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Novel bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* strains with anti-listerial activity isolated from Brazilian artisanal cheese

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ABSTRACT

We isolated and characterized bacteriocin producers *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC from raw milk artisanal cheeses. Their bacteriocins were tolerant to temperatures from 4°C to 100°C and under sterilization conditions (121°C for 15 min). Additionally, the tested bacteriocins remained active after being exposed to pH 2.0 to 10.0 for 2 h. The activity of the bacteriocins was affected by proteolytic enzymes but remained stable after treatment with EDTA, sodium dodecyl sulfate, NaCl, skim milk, and Tween 80. Cell-free supernatants were capable of inhibiting *Listeria innocua* and several strains of *Listeria monocytogenes* obtained from different sources and belonging to different serotypes. When *L. monocytogenes* 211 and *L. monocytogenes* 422 were treated with bacteriocins, growth was completely inhibited over 12 h. Cocultures of bacteriocinogenic strains and *L. monocytogenes* 422 in skim milk showed that *E. hirae* ST57ACC could control the growth of the pathogen in the matrix after 48 h. None of the selected isolates presented positive results on a screening panel for 25 bacteriocin-related genes, however, indicating that both strains might express novel bacteriocins.
Key words: bacteriocin, cheese, *Pediococcus*, *Enterococcus*

INTRODUCTION

Many artisanal cheeses are produced in different regions of Brazil by local small farmers who normally use raw milk in their production (Brant et al., 2007). In general, such cheeses are subjected to ripening, during which their autochthonous lactic acid bacteria (**LAB**) may produce substances with antimicrobial activity, such as organic acids, hydrogen peroxide, diacetyl, CO_2, and bacteriocins (Deegan et al., 2006; Favaro et al., 2015). A variety of LAB can be found in milk and cheese; some have been recognized as producers of bacteriocins and have been used to control pathogens in different food products (Dos Santos et al., 2015; Favaro et al., 2015). *Listeria monocytogenes* is one of the most important foodborne pathogens, because of the high mortality rates associated with listeriosis (Swaminathan and Gerner-Smidt, 2007). The occurrence of *L. monocytogenes* in cheeses is a particular cause for concern, because cheeses are often consumed without further processing, and *L. monocytogenes* has been reported in cheese-manufacturing plants in Brazil (Barancelli et al., 2014).

Some studies in Brazil have demonstrated the presence of bacteriocin producers in raw milk and cheeses, characterizing such products as important sources of novel LAB strains with bacteriocinogenic potential (Ortolani et al., 2010; Moraes et al., 2012; Perin et al., 2012; Tulini et al., 2013; Dos Santos et al., 2015). Bacteriocins are low-molecular-weight polypeptides or proteins that are released extracellularly, genetically encoded, and ribosomally synthesized (Klaenhammer, 1988; de Vuyst and Vandamme, 1994). After binding to surface receptors or entering host cells, bacteriocins can act in the target cell via pore formation, degradation of cellular DNA, and inhibition of peptidoglycan synthesis (de Vuyst and Vandamme, 1994; Heu et al., 2001). Bacteriocins can be used as biopreservatives by adding them directly to a food product, or by adding bacteriocinogenic LAB strains that produce these peptides in situ (Gálvez et al., 1998; Deegan et al., 2006). The latter application is particularly interesting for raw milk cheeses, where adding bacteriocinogenic LAB strains could inhibit spoilage and foodborne pathogens, improving their safety (Ross et al., 2000; Favaro et al., 2015). These qualities suggest a use for novel bacteriocinogenic strains in the dairy industry (Beshkova and Frengova, 2012). However, before using novel LAB strains as biopreservatives in foods, we need to extensively characterize their technological, safety, and virulence features, to ensure their proper use in food (Favaro et al., 2015).

The present study was aimed at isolating bacteriocinogenic LAB from an artisanal cheese produced in

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a specific Brazilian region, and at characterizing the antimicrobial activity of selected LAB strains and their bacteriocins for future use by the dairy and food industries.

