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Potential Control of *Listeria monocytogenes* by Bacteriocinogenic *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC Strains Isolated From Artisanal Cheese

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Abstract
Bacteriocinogenic *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC strains, previously isolated from artisanal cheese, were evaluated for their safety with the aim to determine whether they could be used as beneficial strains, especially in the control of *Listeria monocytogenes*. Both strains survived simulated gastrointestinal conditions and showed high levels of auto- and co-aggregation with *L. monocytogenes*, although the hydrophobicity of cells varied. Using the agar-spot test with 33 commercial drugs from different groups, only anti-inflammatory drugs and drugs containing loratadine and propranolol hydrochloride were able to affect the growth of the tested strains. Both strains were resistant to 3 out of 11 antibiotics tested by the disc diffusion method, and low frequencies of antibiotic resistance-encoding genes were observed by PCR analysis. Tested strains neither presented biogenic amine-related genes nor produced these substances. Aside from some antibiotic resistance characteristics, the tested strains were considered safe as they lack other virulence-related genes. *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC both presented beneficial properties, particularly their ability to survive gastrointestinal conditions and to aggregate with *L. monocytogenes*, which can facilitate the elimination of this pathogen. Further studies should be conducted to better understand these interactions.

Keywords Antibiotic resistance · Beneficial strains · Bacteriocins · LAB · *Listeria monocytogenes*

Introduction

Human listeriosis is one of the most severe foodborne diseases worldwide. The infection is caused by *Listeria monocytogenes* and usually affects at-risk populations with impaired cell-mediated immunity, including elderly people, pregnant woman, newborns and immunocompromised adults [1]. However, healthy children and adults are occasionally infected, and it is estimated that transient asymptomatic intestinal carriage of *Listeria* occurs twice per year in healthy adults [2]. Different clinical manifestations have been noted, including gastroenteritis, septicaemia, meningitis or meningoencephalitis and abortion [3]. Listeriosis morbidity can be considered low worldwide, but its mortality rates reach up to 30%, highlighting this disease as a public health concern [4].

The main contamination route for *L. monocytogenes* transmission is by ingestion of contaminated food, including raw milk and dairy products, vegetables, raw seafood, meat and ready-to-eat products [3]. *L. monocytogenes* is a widespread pathogen in nature, and due to its ability to survive and multiply in harsh conditions, it can persist in food handling environments, reaching ready-to-eat products by cross-contamination during or after processing steps [5, 6]. These characteristics make the rigorous control of *L. monocytogenes* essential to prevent its multiplication in food products and, consequently, reduce the transmission risk of this pathogen.

The development of biopreservation technologies utilising lactic acid bacteria (LAB) and/or their metabolites represents an additional hurdle in the protection of food against microbial contamination as these bacteria produce several anti-microbial substances including organic acids, hydrogen peroxide and

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bacteriocins [7]. Bacteriocins are ribosomally synthesised antimicrobial peptides produced by Gram-negative and Gram-positive bacteria [8]. These bacteriocins can act as antagonists, most often against closely related organisms [7, 9, 10]. The control of *L. monocytogenes* by LAB and their bacteriocins has been widely studied [11–13]. Beyond their direct influence on protecting against microbial contamination of food, several studies have demonstrated that bacteriocinogenic LAB have the potential to control infections due to their effect on microbial interactions in the human intestinal environment [9, 14]. However, the use of LAB for this purpose requires confirmation of the safety of particular strains, as well as their ability to survive the harsh gastrointestinal conditions and their virulent potential, in order to ensure the safety of consumers.

Enterococcus and *Pediococcus* species are commonly part of the microbiota of fermented foods due to their important role in the technological aspects of cheese maturation and contribution to the sensorial characteristics of these foods [15, 16]. *Enterococcus* and *Pediococcus* are known to be able to produce different anti-microbial substances. They are considered a biopreservative tool to control the growth of spoilage-related and pathogenic bacteria, especially *L. monocytogenes* [17, 18].

In a previous study, *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC were described as bacteriocinogenic strains with strong activity against *Listeria* spp. from different serogroups [19]. Considering the anti-microbial potential of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, this study aimed to characterise these strains in regard to their potential beneficial roles as tools to control *L. monocytogenes* development and colonisation, and also to characterise their virulence.

