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Immobilization of antimicrobial peptides from *Lactobacillus sakei subsp. sakei* 2a in bacterial cellulose: Structural and functional stabilization

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ABSTRACT

Lactobacillus sakei subsp. *sakei* 2a is a bacteriocinogenic lactic acid bacterium isolated from Brazilian pork sausage, which produces antimicrobial peptides (bacteriocins) able to control the growth of important foodborne pathogens. These antimicrobial peptides were immobilized by physical entrapment in bacterial cellulose (BC) membranes produced by *Gluconacetobacter xylinus*, aiming at improving their antimicrobial activity against *Listeria monocytogenes*. Scanning Electron Microscopy indicated the BC membranes presented an entangled structure, with void spaces ramndomly distributed throughout the membrane matrix, facilitating the entrappment and immobilization of the bacteriocins. Both free and BC membrane entrapped bacteriocins were applied to an artificial bacterial lawn of *Listeria monocytogenes* grown on solid BHI medium, and incubated at either room (30 °C) or refrigerated (7 °C) temperatures during 24 h or 7 d, respectively. At room temperature, the bacteriocins entrapped within BC membranes were significantly ($p < 0.05$) more efficient in the control of pathogen growth when compared to the free bacteriocins, during the whole timeframe under study. At refrigeration temperature, both free and entrapped bacteriocins led to inactivation of the pathogen after 5 days. These results are good evidence that entrapment of bacteriocins produced by *Lb. sakei* 2a in BC membranes is as a promising strategy for the control of *L. monocytogenes* in foods.

1. Introduction

Bacteriocins are small peptides produced by bacteria, specifically for inhibiting or killing other related and unrelated microorganisms (Collins, Cotter, Hill, & Ross, 2010). These small antimicrobial peptides can be potentially used both in the food and pharmaceutical industries. In the food industry, nisin is the most extensively applied bacteriocin, being utilized in more than 50 countries as a safe and natural antibacterial food preservative (Field et al., 2012). However, other types of bacteriocins have been evaluated aiming at inhibiting undesirable microbial contaminations in foodstuff. *Lactobacillus sakei* subsp. *sakei* 2a is a bacteriocinogenic lactic acid bacterium isolated from Brazilian pork sausage, able to control the growth of microbial pathogens, particularly *Listeria monocytogenes* (De Martinis & Franco, 1998; Liserre, Landgraf, Destro, & Franco, 2002). The bacteriocins produced by this bacterial strain exhibit high thermal stability and are active within a broad range of pH. A previous study has shown that a concentrated acid extract of *Lb. sakei* 2a purified by cation exchange chromatography and reversed-phase chromatography contained three different antimicrobial peptides with molecular weights of 4.4, 6.8 and 9.5 kDa (Carvalho et al., 2010).

Bacteriocins can be added to a particular foodstuff as preservatives either as partially purified or purified concentrates. However, stability issues related to proteolytic degradation and/or interaction with fat and other food components might result in a decrease of their potential antimicrobial activity (Chollet, Sebti, Martial-Gros, & Degraeve, 2008; Glass & Johnson, 2004). Immobilization *via* containment within nanoparticles may improve the antimicrobial efficacy of bacteriocins, by protecting them from interactions with food ingredients and providing a controlled release during storage, thus increasing the shelf life of the product.

Bacteriocins can also be incorporated in food packaging films, creating an active packaging system that maintains the antimicrobial activity during food storage, increasing the safety of the food (Yang, Lin, Sung, & Fang, 2014). Bacterial cellulose (BC) appears as a potential

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candidate to achieve this goal. BC is a non-toxic nanostructured biomaterial, synthesized by several microorganisms such as *Gluconacetobacter xylinus*. BC is an unbranched biopolymer encompassing a three-dimensional network composed of nanofibrils constituted by monomeric glucose units joined by β-(1→4) glycosidic bonds. BC exhibits a high water absorption capability, unique mechanical features, good permeability, and resistance to degradation (Trovatti, Serafim, Freire, Silvestre, & Neto, 2011; Trovatti, Fernandes, Rubatat, Freire et al., 2012; Trovatti, Fernandes, Rubatat, Perez et al., 2012; Yang et al., 2014). Due to these characteristics, BC is potentially applicable both in the food and medical/pharmaceutical industries. For food applications, BC can be used as thickening, gelling, stabilizing and water-binding agent and also incorporated in packaging materials (Yang et al., 2014).

Like most biological molecules, bacteriocins are intrinsically fragile and therefore their full structural and functional stabilization is a *sine qua non* condition for their application as antimicrobial agents (Balcão & Vila, 2015; Balcão, Barreira et al., 2014; Balcão, Glasser et al., 2014; Balcão, Moreira et al., 2013; Balcão, Costa et al., 2013). The nanocontainment technique for stabilization of protein entities is gaining momentum (Balcão & Vila, 2015; Balcão, Costa et al., 2013; Balcão, Glasser et al., 2014), but its application for small proteinaceous molecules, like bacteriocins, has not been fully exploited.

Incorporation of bacteriocins within cellulose membranes possesses a wide applicability in the food industries (Nguyen, Flanagan, Gidley, & Dykes, 2008). The major goal of the research effort entertained herein was to immobilize, by physical entrappment, bacteriocins isolated from *Lactobacillus sakei* subsp. *sakei* 2a in BC membranes produced by *Gluconacetobacter xylinus*, with consequent stabilization of both the structure and function of these protein moieties, and to evaluate *in vitro* their antimicrobial efficacy against *Listeria monocytogenes*.

