Novel bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* strains with antilisterial 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, CO₂, 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 a specific Brazilian region, and at characterizing the antimicrobial activity of selected LAB strains and their

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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 GTG₅). 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 GTATTGGCGCTACTACCGTATC) 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 $10,000 \times g$ 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, plantaricin S, sakacinGA1, sakacin GA2, sakacin X, sakacin A, sakacin Q, sakacin G, sakacin P, sakacin

Table 1. Inhibition zones presented by bacteriocins produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC, against different target microorganisms¹

Target	Isolate code ²	Origin	Serotype	Inhibition zone (mm)	
				ST57ACC	ST65ACC
<i>Listeria monocytogenes</i>	A70	Round	4b	13	18
	A71	Round	4b	12	17
	A72	Round	4b	12	18
	A73	Round	4b	10	19
	A74	Round	4b	13	20
	A75	Round	4b	12	18
	A512	Chuck	4b	18	26
	A522	Chuck	4b	15	24
	A536	Loin	4b	18	25
	A537	Loin	4b	18	26
	A549	Hand	4b	14	19
	A554	Hand	4b	14	24
	A593	Hand	4b	14	20
	A4	Chuck	1/2b	11	18
	A5	Chuck	1/2b	13	19
	A6	Chuck	1/2b	12	18
	A7	Chuck	1/2b	13	20
	A8	Chuck	1/2b	12	18
	A9	Chuck	1/2b	11	18
	A10	Chuck	1/2b	12	18
	A11	Chuck	1/2c	12	17
	A12	Chuck	1/2c	13	20
	A19	Hand	1/2c	14	18
	A20	Hand	1/2c	11	18
	A45	Chuck	1/2c	14	17
	A46	Chuck	1/2c	14	17
	A78	Chuck	1/2c	12	17
	A79	Chuck	1/2c	14	17
	A80	Chuck	1/2c	12	17
	A81	Chuck	1/2c	12	15
	A82	Chuck	1/2c	12	18
	A83	Chuck	1/2c	12	19
	A139	Table	1/2c	12	18
	A140	Table	1/2c	13	18
	A142	Table	1/2c	12	18
	A170	Hand	1/2c	12	18
	A171	Hand	1/2c	14	15
	A172	Table	1/2c	14	15
	A173	Table	1/2c	13	15
	A186	Table	1/2c	12	18
	A187	Table	1/2c	12	17
	A188	Table	1/2c	15	18
	A189	Table	1/2c	13	18
	A190	Hand	1/2c	11	18
	A191	Hand	1/2c	13	17
	A192	Hand	1/2c	12	17
	A193	Hand	1/2c	12	17
	A210	Hand	1/2c	14	18
	A232	Table	1/2c	12	16
	A233	Table	1/2c	11	20
	A234	Table	1/2c	13	17
	A367	Chuck	1/2c	11	18
	A368	Chuck	1/2c	10	18
	A371	Chuck	1/2c	14	18
	A372	Chuck	1/2c	12	18
	A373	Chuck	1/2c	12	17
	A505	Loin	1/2c	13	17
	A506	Loin	1/2c	12	17
	A507	Loin	1/2c	13	14
	A508	Loin	1/2c	14	16
	A509	Chuck	1/2c	12	14
	A510	Chuck	1/2c	17	22
	A511	Chuck	1/2c	16	24

Continued

Table 1 (Continued). Inhibition zones presented by bacteriocins produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC, against different target microorganisms¹

