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Assessment of the inhibitory effect of free and encapsulated commercial nisin (Nisaplin®), tested alone and in combination, on *Listeria monocytogenes* and *Bacillus cereus* in refrigerated milk

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ABSTRACT

The antimicrobial effect of free and encapsulated (carrier agent: gum Arabic) commercial nisin (Nisaplin®) against *Listeria monocytogenes* ATCC 7644 (Lm) and *Bacillus cereus* IAL 55 (Bc) in refrigerated (6 ± 1 °C) milk was determined throughout 21 days (d). Skim and whole milk samples containing free and encapsulated commercial nisin (0.25–1.0 mg/L, alone and combined) were contaminated, individually, with Lm or Bc (vegetative cells and spores) and the microorganisms' counts assessed every 3 d. Encapsulated commercial nisin presented characteristic traits of spray-dried products and stable antimicrobial activity under refrigeration (90 days). In both skimmed and whole milk, free and encapsulated Nisaplin® combined (0.5 mg/L each) exhibited the strongest antilisterial effect (d21 − d0; $P < 0.05$), although Lm resistant cells were observed. Free and encapsulated commercial nisin (0.25 mg/L) were highly effective against Bc spores germination and for the pathogen outgrowth inhibition (d21 − d0; $P < 0.05$) in both types of milk, improving the food product microbiological safety.

1. Introduction

In the last years, an increasing demand by consumers for safe natural food products has been observed worldwide (Lucera, Costa, Conte, & Del Nobile, 2012). In this scenario, biopreservation is an interesting alternative to prevent the growth of pathogenic and spoilage microorganisms in foods and to extend the products shelf life (Acuña, Morero, & Bellomio, 2011; Favaro, Penna, & Todorov, 2015). The approach consists in the use of microorganisms, usually lactic acid bacteria (LAB) and/or their metabolites, which have a GRAS (gerenerally recognized as safe) status (Snyder & Worobo, 2014; Wang & Wang, 2014; Zacharof & Lovitt, 2012). Among them, bacteriocins, defined as antimicrobial ribosomally synthesized peptides notably produced by LAB (Cotter, Ross, & Hill, 2013), are of great importance.

Nowadays, nisin, produced by *Lactococcus lactis* subsp. *lactis* strains, is the only bacteriocin used worldwide as a food preservative, recognized as safe by the World Health Organization (WHO) since 1969, and accepted by the American Food and Drug Administration since 1988 (Oshima et al., 2014; Ross, Morgan, & Hill, 2002). Nisin is commercially available under the trade name of Nisaplin®, a heat-stable and water-soluble product (Oshima et al., 2014). The bacteriocin presents bactericidal activity against important Gram-positive pathogens, including *Listeria monocytogenes* and *Bacillus cereus*, and presents bacteriostatic activity against bacterial spores (Oshima et al., 2014).

L. monocytogenes is a psychrotrophic pathogen that causes listeriosis, an infection that exhibits high mortality rates (up to 30%) and, therefore, is considered an important public health concern (Buchanan et al., 2004; Gandhi & Chikindas, 2007; Carpentier & Cerf, 2011). *L. monocytogenes* occurs in a variety of foods, including milk and dairy products (Karthikeyan, Gunasekaran, & Rajendhran, 2015; Santorum, Garcia, Lopez, & Martinez-Suarez, 2012). The selection of nisin-resistant *L. monocytogenes* strains has been evidenced by several researchers (Collins, Curtis, Cotter, Hill, & Ross, 2010; Davies & Adams, 1994; Harris, Fleming, & Klaenhammer, 1991; Malheiros, Sant'Ana, Barbosa, Brandelli, &

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Franco, 2012a; Malheiros, Sant'Ana, Utpott, & Brandelli, 2012b; Mazzotta & Montville, 1997), which demonstrates the need for an adequate/optimized use of the bacteriocin for food biopreservation.

B. cereus is widely distributed in nature due to its spores' high resistance against environmental stresses (Merzougui, Lkhider, Grosset, Gautier, & Cohen, 2014). The microorganism is considered the most important aerobic spore-forming bacterium found in milk and dairy products (Penna, Moraes, & Fajardo, 2002), being responsible for two diseases, the emetic and the diarrheic syndromes. The report of nisin-resistant *B. cereus* strains in scientific literature is not frequent; however, an interesting study performed by Jarvis (1967) showed that the cell-free supernatant from the microorganism had anti-nisin activity. This phenomenon is especially important in foods with extended shelf-life, where low concentrations of resistant microorganisms can multiply and reach high levels, leading to the spoilage of the product or even causing foodborne diseases outbreaks (Zhou, Fang, Tian, & Lu, 2014).

For food application, technologies that enable the production of low-cost, large-scaled, particulate systems are essential (Xiao, Davidson, & Zhong, 2011). In this context, spray drying deserves special attention. The method can be briefly described as the atomization of a solid-fluid mixture, which is submitted to hot air flow (co-current, contra-current or a combination of both) in a drying chamber, leading to the formation of a dried product. Then, the solid particles are separated by a cyclone and finally collected (Guarsallaoui, Roudaut, Chambin, Volley, & Saurel, 2007). Spray drying has been successfully used in the last years for the controlled delivery of a number of food ingredients, including antimicrobial agents, antioxidants, oils, flavorings, bioactives, and nutrients (Dias, Ferreira, & Barreiro, 2015; Garti & McClements, 2012; Murugesan & Orsat, 2012). Gum Arabic is a natural resin composed by polysaccharides and glycoproteins extracted from two Acacia species (*Acacia senegal* and *Acacia seyal*) found in the sub-Saharan Africa territory. The substance is non-toxic, odorless, tasteless, and extensively used as carrier agent for spray drying due to its adequate emulsifying ability and low viscosity under aqueous solution (Guarsallaoui et al., 2007; Righetto & Netto, 2005). Encapsulated (spray-dried) nisin can allow a controlled (delayed) release of the antimicrobial peptide into food matrices and, therefore, possibly minimize the selection of resistant foodborne pathogens.