Materials and Methods

Strains and Culture Conditions

E. hirae ST57ACC and *P. pentosaceus* ST65ACC used in the present study were isolated from artisanal cheeses, and were previously characterised as bacteriocinogenic by Cavicchioli et al. [19]. *L. monocytogenes* L711, L422 and L637 were used as target strains in the present study.

Stock cultures were maintained in de Man, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA), and *L. monocytogenes* isolates were maintained in brain-heart infusion (BHI) (Oxoid Ltd., Basingstoke, England). All cultures were kept at − 20 °C in MRS broth or BHI supplemented with 20% (*w*/*v*) glycerol. The isolates were streaked onto their respective agar media to obtain single colonies. These were cultivated overnight at 37 °C in each respective broth for use in subsequent tests.

Colonisation Ability

Resistance to Conditions Simulating the Gastrointestinal Tract

Cultures of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were diluted until a turbidity similar to tube 1 from the McFarland scale (approximately 3×10^8 CFU/mL) and diluted 10-fold in NaCl 0.85% (*w*/*v*). Selected dilutions were plated on MRS agar (BD) and incubated at 37 °C for 48 h, when colonies were enumerated and the results expressed as log CFU/mL at time 0. Resistance to the simulated gastrointestinal conditions was assessed according to Santos et al. [20]. Briefly, 6-mL aliquots of test culture cell suspensions were diluted in 10 mL of an artificial gastric fluid consisting of a sterile electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$; pH 2.5) supplemented with 0.3% pepsin (all chemicals from Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37 °C under continuous agitation (150 rpm). Populations of both tested cultures were enumerated as described above; then, 2 mL of gastric-simulated cultures was diluted in 8 mL of an artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salts and 0.1% pancreatin (all chemicals from Sigma-Aldrich) and incubated for 3 h at 37 °C under continuous agitation (150 rpm), after which populations of tested cultures were enumerated as described above.

The survival rates (SR) of tested strains after gastric- and enteric-simulated passage were calculated considering both steps using the equation provided by Santos et al. [20] based on their log populations: SR (%) = [log CFU N/log CFU N_0] × 100, where N_0 and N are the population values before and after the assay, respectively. The tests were performed in duplicate.

Cell Surface Hydrophobicity

Cell surface hydrophobicity of the tested strains was assessed as described by Santos et al. [21]. Briefly, overnight cultures of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were centrifuged at 7000×*g* for 5 min at 4 °C, washed twice with NaCl 0.85% (*w*/*v*) and resuspended in the same solution until a turbidity of 1.0 was reached, measured by the optical density (OD) at λ = 560 nm (Spectrophotometer UV-M51; BEL Engineering s.r.l., Monza, Italy), which corresponded to the absorbance at time 0 (A0). N-hexadecane (Sigma-Aldrich) was added to the cell suspension at a ratio of 1:5, and the mixture was homogenised for 2 min by vortex. After 1 h of incubation at 37 °C, the OD of the aqueous layer (A) was measured at λ = 560 nm. Cell surface hydrophobicity was calculated according to the equation provided by Santos et al. [21]: %H = [(A0 − A)/A0] × 100, where A0 and A are the absorbance values before and after extraction with the organic solvent, respectively. This test was performed in duplicate.

Auto-Aggregation and Co-Aggregation With *L. monocytogenes*

For the auto-aggregation test, *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were grown in MRS broth (BD) for 24 h at 37 °C, centrifuged at 7,000×*g* for 10 min at 20 °C, then washed twice with 0.85% NaCl (*w*/*v*). Cultures were then diluted in 0.85% NaCl (*w*/*v*) until a turbidity of 0.3 was reached, with the OD measured at λ = 660 nm (OD_0; UV-M51, BEL). Cultures were incubated at 37 °C for 60 min, then centrifuged at 300×*g* for 2 min at 20 °C, after which the ODs at λ = 660 nm were recorded (OD_{60}; UV-M51, BEL). Auto-aggregation was determined based on the equation proposed by Todorov et al. [22]: %auto-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$. Experiments were conducted in duplicate.

