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Antimicrobial activity of nanoliposomes co-encapsulating nisin and garlic extract against Gram-positive and Gram-negative bacteria in milk

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ABSTRACT

Nisin and garlic extract (GE) were co-encapsulated into phosphatidylcholine nanoliposomes. The mean diameter and zeta potential of the nanoparticles were 179 nm and −27.7 mV, respectively, with an entrapment efficiency of about 82% and 90% for nisin and GE, respectively. The efficiency of free and encapsulated nisin-GE to control the development of *Listeria monocytogenes*, *Salmonella* Enteritidis, *Escherichia coli* and *Staphylococcus aureus* was assessed over time in whole milk at 37 °C. At such abuse temperature conditions, both free and liposomal nisin-GE resulted a difference of 1–4 log CFU/ml against the strains tested, when compared with free nisin and GE separately. A difference of 5–6 log CFU/ml in viable counts of Gram-positive strains and 3–4 log CFU/ml for Gram-negative bacteria was observed for treatments with nisin-GE when compared to the control. The effect of nisin-GE on *L. monocytogenes* was evaluated under refrigeration (7 ± 1 °C) for up to 25 days. Viable counts for treatments with free and encapsulated nisin-GE were 4–5 log CFU/ml lower than the values reached by the control. Liposome encapsulation of natural antimicrobials with synergistic effect may be important to overcome stability issues and undesirable interaction with food components. The results of this study indicated that nanoliposome-encapsulated nisin-GE has potential as an antimicrobial formulation for food use.

Industrial relevance: There is increased interest for minimally processed foods and natural additives, which agrees with the use of natural antimicrobials as food preservatives. Co-encapsulation of antimicrobials may extend the inhibitory spectrum and effectiveness in controlling food pathogens. The use of nanoliposomes for delivery of natural antimicrobials in dairy products represents an interesting alternative for controlled release of biopreservatives and improvement of food quality and shelf life.

1. Introduction

The consumption of contaminated food can cause serious illness. Literature has reported that food contaminated with pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli, Salmonella* spp. and *Staphylococcus aureus* represents a serious public health risk. In the United States it has been estimated that 76 million people, annually, are victims of foodborne diseases. Known pathogenic organisms cause 9.4 million infections per year and the leading causes of death include foodborne bacteria like *Salmonella* spp. (28%) and *L. monocytogenes* (19%) (Scallan et al., 2011).

L. monocytogenes is a foodborne pathogen of concern to the dairy industry due to its capability to grow under refrigeration temperature (Gandhi & Chikindas, 2007). This bacterium also grows at higher rates due to temperature abuses, which may be encountered during warehouse storage, transportation, retail display, consumer transportation and consumer storage at home. Salmonellosis is an important foodborne illness usually associated with the consumption of foods of animal origin. In many countries, the majority of the recorded foodborne outbreaks are attributed to salmonellosis (Rabsch, Tschäpe, Andreas, & Bäumler, 2001). In addition, food is a main source of *E. coli* infections, which are estimated to account for over 2000 hospitalizations in the United States each year (Scallan et al., 2011). In human and animal intestines, shiga toxin-producing strains of *E. coli* live naturally and are responsible for most food-related *E. coli* infections. *S. aureus* also causes foodborne illness; it can multiply rapidly in food held at room temperature, producing a toxin that is very resistant to heat, refrigeration or freezing (Doyle, 2013). Thus, the control of these bacteria that cause decay and toxicity in food products is important to public health. Although different chemicals can inhibit foodborne pathogens in foods, the increasing demand of the consumers for more natural and safe foods directs to the use of natural antimicrobials.

Garlic (*Allium sativum*) is a widely distributed plant used in all parts of the world not only as a spice but also as a popular medicine for several diseases. A wide range of microorganisms including bacteria, fungi, protozoa and viruses has shown to be sensitive to garlic extracts (Goncagul & Ayaz, 2010; Wallock-Richards et al., 2014). Allicin is considered as the

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main responsible for the biological activities of garlic and is almost exclusively responsible for the antimicrobial activity of freshly crushed garlic (Borlinghaus, Albrecht, Gruhlke, Nwachukwu, & Slusarenko, 2014). Only when the garlic clove is crushed, allicin is formed from alliin by the action of the enzyme alliinase, since the enzyme and substrate are located in different compartments of the clove (Arzanlou & Bohlooli, 2010). Therefore, the inhibitory effect of garlic extracts against *L. monocytogenes* is likely to be expected by allicin.

Nisin is a well-known bacteriocin active against a broad spectrum of Gram-positive foodborne pathogens, including effective growth inhibition of *Bacillus* and *Clostridium* spores. However, its use as a food biopreservative is limited by the lack of effect against Gram-negative bacteria. Moreover, the development of nisin resistance by some pathogens has been described (Zhou, Fang, Tian, & Lu, 2014). Some studies indicate that reduction of bacteriocin resistance in target strains and extended spectrum of activity to Gram-negative bacteria may be achieved by combination with other antimicrobials or other preservation strategies (Branen & Davidson, 2004; Sobrino-López & Martín-Belloso, 2008). Some reports describe the synergistic antimicrobial effect of nisin and other agents, such as lysozyme (Chung & Hancock, 2000), thymol (Ettayebi, Yamani, & Rossi-Hassani, 2000), carbon dioxide (Nilsson et al., 2000), ethanol (Phongphakdee & Nitisinprasert, 2015) and garlic extract (Kim, Choi, Bajpai, & Kang, 2008).