Target	Isolate code ²	Origin	Serotype	Inhibition zone (mm)	
				ST57ACC	ST65ACC
	A517	Hand	1/2c	18	24
	A518	Hand	1/2c	20	22
	A519	Loin	1/2c	14	20
	A520	Loin	1/2c	16	22
	A521	Chuck	1/2c	17	22
	A523	Chuck	1/2c	15	23
	A524	Chuck	1/2c	15	21
	A525	Chuck	1/2c	14	21
	A526	Loin	1/2c	18	22
	A527	Loin	1/2c	10	21
	A528	Loin	1/2c	19	23
	A529	Loin	1/2c	15	16
	A530	Hand	1/2c	16	22
	A531	Hand	1/2c	13	20
	A532	Hand	1/2c	18	22
	A534	Chuck	1/2c	16	20
	A535	Chuck	1/2c	19	20
	A539	Loin	1/2c	18	21
	A540	Loin	1/2c	13	18
	A550	Hand	1/2c	12	18
	A581	Round	1/2c	13	17
	A582	Round	1/2c	18	18
	D110	Tenderizer	1/2c	15	20
	D58	Tenderizer	1/2c	14	18
	D121	Grinder	1/2c	14	18
	D210	Tenderizer	1/2c	8	14
	D123	Grinder	1/2c	14	16
	D206	Grinder	1/2c	0	0
	D184	Knife	1/2c	12	18
	D136	Hand	1/2c	15	19
	L101	Chicken		12	18
	L211	Chicken		10	18
	L409	Chicken		0	0
	L703	Chicken		12	18
	L422	Chicken		18	21
	L506	Chicken		19	22
<i>Listeria innocua</i> D102		Table		17	20
<i>Listeria innocua</i> D107		Grinder		14	20
<i>Enterococcus faecium</i> 159		Goat cheese		0	14
<i>Enterococcus faecium</i> ATCC 19443		Reference		10	16
<i>Enterococcus faecium</i> ET05		Salmon		0	0
<i>Enterococcus faecium</i> ET12		Salmon		0	0
<i>Enterococcus faecium</i> ET88		Salmon		0	18
<i>Enterococcus mundtii</i> CRL35		Cheese		15	15
<i>Enterococcus</i> spp. 13Lb1		Goat milk		0	15
<i>Enterococcus</i> spp. 1En2		Goat milk		0	15
<i>Lactobacillus curvatus</i> ET06		Salmon		0	0
<i>Lactobacillus curvatus</i> ET30		Salmon		0	0
<i>Lactobacillus curvatus</i> ET31		Salmon		0	0
<i>Lactobacillus delbrueckii</i> ET32		Salmon		0	0
<i>Lactobacillus fermentum</i> ET35		Salmon		0	0
<i>Lactobacillus plantarum</i> LP08AD		Donkey milk		0	0
<i>Lactobacillus sakei</i> ATCC 15521		Reference		15	20
<i>Lactobacillus sakei</i> MK02R		Rocket salad		0	0
<i>Lactobacillus sakei</i> ST152Ch		Salpicao		0	0
<i>Leuconostoc mesenteroides</i> UCV		Baby food		0	0
<i>Pediococcus acidilactici</i> ET34		Salmon		0	0
<i>Pediococcus pentosaceus</i> A3		Cheese		0	0
<i>Streptococcus infantarius</i> L13		Goat milk		20	24
<i>Streptococcus mucosae</i> L21		Goat milk		0	0

¹*Listeria* spp. were cultured in brain heart infusion agar at 37°C. Lactic acid bacteria were cultured in de Man, Rogosa and Sharpe broth at 37°C.²Isolate code refers to laboratory identification of isolates.

Table 2. Effect of proteolytic enzymes, temperature, and pH on activity of the bacteriocin produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC¹

Treatment	<i>E. hirae</i> ST57ACC	<i>P. pentosaceus</i> ST65ACC
Enzyme		
Proteinase K	—	—
Trypsin	—	—
Pepsin	—	—
α-Chymotrypsin	—	—
Protease type XIV	—	—
α-Amylase	+	+
Lipase	+	+
Temperature (time)		
4–100°C (60 min)	+	+
121°C (15 min)	+	+
pH		
2.0–6.0	+	+
8.0	+	+
10.0	+	+

¹Where — represents loss of bacteriocin activity; + represents stable activity.

Tα, sakacin Tβ, lactacin 481, lactacin A, lactacin M, lactacin 3147, lactacin GQ, and leuconocin 972. We prepared the PCR reactions using primers at a concentration of 10 pmol/μL and the conditions described previously (Aymerich et al., 1996; Cintas et al., 1998; Du Toit et al., 2000; Barbosa et al., 2014; Furtado et al., 2015; de Souza Barbosa et al., 2015; Favaro et al., 2015; Barbosa et al., 2016). For each PCR reaction, we adjusted the annealing temperatures according to the specifications of the primers used. The amplified products were separated by electrophoresis on agarose gels in 0.5× TAE buffer. We stained agarose gels in 0.5× TAE buffer in the presence of GelRed (Biotium Inc.) and visualized them on an LPIX transilluminator (Loccus Biotecnologia, São Paulo, Brazil).

Bacteriocin Production by Selected LAB

We treated the cell-free supernatants from the selected isolates, obtained as described above, with 0.1 mg/mL (final concentration) of selected proteolytic enzymes, α-amylase, and lipase (Table 2) at 37°C for 1 h, and then deactivated enzymes by thermal treatment at 98°C for 3 min. We determined antimicrobial activity as described above against *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521, and *E. faecalis* ATCC 19443. Cell-free supernatants with no enzyme treatments served as controls.