2. Material and methods

2.1. Bacterial cultures and microbiological media

Lb. sakei subsp. *sakei* 2a (*Lb. sakei* 2a), a bacteriocin-producing isolate from Brazilian pork sausage (De Martinis and Franco et al., 1998), was grown at 30 °C for 18 h in MRS broth (Difco, Detroit MI, U.S.A.). *Listeria monocytogenes* Scott A, used as indicator of the bacteriocins antimicrobial activity, was grown in BHI broth (Oxoid, Basingstoke, U.K.) at 37 °C for 24 h. Both strains were maintained at −80 °C in BHI broth with 20% (v/v) glycerol and subcultured periodically. *Gluconacetobacter xylinus* ATCC 53582, used for the production of bacterial cellulose membranes, was grown at 30 °C for 96 h in Hestrin and Schramm broth (HS) medium composed by glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L, anhydrous sodium phosphate 2.7 g/L, and citric acid monohydrate 1.5 g/L.

2.2. Production of bacteriocins by Lb. sakei 2a

Six liters of MRS broth supplemented with 5.5 g/L glucose and 1.05% (w/w) Tween 20, at an initial pH of 6.28, were inoculated with 1% (v/v) of an overnight culture of *Lb. sakei* 2a, and incubated at 25 °C for 18 h (Malheiros, Daroit, Silveira, & Brandelli, 2010). The pH of the bacterial culture was adjusted to 6.5 with NaOH 10 M and heated at 80 °C for 30 min. Bacterial cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C, washed with 2-(N-morpholin)-ethanesulphonate (MES) buffer (pH 6.5, 5 mmol/L) and suspended in a solution of NaCl (100 mmol/L and adjusted to pH 1.5 with phosphoric acid). The cell suspensions were maintained under magnetic stirring (200 rpm) at 4 °C during 1 h, and then centrifuged at 10,000 x g for 20 min at 4 °C. The supernatants were carefully collected, concentrated by ultra-filtration in an Amicon filter system (Millipore, Bedford MA, U.S.A.) with a 1000 Da molecular weight cut-off membrane (MWCO) (Carvalho et al., 2010), and lyophilized. Prior to use, the lyophilized bacteriocins were resuspended in ultrapure water and sterilized by filtration using 0.22 µm pore diameter filter membranes (Millipore, Bedford MA, U.S.A.).

2.3. Antimicrobial activity assay

The antimicrobial activity of the concentrated bacteriocins was determined as described by Rosa, Franco, Montville, and Chikindas (2002). Briefly, the suspension containing the concentrated bacteriocins was diluted two-fold in sterile distilled water (*viz.* 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128), and 10 µL of each dilution were applied to the surface of Petri plates containing solid bacteriological agar overlaid with 5 mL of semi-solid BHI agar containing 50 µL of a *L. monocytogenes* culture (8 log CFU/mL). The plates were incubated at 37 °C for 24 h, and checked for inhibition zones around the spotted materials. The antimicrobial activity was expressed as activity units (A.U.) per milliliter and corresponded to the reciprocal of the highest dilution producing an inhibition zone.

2.4. Production of bacterial cellulose membranes

Bacterial cellulose (BC) membranes were produced in 24-well plates (JetBiofil® Guangzhou Jet Bio-Filtration Co., Ltd., Guangdong, China), as described by Jozala et al. (2015). Each well of the plates was filled with 1 mL of Hestrin and Schramm broth (HS) containing a suspension of *Gluconacetobacter xylinus* ATCC 53,582 (6 log CFU/mL). The plates were incubated at 30 °C for 96 h, under static conditions, for production of the BC membranes. The resulting membranes were removed from the plates with the aid of sterile tweezers, rinsed with ultrapure water and immediately immersed in a 1 M NaOH solution at 60 °C during 90 min. The BC membranes were thoroughly rinsed with distilled water and sterilized by autoclaving at 121 °C for 15 min.

2.5. Scanning Electron Microscopy analyses

The surface and microstructure of the bacterial cellulose (BC) membranes were monitored by scanning electron microscopy using a Scanning Electron Microscope from JEOL (model JSM 6301 F from JEOL, Tokyo, Japan), equipped with a lower secondary electron in-lens (LEI) detector, operating at 1.0 kV, and coupled with an Ion Sputter-Coater JSC1100 (JEOL, Tokyo, Japan). The microscope was also equipped with a high-resolution CCD camera for the acquisition of digital images. BC membrane samples were sputter coated with colloidal gold/palladium under vacuum, and placed in the microscope chamber. Microphotographs were taken using electron beams with energy and acceleration speeds of 1.0 keV. The samples were randomly scanned and photomicrographed at magnifications of x2500 and x5000.

2.6. Immobilization of bacteriocins in the BC membranes

The bacteriocins produced by *Lb. sakei* 2a were entrapped in the BC membranes in 12-well JetBiofil® plates (Guangzhou Jet Bio-Filtration Co. Ltd., Guangdong, China), by immersion of the membranes in 1 mL of the bacteriocins suspension added to each well. The plates were incubated at 30 °C for 4 h under stirring at 100 rpm. The BC membranes were removed from the wells, placed on sterile filter papers for drying, transferred to the center of Petri plates containing bacteriological agar overlaidwith 5 mL of semi-solid BHI agar containing 50 µL of a *L. monocytogenes* culture (8 log CFU/mL) and incubated at 37 °C for 24 h. A 50 µL-droplet of free (non-immobilized) bacteriocin suspension was also spotted in the center of a Petri plate containing bacteriological agar overlaidwith 5 mL of semi-solid BHI agar containing 50 µL of a *L. monocytogenes* culture (8 log CFU/mL) as control. Growth inhibition halos around the membranes were measured with a digital caliper rule.