Bacteriocins or bacteriocin-like substances can lose their antimicrobial activity in food products for a variety of reasons. Interference and cross-reactions of the antimicrobial with food constituents, such as protein and fat, are difficult to overcome and often require large amounts of antimicrobial in order to gain significant reductions in the pathogen load in a product (Sobrino-López & Martín-Belloso, 2008). Liposomes are under investigation as micro- and nanocarrier systems for the protection and delivery of bioactive agents and may represent an interesting alternative to meet the challenges in developing healthy foods, which are aimed to reduce the risks of target diseases in a population (Brandelli & Taylor, 2015). In food industry, liposomes can be useful to encapsulate nutraceuticals, flavors, colorings, and antimicrobials (Keller, 2001; Taylor, Davidson, Bruce, & Weiss, 2005).

The aim of the present study was to assess the effect of liposome-encapsulated nisin in combination with garlic extract (GE) against different Gram-positive and Gram-negative foodborne pathogens in milk, as a nanostructure with potential use as biopreservative in the food industry.

2. Materials and methods

2.1. Bacterial strains and media

Listeria monocytogenes ATCC 7644, *Salmonella* Enteritidis SE86, *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 1901 were used as the indicator organisms for antimicrobial activity assays. Strain were maintained on Brain Heart Infusion (BHI; Oxoid, Basingstoke, UK) agar plates at 4 °C, and subcultured periodically. Before each experiment, strains were grown in BHI medium at 37 °C for 24 h in a rotary shaker (125 rpm).

2.2. Preparation of garlic extract (GE) and nisin

Garlic cloves were purchased from a local market in Porto Alegre, Brazil. The samples were prepared by the method of Kim, Choi, and Kang (2007), with some modifications. The collected samples of garlic cloves were washed in running water followed by rinsing in distilled water. For sample preparation, the garlic clove samples were mixed with distilled water at the ratio of 1:1 (w/w) and processed to obtain a garlic extract (GE). The product was sonicated for 15 min and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant of GE was collected, passed through a 0.22-μm membrane (Millipore, Billerica, MA, USA) and stored at 4 °C until further analysis.

Commercial nisin (Nisaplin®) was purchased from Danisco (Vargem Grande, Brazil). Nisin solution was prepared with 0.01 M HCl to obtain a 12.5-mg/ml stock solution, filter sterilized through 0.22 μm membranes, and further diluted in 10 mM phosphate buffer pH 7.0 to reach working concentrations. Nisin is expressed as levels of pure nisin (μg/ml or μg/g). To convert these units to International Units (IU/ml or IU/g) or to equivalent Nisaplin® levels (mg/l, mg/kg), the levels of pure nisin should be multiplied by 40 (Thomas, Clarkson, & Delves-Broughton, 2000).

2.3. Antimicrobial activity assay

Antimicrobial activity was determined essentially as described elsewhere (Motta & Brandelli, 2002). Nisin was diluted to reach (16, 8, 4 μg/ml) and tested alone and combined with GE (1:1 ratio). An aliquot of 10-μl each sample dilution was applied onto BHI agar plates inoculated with a swab submerged in every strain culture (approximately 7 log CFU/ml). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and was expressed as activity unit per milliliter (AU/ml).

2.4. Liposome production by film hydration

Encapsulation of nisin and GE in phosphatidylcholine liposomes was carried out by the thin-film hydration method (Malheiros, Micheletto, Silveira, & Brandelli, 2010a). Briefly, 0.076 g of phosphatidylcholine was dissolved with 15 ml chloroform in a round-bottom flask and the organic solvent was removed by a rotary evaporator to 40 °C until a thin film was formed on the flask walls. Flasks were stored for 24 h in a desiccator to remove traces of organic solvent. The resulting dried lipid film was dispersed by the addition of 5.0-ml nisin-GE mixture (2.5 ml GE plus 2.5 ml nisin at 8 μg/ml, dissolved in 10 mM phosphate buffer pH 7.0). Then, the system was mixed exceeding the phase transition temperature of lipids (55 °C) before sonication using a probe-type sonicator (Unique OF S500, frequency 20 kHz, power 250 W) for 5 min in an ice bath. Liposomes with 10 mM phosphate buffer pH 7.0 were prepared as control. Shortly after preparation the liposomes were filtered using a 0.22-μm membrane.

2.5. Characterization of liposomes

The size of nanoliposomes was determined by dynamic light scattering as described elsewhere (Teixeira, Santos, Silveira, & Brandelli, 2008). The zeta (ζ) potential analyses of liposomes were carried out after dilution of the formulations in 1 mM phosphate buffer pH 7.0 using a Zetasizernano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany). The liposome morphology was observed by transmission electron microscopy (TEM). Samples were dropped onto a Formvar-coated 300 mesh copper grids and was leaved for 1 min, then stained in 25-g/l uranyl acetate for 30 s and dried. Prepared samples were visualized using a transmission electron microscope (JEM-1200, Jeol Ltd., Tokyo, Japan).

2.6. Entrapment efficiency

Encapsulation efficiency (EE) of liposomes was determined using freshly prepared samples. Liposome vesicles were separated from the solution by ultrafiltration using a 10-kDa membrane (Ultracel YM-10; Millipore, Billerica, MA, USA). The concentrations of nisin and allicin (GE) in the filtrate were determined using HPLC analysis. The EE values were calculated separately for nisin and GE (allicin) according to the following equation:

$$\%EE = \frac{[N/A]_s - [N/A]_{filtrate}}{[N/A]_s} \times 100$$