Co-aggregation was assessed with *L. monocytogenes* L711, L422 and L637. Aliquots of cultures of the target strains were transferred to BHI (Oxoid) and incubated at 37 °C for 24 h. *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC cells were obtained as described above and diluted in 0.85% NaCl (*w*/*v*) until a turbidity of 0.3 was reached, measured by the OD at λ = 660 nm (UV-M51, BEL). Then, 750 μL of each bacteriocinogenic LAB suspension was mixed with 750 μL of prepared cultures of each target strain and the OD was measured at λ = 660 nm (OD0; UV-M51, BEL). Combined cultures were incubated at 37 °C for 60 min, and then, the supernatant was obtained by centrifugation at 300×*g* for 2 min at 20 °C and the OD was measured at λ = 660 nm (OD_{60}; UV-M51, BEL). Co-aggregation was calculated based on the equation proposed by Todorov et al. [22]: %co-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$. Experiments were conducted in duplicate.

Effect of Commercial Drugs on Growth of the Bacteriocinogenic Strains

The growth of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC in the presence of 33 commercial drugs from 19 distinct groups was tested according to Paula et al. [23]. Drugs were solubilised in Milli-Q water (Merck KGaA, Darmstadt, Germany) 24 h prior to the tests in order to obtain solutions at different concentrations (Supplementary Table 1). Overnight cultures of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were inoculated in 15 mL MRS agar (BD) to reach a final concentration of 10^6 CFU/mL. After solidification, 10 μL of the solubilised drugs was spotted on to the surface of the agar and incubated at 30 °C for 24 h. Inhibition zones around the spotted drugs were checked, and those which presented inhibition zones larger than 2 mm in diameter were tested to determine the minimal inhibitory concentration (MIC). For this test, a serial two-fold dilution of the drugs was prepared in Milli-Q water (Merck), and 10 μL of each dilution was spotted on to the surface of the MRS agar plates, which were prepared as previously described. The plates were incubated at 30 °C for 24 h and observed for the presence of inhibition zones around the spotted drug. The MIC corresponded to the highest dilution that resulted in an inhibition halo at least 2 mm in diameter.

Safety Characteristics

Antibiotic Resistance

E. hirae ST57ACC and *P. pentosaceus* ST65ACC were tested to assess their susceptibility to 11 antibiotics (ampicillin, penicillin G, oxacillin, clindamycin, erythromycin, imipenem, rifampicin, chloramphenicol, tetracycline, trimethoprim/sulfamethoxazole and vancomycin), selected based on the recommendations of the Clinical and Laboratory Standards Institute, as well as the concentrations tested by the disc diffusion method [24]. Cultures in the exponential phase in MRS broth (BD) were diluted in 0.85% NaCl (*w*/*v*) until a turbidity similar to tube 0.5 of the McFarland scale was reached. Diluted cultures were swabbed on to the surface of the Mueller-Hinton agar (Oxoid) and antibiotic discs (Oxoid) were added (three discs per plate). After incubation at 37 °C for 24 h, halos around each anti-microbial disc were measured and the results were classified as susceptible or resistant according to the diameters of the halos [24].

Phenotypic Virulence Factors

To identify the virulence activity related to haemolysis and the production of hydrolytic enzymes (lipase, gelatinase and DNAse), cultures of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were subjected to phenotypic tests according to Perin et al. [25].

Haemolytic activity was assessed by streaking the cultures onto trypticase soy agar (Oxoid) supplemented with 5% (*v*/*v*) of defibrinated horse blood and incubating at 37 °C for 24 h. The pattern of haemolysis exhibited by each isolate was classified according to the degree of destruction of erythrocytes as total or β-haemolysis, partial or α-haemolysis and absent or γ-haemolysis.

Lipase production was assessed by spotting 1 μL of the 18-h cultures on to plates containing Luria-Bertani (LB) agar (10% tryptone, 5% yeast extract and 10% NaCl; pH 7.0) supplemented with 0.2% (*w*/*v*) $CaCl_2$ (Sigma-Aldrich) and 1% (*v*/*v*) Tween 80 (Sigma-Aldrich) and incubated at 37 °C for 48 h. The formation of clear halos around the colonies was recorded as lipase production.

Gelatinase production was identified by spotting 1-μL aliquots of the 18-h cultures on to the surface of LB agar supplemented with 3% (*w*/*v*) of gelatine (BD) and incubated at 37 °C for 48 h. After incubation, the plates were maintained at

4 °C for 4 h, after which the hydrolysis of gelatine was recorded by the formation of opaque halos around the colonies.

