/g product for *S. aureus*; these values would deem the product as "unacceptable" under current European legislation (specifically Council Directives 88/657/EEC (European Parliament, 1988) and 94/65/EC (European Parliament, 1994)).

3.3. Endotoxin level determination

In Europe, the general principles required by the Regulation (EC) Nº 1935/2004 for food contact materials state that these materials must be safe, i.e. 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 (FCM) must be manufactured using good manufacturing practices (European Parliament, 2004, 2006). In the USA, the Food, Drug and Cosmetic Act (Federal Food, 1958) contains the basic regulation on FCM. Briefly, FCM are regulated in the Code of Federal Regulations (CFR.), specifically under Title 21 on Food and Drugs, and Parts 176 to 186 (e-CFR, 2016).

Several toxicological data are necessary to provide information regarding the safety of the food packaging. In this work, an initial assessment on the safety of BC films containing bLF was done. Lipopolysaccharide (LPS) is a characteristic pathogen-associated molecular pattern (PAMP) present in the 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 (Akira, Uematsu, & Takeuchi, 2006; Karima, Matsumoto, Higashi, & Matsushima, 1999). The BC films herein produced are intended to be used as FCM, thus the absence of LPS must be assured since the BC membranes are produced by a Gram-negative prokaryote. While BC is not absorbed by the intestinal tract, LPS may be released and absorbed, consequently entering the blood stream. The endotoxin values measured on the BC membranes were 0.851 ± 0.292 and 2.027 ± 0.068 EU/L for BC1 and BC2, respectively. These values are considerably below the maximum threshold of acceptance determined by the Food and Drug Administration (FDA) for general medical devices materials, which is 500 EU/L (FDA, 1987).

3.4. Cell viability assessment

According to the regulatory aspects covered in the previous section, an essential assumption for a film to be considered edible is its absence of cytotoxicity to the human host. Therefore, the new