We also tested cell-free supernatants for the effects of pH, temperature, and enzymes on the stability of the antimicrobial substance(s) produced (Table 2), as described by Todorov and Dicks (2006). The effect of pH on the bacteriocins was determined by adjusting

the cell-free supernatants from pH 2.0 to 10.0 with sterile 1 N HCl or 1 N NaOH. After 2 h of incubation at 30°C, the samples were readjusted to pH 6.5 and the activity was tested as described before. The effect of temperature on the bacteriocins was tested by cooling or heating the cell-free to 4, 20, 25, 30, 37, 45, 60, 80, and 100°C, respectively. Residual bacteriocin activity was tested after 15, 30, 60, and 120 min at each of these temperatures as previously described. Additionally, the activity was tested after exposing the bacteriocins to 121°C for 15 min. The effect of detergents and chemicals (EDTA, SDS, NaCl, skim milk, Tween 80) on the bacteriocins was assessed by adding 1% (wt/vol, final concentration) to the cell-free supernatants. Untreated cell-free supernatants and surfactants at the latter concentrations served as controls. All treated supernatants were incubated at 37°C for 3 h, and then we tested activity as previously described. For these tests, we used *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521, and *E. faecalis* ATCC 19443 as target organisms and non-treated cell-free supernatants as controls.

Inhibitory Activity of Bacteriocins

Effect of Cell-Free Supernatant on Actively Growing Cultures. One hundred milliliters of BHI broth was inoculated with 1% (vol/vol) overnight culture of *L. monocytogenes* 211 and *L. monocytogenes* 422, respectively, and incubated the culture at 37°C for 3 h. Then, we added 20 mL of filter-sterilized cell-free supernatant containing bacteriocins of selected LAB to the culture and recorded changes in optical density at 600 nm every hour for 12 h. We recorded the cell counts of *L. monocytogenes* 211 and *L. monocytogenes* 422 by pour-plating aliquots of the cultures on BHI supplemented with 2% agar and incubated at 37°C for 48 h. Results were expressed in colony-forming units per milliliter.

Coculture with *Listeria monocytogenes* in Milk. After optimizing bacteriocin production, we individually inoculated overnight cultures of selected bacteriocinogenic LAB strains onto 10% (wt/vol) reconstituted skim milk at concentrations of 2.0% (vol/vol), as equivalents of approximately 10⁷ cfu/mL. We incubated mixed cultures at 37°C for 48 h, and at 3 h intervals, we obtained aliquots and measured pH (W3B pH meter; BEL, Piracicaba, SP, Brazil). We obtained *Listeria* counts by surface plating aliquots onto Chromogenic *Listeria* agar (Oxoid, Basingstoke, UK) and incubating at 35°C for 48 h. We obtained LAB counts by pour-plating aliquots on MRS agar and incubating at 37°C for 24 h. Microbial counts were expressed as log colony-forming units per milliliter. In parallel, we

tested aliquots for the presence of bacteriocins, as described above, using *L. monocytogenes* 422 as the target organism.

RESULTS AND DISCUSSION

LAB Selection from Artisanal Cheese

Cheese production is a traditional part of the Brazilian dairy industry, and it includes artisanal practices. The safety of artisanal cheeses is an important issue, because they are widely consumed by the public, and alternative methods to ensure their safety are needed. The Brazilian Ministry of Health and the Brazilian Ministry of Agriculture, Livestock, and Food Supply have established production, quality, and safety standards for these cheeses to ensure consumer health (Brazil, 2001, 2011). Different quality-assurance tools can be adopted by dairy industries to ensure the safety and quality of end products (Cusato et al., 2013, 2014), and bacteriocinogenic strains, bacteriocins, or both, can be among these tools.

The cheese samples included in this study presented LAB populations ranging from 7.36×10^4 to 2.21×10^6 cfu/g. However, after adding the agar overlay to the target organisms, only 1 sample presented colonies with inhibitory activity. Following this step, we selected 84 colonies due to their inhibitory activity and tested them for the production of bacteriocins. Based on the results obtained, 2 isolates, both gram-positive cocci, presented bacteriocinogenic activity, and were catalase- and oxidase-negative.

Based on the RAPD-PCR and rep-PCR profiles, we determined that the isolates were different strains. After 16S rRNA analysis, we identified them as *Enterococcus hirae* (ST57ACC) and *Pediococcus pentosaceus* (ST65ACC). Because 16S rRNA analysis does not usually provide accurate data for identifying *Enterococcus* spp. strains (Costa et al., 1993; Robredo et al., 1999), we performed additional PCR targeting specific genes for *Enterococcus durans* and *Enterococcus faecium*, generating negative results and confirming our finding that isolate ST57ACC was *E. hirae*.