MATERIALS AND METHODS

LAB Selection from Artisanal Cheese

Cheese Samples, LAB Isolation, and Identification. Four samples of artisanal cheeses, produced from unpasteurized cow's milk in Nova Venécia, Espírito Santo, Brazil, were homogenized at a 1:10 ratio with saline solution (0.85% NaCl, wt/vol). The obtained suspensions were diluted 10-fold in saline solution, plated on multiple plates containing 10 mL of de Man, Rogosa, and Sharpe agar (**MRS**; Becton, Dickinson and Co., Franklin Lakes, NJ), and incubated at 37°C for 24 h. We used the triple-layer method described by Todorov et al. (2010) and test organisms (*L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lactobacillus sakei* ATCC 15521, and *Enterococcus faecalis* ATCC 19443) to preselect for potential producers of antimicrobial LAB. Briefly, we overlaid colonies on MRS agar plates with a second layer of agar. Then, we took plates with fewer than 50 colonies and overlaid them with 10 mL of semisolid brain heart infusion (**BHI**) agar (Becton, Dickinson and Co.) containing active growing cells from the test organisms (approximately 10^6 cfu/mL) and incubated them at 37°C for 24 h. We selected colonies with evident inhibition zones for isolation and examined them for purity, Gram staining, catalase and oxidase reactions, and the production of antimicrobial compounds, as described in the next section. All cultures (bacteriocinogenic LAB and test organisms) were stored in MRS or BHI plus 20% glycerol (vol/vol) at −80°C.

Total genomic DNA of selected isolates was extracted by using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA). The extracted DNA was quantified by a NanoDrop (Thermo Fisher Scientific, Waltham, MA) and isolates were differentiated by random amplification of polymorphic DNA (**RAPD**)-PCR, using primers OPL-01 (GGCATGACCT), OPL-14 (GTGACAGGCT) and OPL-20 (TGGTGGACCA) and by repetitive element palindromic (**rep**)-PCR (using the primer GTG_5). Amplification reactions were performed according to Todorov et al. (2010) for RAPD-PCR as follows: 45 cycles of 1 min at 94°C, and 1 min at 36°C, followed by an increase to 72°C over 2 min. Extension of the amplified product was at 72°C for 5 min. Conditions for rep-PCR were 5 min at 95°C, 30 cycles of 30 s at 95°C; 30 s at 40°C and 8 min at 65°C, and final extension of 16 min at 65°C, according to Perin and Nero (2014). The amplified products were separated by electrophoresis in 1.4% (wt/vol) agarose gels in 0.5× Tris-acetate-EDTA (**TAE**) buffer at 100 V for 2 h. Gels were stained in TAE buffer containing GelRed (Biotium Inc., Hayward, CA). Banding patterns were analyzed using Gel Compare (version 4.1; Applied Maths, Kortrijk, Belgium).

We identified isolates by amplifying 16S rRNA genes from the genomic DNA with the universal primers 8F (CACGGATCCAGACTTTGATYMTGGCTCAG) and 1512R (GTGAAGCTTACGGYTAGCTTGTTACGACTT) (Felske et al., 1997). We purified the amplified fragments using the QIAquick PCR Purification Kit (Qiagen, Venlo, the Netherlands). Purified fragments were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo, Brazil, and compared with sequences in GenBank using the Basic Local Alignment Search Tool (**BLAST**).

As recommended by Robredo et al. (1999) and Costa et al. (1993), we tested for the presence of genes related to the identification of *Enterococcus durans* (AACAGCTTACTTGACTGGACGC and GTATTGGCGCTACTACCCGTATC) and *Enterococcus faecium* (GCGGTAGCAGCGGTAGACCAAG and GCATTTGGTAAGACACCTACG) using PCR.

Bacteriocin Production Test and Spectrum of Activity. For individual colonies that presented an inhibitory zone in the previous test, we determined the proteinaceous nature of the antimicrobial compound as described by Dos Santos et al. (2015). Selected isolates were grown on MRS at 37°C for 24 h and cell-free supernatant was obtained by centrifugation at 10,000 × *g* for 10 min. The pH of the supernatant was adjusted to 6.0–6.5 with 1 *M* NaOH and treated for 10 min at 80°C. Agar-spot test using *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521, or *E. faecalis* ATCC 19443 at a final concentration of 10^5 cfu/mL was performed, and inhibition zones >2 mm in diameter were considered positive.

Isolates that were identified as bacteriocinogenic were grown as described above. Then, their cell-free supernatants were obtained and treated as described above, and used to assess the inhibitory spectrum of isolates, considering as targets, strains of *L. monocytogenes* (different serological groups) and LAB (Table 1). Culture conditions (growth medium and incubation temperature) and the origin of the test microorganisms used are also specified in Table 1.

Bacteriocin Genes. We subjected DNA from selected strains to PCR reactions to check for the presence of the following bacteriocins: enterocin A, enterocin P, enterocin B, enterocin L50B, pediocin PA-1, nisin, plantaricin W, plantaricin NC8, plantaricin A,