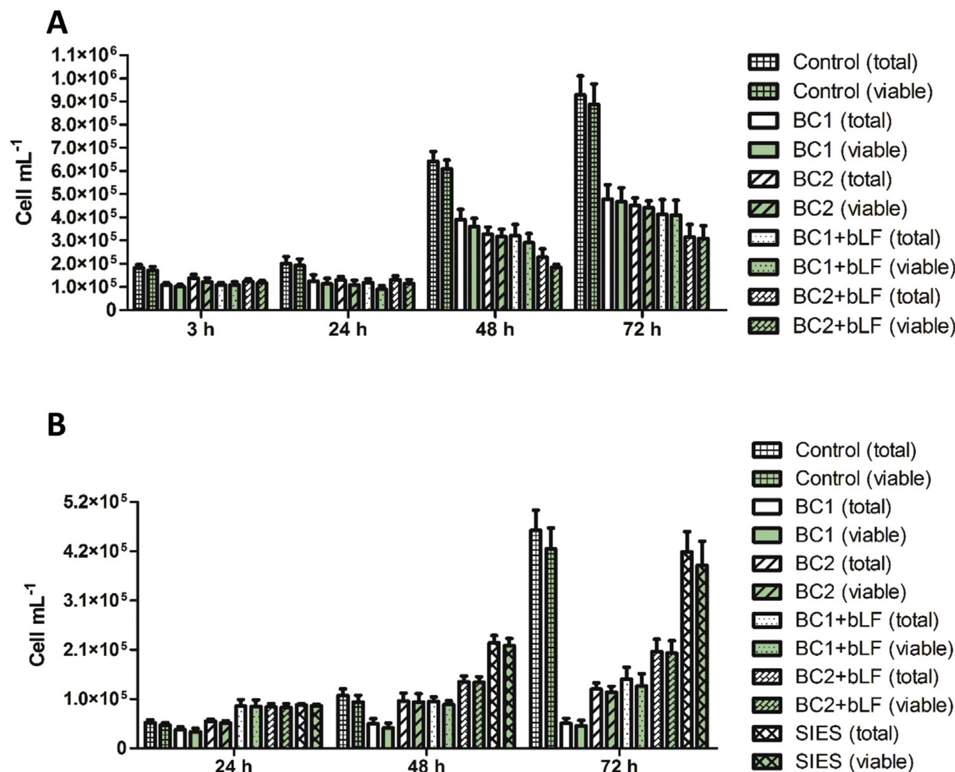


Fig. 9. Cytotoxicity analysis of A – samples placed on the bottom of the wells and B – cells in contact with solutions that resulted from the dynamic *in vitro* gastrointestinal digestion of: "" "Control total" total cells in culture plate polystyrene surface, "" "Control viable" viable cells in culture plate polystyrene surface, "" "BC1 total" total cells in bacterial cellulose from ATCC 53582 (BC1), "" "BC1 viable" viable cells in BC1 "" ""BC2 total" total cells commercial bacterial cellulose (BC2), "" "BC2 viable" viable cells in BC2, "" "BC1 + bLF total" total cells in BC1 with adsorbed bovine lactoferrin (bLF), "" "BC1 + bLF viable" viable cells in BC1 with adsorbed bovine lactoferrin, "" "BC2 + bLF total" total cells in BC2 with adsorbed bLF, "" "BC2 + bLF viable" viable cells in BC2 with adsorbed bLF, "" "SIES total" total cells in contact with small intestinal electrolytic solution (SIES), "" "SIES viable" viable cells in contact with SIES. Results are expressed in mean ± standard error, obtained from six independent assays (n = 6). The assays were performed using mammalian mouse embryo fibroblasts 3T3 (ATCC CCL-164) at 3 72 h of incubation at 37°, 5% CO_2 humidified atmosphere.

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 mimetic *in vitro* dynamic gastrointestinal system. Direct contact with 3T3 cells showed a slight impairment of the cell proliferation after 24 h 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 reported by our group (Fig. 9A) (F. K. Andrade, Moreira, Domingues, & Gama, 2010; Pertile, Andrade, Alves Jr, & Gama, 2010). Among BC films, cell proliferation was identical with similar values of total cells count in films with and without bLF. Cell viability did not suffer any significant impact (Fig. 9A), which is in accordance with other studies reported in the literature. Indeed, no cytotoxicity was expected, since 10 mg mL^{-1} of bLF has been reported to be non-toxic to CaCO-2 cells (Atef Yekta et al., 2010).

After digestion of the BC films (with and without bLF) in the mimetic dynamic gastrointestinal system, the 3T3 cells were exposed to the several products resultant from the simulated digestion process (Fig. 9B). The production of an accurate control would imply a gastrointestinal digestion without the ingestion of any produced BC films, which was not feasible. The ileum's main fluid is SIES and it was used as the control sample. Significant differences in the total 3T3 cell counts were only visible after 72 h of incubation (Fig. 9B). Cell number in BC1 remained identical throughout the experiment, although the minimum percentage of viability was 80%. BC2 + bLF samples presented the highest percentage of viability and demonstrated a significant increase of the cell number after 72 h of incubation, which may be due to the higher amount of adsorbed bLF. Cells cultured with SIES samples showed similar behaviour to the cells growing with the diluted degradation products; however SIES lacks several of the gastrointestinal components. Overall, the viability was above 70% in all cases and therefore, non-cytotoxic BC1 + bLF and BC2 + bLF films could have actually promoted cell growth, since bLF has also been reported to act as a growth factor in cell culture (Britton & Koldovsky, 1989).

4. Conclusions

Fully bio-based edible antimicrobial BC films were developed using bLF as the active component. Physicochemical differences were observed between the two different BC, before and after activation with the protein (BC1 + bLF and BC2 + bLF). The WVP of the pig's small intestine casing retrieved from fresh sausages was 5-fold higher than the one displayed by the activated BC films, even though the later possess nearly half of the thickness. The adsorption of bLF caused a shift in the surface properties of the BC films towards their interaction with water, denoting the relevance of the impact of the protein in the BC films surface properties. Although the mechanical properties of the BC films were slightly impaired after bLF absorption, the tensile strength remained much higher than that of the pig's small intestine casing.

BC1 + bLF and BC2 + bLF were able to reduce *in vitro* the CFU viability of both *E. coli* and *S. aureus*, reaching 1-log reduction in the *S. aureus*. Contrarily, in the fresh sausage, the bactericidal efficiency was higher for *E. coli* than for *S. aureus*. All BC edible films *per se* and the products resulting from their simulated digestion did not

exhibit cytotoxicity against fibroblasts. The developed films were found to be bactericidal (even under an unlikely extreme contamination scenario), non-toxic, as well as to have the appropriate technological characteristics to be used as a bio-based meat product casing.

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