The presence of *Enterococcus* spp. in raw milk products is not surprising, particularly in cheeses. Different *Enterococcus* spp., including bacteriocin producers, have previously been isolated from artisanal cheeses from different regions of Brazil, as well as worldwide (Vera Pingitore et al., 2012; Rehaïem et al., 2012; Tuncer et al., 2013; Dos Santos et al., 2015; Kırmacı et al., 2015).

The presence of *P. pentosaceus* was surprising, given that strain ST65ACC could not use lactose in its metabolic pathway (data not shown). However, *Pediococcus* spp. have been identified in many cheeses from different

regions (Gerasi et al., 2003; Gurira and Buys, 2005; El-Baradei et al., 2007). Carafa et al. (2015) characterized the LAB from traditional Mountain Malga cheese, which is made in small farms in Italy using raw cow milk and spontaneous fermentation, and they found that *P. pentosaceus* was among the dominant species. In addition, the antimicrobial activity of *Pediococcus* spp. isolated from cheeses has already been reported, and their inhibitory activity against *Listeria* species have been previously demonstrated (Gurira and Buys, 2005; Altuntas et al., 2014).

Based on screening for the presence of different bacteriocin genes, none of the selected isolates presented positive results for the targets considered in this study. These findings indicate that both strains likely expressed novel bacteriocins, different from those in the gene screening panel. The presence of genes for bacteriocin production is often incorrectly interpreted as evidence for the expression of these genes.

Bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC inhibited all tested *L. monocytogenes* strains (99 in total) and 2 strains of *Listeria innocua* used in this study (Table 1). It is important to underline that the *L. monocytogenes* tested were from different serological groups and isolated from various ecological niches, including food-processing environments. Both of the studied bacteriocins expressed only limited activity against the LAB tested as sensitive strains. These results are relevant for food industries, because they indicate that the bacteriocins produced by these strains were not active against potential starter cultures added to fermented products, highlighting the bacteriocins' potential as biopreservatives.

Bacteriocin Production by Selected LAB

After contact with proteolytic enzymes, both cell-free supernatants lost activity, pointing to the protein nature of the antimicrobial compounds produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC (Table 2). Moreover, the presence of α -amylase or lipase did not affect bacteriocin activity (Table 2), indicating that their antimicrobial characteristics were not related to the presence of a glycolytic or lipolytic moiety of the bacteriocin molecule.

Both of the studied bacteriocins demonstrated significant stability after treatment for up to 2 h at different temperatures (Table 2). Similar findings have been described for other bacteriocins produced by *Enterococcus* spp. and *Pediococcus* spp. (Gurira and Buys, 2005; Altuntaş et al., 2014; Cavicchioli et al., 2015). The thermal stability of bacteriocins is considered to be a positive feature, allowing them to be applied for biopreservation in products that will be exposed to

Figure 1. Effect of bacteriocins produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC on growth of (A) *Listeria monocytogenes* 211, and (B) *L. monocytogenes* 422, by optical density at 600 nm. ● = *L. monocytogenes* growth (control, no bacteriocins added); ■ = *L. monocytogenes* growth with bacteriocin from *E. hirae* ST57ACC; □ = *L. monocytogenes* growth with bacteriocin from *P. pentosaceus* ST65ACC. Arrows indicate the addition of cell-free supernatant containing bacteriocins.

pasteurization or other thermal treatments to ensure the safety and extended shelf life of food products. For potential medical (human and veterinary) applications of bacteriocins, sterile applied products are necessary, and the stability of bacteriocins after exposure to sterilization processes is essential.

When cell-free supernatants produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were corrected to different pH values (from 2.0 to 10.0), we observed only a low-level reduction of antimicrobial activity at pH 9.0 and 10.0 for both bacteriocins. In the rest of the experiments, we observed no changes in antimicrobial activity compared with the untreated cell-free supernatants. Such results were not unusual, because most known bacteriocins, including nisin, are known to be stable at both neutral and acidic pH (Barbosa et al., 2014; de Souza Barbosa et al., 2015).

We observed that EDTA, SDS, NaCl, skim milk, and Tween 80 had no effect on the stability of bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, as reported by other authors (Gálvez et al., 1998; Ivanova et al., 2000; Todorov and Dicks, 2005).

Inhibitory Activity of Bacteriocins

The growth of *L. monocytogenes* 211 and *L. monocytogenes* 422 treated with cell-free supernatants containing bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, was completely inhibited over a period of 12 h (Figure 1). Over the same period, controls of *L. monocytogenes* 211 and *L. monocytogenes* 422 reached growth of approximately 10^7 cfu/mL (Figure 1). The cell count of *L. monocytogenes* 211 and *L. monocytogenes* 422 in the treated samples was lower than the detection threshold.