Until now, to the best of our knowledge, no published researches in peer-reviewed scientific journals have tested the antimicrobial activity of encapsulated nisin using gum Arabic as carrier agent and compared its efficacy with free nisin in refrigerated milk. Therefore, our study aimed to evaluate the inhibitory effect of individual and combined free and encapsulated (spray-dried) commercial nisin (Nisaplin®) on *L. monocytogenes* ATCC 7644 and *B. cereus* IAL 55 (vegetative cells and spores) inoculated in refrigerated UHT (ultra-high temperature) milk, throughout 21 days of storage.

2. Materials and methods

2.1. Microbial strains and preparation of spores' suspension

Pure cultures of *L. monocytogenes* ATCC 7644 and *B. cereus* IAL 55 were kept at −80 °C in BHI (Brain Heart Infusion – Oxoid, Basingstoke, United Kingdom) broth and TSB (Trypticase Soy Broth – Oxoid), respectively, both added of 20% (v/v) of glycerol. For application in milk samples and evaluation of free and encapsulated commercial nisin inhibitory activity, *L. monocytogenes* ATCC 7644 and *B. cereus* IAL 55 (vegetative cells) were grown, respectively, in BHI broth and TSB (2%, v/v; 37 °C for 24 h, aerobic conditions), transferred to fresh BHI broth and TSB (5%, v/v) and aerobically incubated at 37 °C for 24 h. Following, the suspensions were centrifuged (8,000 *g* for 10 min, 4 °C) (RC5C Sorvall Instruments Du Pont, Newtown, CT, United States of America), and the pellets were washed twice with sterile physiological saline solution (NaCl, 0.85% w/v) (Dinâmica, Diadema, SP, Brazil) and ressuspended in appropriated volumes using the same diluent.

For preparation of *B. cereus* IAL 55 spores' suspension, the method used was previously described by Peña et al. (2014). *B. cereus* IAL 55 was cultivated in nutrient broth (Kasvi, Curitiba, PR, Brazil) added of manganese sulfate (Synth, Diadema, SP, Brazil) (10 ppm/L) under incubation at 30 °C for 24 h. Following, the strain was inoculated in Roux bottles (Uniglass, São Paulo, SP, Brazil) containing nutrient agar (Kasvi) added of manganese sulfate (10 ppm/L). The bottles were incubated at 30 °C for 30 days and the sporulation process was evaluated daily using a *malachite green* solution (Synth, Diadema, SP, Brazil) for spores staining. The spores washing procedure was carried out with 90% of sporulated cells using sterile distilled water and centrifugation (1,500 *g* for 20 min, 4 °C) (RC5C Sorvall Instruments Du Pont). The spores' suspension obtained was stored at −20 °C and ressuspended in appropriate volumes of sterile physiological saline solution before inoculation in milk.

2.2. Nisaplin® encapsulation with spray drying method

A commercial preparation of nisin (Nisaplin®, Danisco, Grindsted, Denmark), which contains 2.5% (w/w) of the antimicrobial peptide itself plus NaCl and proteins (solids) from dairy origin was used. Arabic gum (Instantgum BA®, Colloides Naturels Brasil, São Paulo, SP, Brazil) was the carrier agent used as core or bioactive compound at a proportion of 90:10 (w/w) with Nisaplin®. The encapsulation procedure was carried out using the spray drying method with the SD 5.0 equipment (Labmaq, Ribeirão Preto, SP, Brazil). The dispersion obtained was pumped with a peristaltic pump set a flow rate of 10 mL/min. The drying step was done at concurrent mode. The solutions were atomized at room temperature (ca. 23 °C) using compressed air (30 L/min). The temperatures used for air inlet and outlet were, respectively, 130 °C and 110 °C. The powdered product obtained was collected at the bottom of the drying cyclone with an air flow set at 2.5 m/s. Finally, the encapsulated material was stored in a sterile glass bottle covered with aluminum foil, and kept under refrigeration.

2.3. Characterization of the encapsulated material

2.3.1. Particle size and distribution measurement

The size and distribution of the encapsulated particles obtained with spray drying method was determined using a laser light diffraction equipment (Shimadzu Sald-201V, Kyoto, Japan). Thus, samples of approximately 1 g of the powdered material obtained were previously resuspended in MilliQ® water and the results were expressed as mean diameters (μm) ± standard deviation for volume and area.

2.3.2. Morphology

The morphological features of the microencapsulated particles were observed using a scanning electronic microscope (TM300, HITACHI, Tokyo, Japan). The powder was scattered on *double sided carbon tape* and evaluated under 15 Kv acceleration voltage. The metallic coverage of the samples, a procedure normally performed with gold or palladium, was not necessary since the equipment works on low vacuum.

2.4. Maintenance of the biological activity of commercial versus encapsulated nisin under cold storage

The antimicrobial activity of encapsulated Nisaplin® was