DNAse activity was assessed by spotting 1 μL aliquots of the 18-h cultures on to the surface of DNAse methyl green agar (BD) and incubating at 37 °C for 48 h. Positive results were identified by the formation of clear halos around the colonies. All tests were performed in duplicate.

E. hirae ST57ACC and *P. pentosaceus* ST65ACC were also subjected to the protocol described by Bover-Cid, Holzapfel [26], to detect the production of biogenic amines. The bacteriocinogenic strains were cultivated in MRS broth (BD) supplemented with 0.005% (*w*/*v*) pyridoxal-5-phosphate (Sigma-Aldrich), to which each of the biogenic amine precursors, including tyrosine free base (for tyramine), histidine monohydrochloride (for histamine), ornithine monohydrochloride (for putrescine) and L-lysine (for cadaverine; all from Sigma-Aldrich), were added at 0.1% (*w*/*v*) to induce the production of decarboxylase. After five consecutive passages in these media, each culture was streaked on to MRS decarboxylase agar supplemented with 1% (*w*/*v*) of each amino acid precursor of the biogenic amines, performed as described above and according to Joosten, Northolt [27]. The plates were incubated at 37 °C for 4 days, and positive results were recorded by a change in colour of the medium from yellow to purple. Tests were performed in duplicate.

Genotypic Virulence Determinants

Total DNA from *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were obtained using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. All PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, CA, USA). Both isolates were tested for the presence of 41 genes related to safety, including anti-microbial resistance, virulence and biogenic amine production (Supplementary Table 2). The amplified products were separated by electrophoresis in 1.5% (*w*/*v*) agarose gels in 0.5× TBE buffer containing GelRed (Biotium Inc., Hayward, CA, USA) at 100 V for 2 h and visualised on a LPIX transilluminator (Loccus Biotecnologia, São Paulo, SP, Brazil).

Results

Colonisation Ability

Both tested bacteriocinogenic strains were able to resist and survive when subjected to simulated gastric and/or intestinal conditions, with the SR of their log populations ranging from 79.1% (*E. hirae* ST57ACC after gastric and enteric conditions) to 92.6% (*P. pentosaceus* ST65ACC after gastric condition; Table 1). The population of *E. hirae* ST57ACC decreased from 7.8 to 6.2 log CFU/mL, whilst *P. pentosaceus* ST65ACC decreased from 7.6 to 6.2 log CFU/mL, demonstrating their resistance to the simulated gastrointestinal conditions (Table 1).

The results obtained for cell surface hydrophobicity, auto-aggregation and co-aggregation with *L. monocytogenes* strains are presented in Table 2. Both bacteriocinogenic strains presented similar results for cell hydrophobicity of about 40%, but the aggregation rates varied according to strain, where *E. hirae* ST57ACC aggregation rates varied from 17.3% (with *L. monocytogenes* L711) to 51.6% (auto-aggregation), whilst *P. pentosaceus* ST65ACC aggregation rates varied from 48.1% (with *L. monocytogenes* L711) to 71.8% (auto-aggregation; Table 2). For both strains, *L. monocytogenes* L711 presented a lower aggregation capacity when compared to *L. monocytogenes* L422 and L637.

Both strains were able to survive in the presence of most of the tested commercial drugs (Table 3). Amongst the evaluated drugs, the growth of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were affected by only five and three drugs, respectively. The strains were inhibited by anti-inflammatory drugs containing diclofenac potassium, aceclofenac, nimesulide or ibuprofen (Table 3). Moreover, *E. hirae* ST57ACC was inhibited by the anti-histamine loratadine and the anti-hypertensive propranolol. Diclofenac potassium showed the lowest MIC amongst all evaluated drugs of 0.5 and 0.12 mg/mL for *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, respectively. In contrast, the MIC of ibupril, containing ibuprofen, was highest (120 mg/mL) against *P. pentosaceus* ST65ACC. For propranolol hydrochloride, the MIC observed against *E. hirae* ST57ACC was 8 mg/mL, whilst for loratadine, the MIC observed against the same strain was 2 mg/mL.