When both strains were cultured in skim milk at 25, 30, and 37°C, we recorded similar growth results at 12, 24, and 36 h. However, bacteriocin production was higher at 37°C (data not shown). We considered these results to be a preliminary test for choosing the ideal temperature and incubation time for the study of bacteriocin production by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC in skim milk. Results for cocultures of selected LAB strains and *L. monocytogenes* in milk are presented in Figure 2. Cell numbers of *L. monocytogenes* 422 decreased from log 4.37 cfu/mL (time 0) to log 1.48 cfu/mL at 36 h and to undetectable levels after 48 h, highlighting the high antilisterial activity of bacteriocins produced by *E. hirae* ST57ACC in these matrices. *Enterococcus* spp. are important in the field of food microbiology, mainly because of their contribution to different fermentation processes; their functionality has been observed in dairy and meat products (Giraffa, 2003; Vera Pingitore et al., 2012). According to Achemchem et al. (2006), coculture experiments with *E. faecium* F58 and *L. monocytogenes* in goat's milk did not eliminate the pathogen, but did promote reductions in its populations of log 1 to 4. In addition, when *L. monocytogenes* contamination was reported after 12 h of *E. faecium* F58 growth in milk, the pathogen was eliminated after 130 h of coculture. In another experiment, *E. faecalis* EJ97 was also able to produce enterocin during cocultivation with *L. monocytogenes* in skim milk; however, its capacity to control the pathogen was limited to populations of 10^3 cfu/mL or lower (García et al., 2003). In our experiment, we demonstrated that *E. hirae* ST57ACC could eliminate *L. monocytogenes* in milk after 48 h of inoculation, demonstrating its potential for controlling this pathogen in milk (Figure 2).

Regarding the counts of control and cocultures of *P. pentosaceus* ST65ACC and *L. monocytogenes* 422, we

Figure 2. Growth curves and bacteriocin production of (A) *Enterococcus hirae* ST57ACC, and (B) *Pediococcus pentosaceus* ST65ACC cocultured with *Listeria monocytogenes* 422 in skim milk 10%. ● = growth (log cfu/mL) of (A) *E. hirae* ST57ACC and (B) *P. pentosaceus* ST65ACC; ▲ = growth of *L. monocytogenes* 422 (log cfu/mL) without bacteriocin added; Δ = growth of *L. monocytogenes* 422 (log cfu/mL) with bacteriocin added; gray bars = bacteriocin activity (arbitrary units/mL).

had the opposite results. The cell numbers of *L. monocytogenes* 422 increased from log 3.94 cfu/mL (time 0) to log 7.26 cfu/mL after 36 h and log 7.11 cfu/mL after 48 h (Figure 2). Therefore, *P. pentosaceus* did not show

antilisterial activity in the tested milk matrix, probably due to the limitation of the fermentation of lactose and low levels of bacteriocins detected. However, the inhibitory profile of *P. pentosaceus* in other products (such

as meat or fermented fruit products) may be different, and should be evaluated in future studies.

Cheese production involves a combination of microbiological and biochemical processes that can be simplified as precipitation of milk proteins and maturation. Starter and non-starter cultures have specific roles in the organoleptic characteristics of cheeses (Ross et al., 2000), but special attention has been given to their antimicrobial potential to prevent foodborne pathogen growth and spoilage (Favaro et al., 2015). Considering these goals, the search for autochthonous LAB from different cheeses that are capable of producing antimicrobial substances as bacteriocins may be an answer for controlling spoilage and pathogenic microorganisms, ensuring the quality and safety of dairy products. Many examples of bacteriocinogenic LAB isolated from cheeses have already been reported (Ross et al., 2000; Dos Santos et al., 2015; Favaro et al., 2015). However, selecting novel bacteriocinogenic LAB strains may be a way to find alternatives for biopreservation in the dairy and other food industries (Beshkova and Frengova, 2012). Still, these LAB strains need to be carefully examined for their technological and biopreservation properties, and for their safety, considering virulence factors, antibiotic resistance, and the production of biogenic amines.

CONCLUSIONS

We isolated bacteriocinogenic *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC from raw milk cheese, showing strong antilisterial activity with bacteriocins resistant to conditions that can be found in food processing. These isolates could affect the development of new industrially important cultures to control *L. monocytogenes* in the manufacture of food products. As these peptides can be new bacteriocins, future studies are required to identify them.

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