Safety Characteristics

Both strains evaluated in this study were sensitive to the majority of tested antibiotics, presenting low frequencies of antibiotic resistance-encoding genes (Table 4). From the three target genes evaluated for erythromycin resistance (*ermA*, *ermB* and *ermC*), only *E. hirae* ST57ACC amplified a fragment corresponding to *ermB*. Despite the presence of the *ermB* gene in this isolate, a correlation with phenotypic resistance was not observed (Table 4). For *P. pentosaceus* ST65ACC, amplicons corresponding to *bcrB*, *tetO* and *vatE* genes, related to bacitracin, tetracycline and streptogramin resistance, respectively, were detected. Vancomycin-encoding genes were detected in both strains. In *E. hirae* ST57ACC, *vanC1* and *vanC2* were observed, whilst *vanA* and *vanC1* were amplified in *P. pentosaceus* ST65ACC. In both cases, phenotypic

Table 1 Populations (log CFU/mL) and survival rates (%) based on the log populations of the bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC after simulating conditions of the gastrointestinal tract

Tested strain	Population (log CFU/mL)			Survival rate (%)[a]		
	Initial (T_0)	After gastric phase (T_1)	After enteric phase (T_2)	Gastric phase	Enteric phase	Entire assay
E. hirae ST57ACC	7.8 ± 0.11	7.1 ± 0.07	6.2 ± 0.03	91.0	86.9	79.1
P. pentosaceus ST65ACC	7.6 ± 0.17	7.0 ± 0.12	6.2 ± 0.09	92.6	88.7	82.2

[a] Survival rates calculated as described in the "Materials and Methods" section, and based on equation proposed by Santos et al. [20]

resistance to vancomycin was observed, as well as for trimethoprim and sulfa and oxacillin (Table 4).

Regarding virulence factors, phenotypic tests showed that neither *E. hirae* ST57ACC nor *P. pentosaceus* ST65ACC produced gelatinase, DNAse or lipase, and did not induce haemolysis. Considering the 15 virulence-related genes assessed in this study, *E. hirae* ST57ACC presented amplification only for the *fsrB* gene, whilst *P. pentosaceus* ST65ACC amplificated only *ccf* out of the three genes tested, which is related to sex pheromones.

Biogenic amine production was not detected in the phenotypic tests performed with *E. hirae* ST57ACC and *P. pentosaceus* ST57ACC, and genes related to the expression of histamine, tyramine and putrescine were absent.

Discussion

The ability to survive and remain viable after passage through the gastrointestinal tract is the most important feature when searching for beneficial strains. Hydrophobicity and auto-aggregation are also relevant and desirable traits, as they are required for the initial steps of intestinal adhesion and are also related to the ability of the bacteria to adsorb toxic substances like mycotoxins [28]. Hydrophobic interactions precede the subsequent adhesion processes mediated by specific mechanisms involving cell surface proteins and lipoteichoic acids, whilst auto-aggregation is an important criterion for biofilm formation, which can aid in colonisation of the intestine and help bacteria bind effectively to the intestinal epithelium, thereby preventing pathogen adhesion [29, 30]. The ability of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC to survive passage through the simulated gastrointestinal tract was demonstrated in this study (Table 1), in addition to their hydrophobic and auto-aggregation potential (Table 2). Due to these characteristics, these strains can be considered beneficial microorganisms.

Another characteristic considered positive for a beneficial strain is its ability to interact with pathogens and prevent gastrointestinal infections. Co-aggregation of beneficial strains with pathogens and the close proximity between them facilitates interactions amongst these microorganisms, leading to elimination of the pathogens through a variety of mechanisms including the production of antimicrobial compounds such as bacteriocins [31]. In a previous study, both *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC showed strong bacteriocinogenic activity against *L. monocytogenes* [19]. The bacteriocins produced by these isolates were able to inhibit the growth of *L. monocytogenes* after 12 h, and *E. hirae* ST57ACC was also found to efficiently reduce the population of this pathogen in skim milk [19]. Considering the earlier findings and the results from this work with *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC (Table 2), it is possible to conclude that these isolates have potential for the control of *L. monocytogenes*. As the pathogenesis of *L. monocytogenes* begins with binding to intestinal epithelial cells, beneficial strains may interact with the pathogen before this adhesion process, preventing invasion of the host intestine. Moreover, close contact between *L. monocytogenes* and beneficial isolates allows the bacteriocins produced by these strains to promptly act on the target cells prior to being inactivated by the proteolytic enzymes present in the intestinal environment.

Drugs commonly used by patients for the treatment of different diseases may affect the viability of live cultures

Table 2 Cell surface hydrophobicity (%), auto-aggregation (%) and co-aggregation with *Listeria monocytogenes* strains (%) of the bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC

Tested strain	Cell surface hydrophobicity (%)[a]	Auto-aggregation (%)[a]	Co-aggregation (%) with[a]		
			L. monocytogenes L711	*L. monocytogenes* L422	*L. monocytogenes* L637
E. hirae ST57ACC	41.0 ± 0.13	51.6 ± 0.01	17.3 ± 0.01	42.5 ± 0.06	48.1 ± 0.02
P. pentosaceus ST65ACC	46.5 ± 0.04	71.8 ± 0.02	48.1 ± 0.05	71.7 ± 0.08	71.0 ± 0.02

[a] Values were calculated as described in the "Materials and Methods" section, based on equations proposed by Santos et al. [20] and Todorov et al. [22]

Table 3 Effect of commercial drugs on growth of *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC in MRS agar, presented as diameter of inhibition zones (mm) and minimal inhibitory concentration (MIC; in mg/mL)

Medical group	Commercial name	Inhibition[a]	
		E. hirae ST57ACC	*P. pentosaceus* ST65ACC
Analgesic and anti-pyretic	Anador	0	0
	Paracetamol	0	0
	Saridon	0	0
Analgesic and anti-spasmodic	Buscopan composto	0	0
Analgesic, anti-inflammatory and muscle relaxant	Doralflex	0	0
Anti-acid	Sonrisal	0	0
Anti-allergic	Loratadina	6 (2)	0
Anti-asthmatic	Aminofilina	0	0
	Fumarato de cetotifeno	0	0
Anti-diarrheic	Lopedium	0	0
Anti-emetic	Bromoprida	0	0
Anti-flu	Perfenol	0	0
	Trimedal	0	0
Anti-histaminic	Allergosan	0	0
	Dramin	0	0
Anti-hypertensive	Cloridrato de propanolol	10 (8)	0
	Lisinopril	0	0
	Press Plus	0	0
Anti-inflammatory	Aceclofenaco	15 (20)	14 (20)
	Ácido mefenâmico	0	0
	Diclofenaco de potássio	11 (0.5)	20 (0.12)
	Ibupril	0	12 (120)
	Nisoflan	30 (20)	0
Antitussive	Libexin	0	0
Anti-ulcers	Pratiprazol	0	0
Anti-vertiginous and vasodilatory	Cinarizina	0	0
Digestive stimulant	Hepatilon	0	0
Laxative	Dulcolax	0	0
Mucolytic	Acetilcisteína	0	0
	Fluteína	0	0
Vitamin complex	Complexo Ômega A–Z	0	0
	Dayvit	0	0

[a] Inhibition presented as diameter of inhibition zones (mm), followed by recorded MIC (mg/mL). Concentrations of the tested commercial drugs are detailed in the Supplementary Table 1

delivered as beneficial organisms in food products or as prescribed probiotic cultures. In this sense, it is important to evaluate possible interactions between drugs and beneficial strains with the aim to avoid negative interactions. Anti-inflammatory drugs containing diclofenac potassium and its analogues have been previously reported to interfere with the viability of beneficial LAB [23, 32, 33]. In the same way, inhibition by medications containing loratadine, propranolol hydrochloride and other similar anti-allergics and anti-hypertensives were also reported to be associated with LAB inhibition [23, 32, 34]. It is important to note that the effectiveness of these drugs depends on the amount of the active compound that reaches the gastrointestinal tract, and correct evaluation of possible interactions between drugs and strains depends on determining the MIC of these compounds [32]. Depending on the period of treatment, drugs may accumulate in the gastrointestinal environment, and as a result, concentrations sufficient to inhibit beneficial cultures can be easily reached, affecting their viability. Only a few drugs considered in this study interfered with the growth of the

Table 4 Antibiotic resistance profiles of *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC evaluated by disc diffusion method and PCR

Antimicrobial group	Anti-microbial (μg per disc)	*E. hirae* ST57ACC		*P. pentosaceus* ST65ACC	
		Disc	PCR[a]	Disc	PCR[a]
Chloramphenicol	Chloramphenicol (30)	30 (S)	None	30 (S)	None
Glicopeptides	Vancomycin (30)	0 (R)	*vanC1*, *vanC2*	0 (R)	*vanA*, *vanC1*
Tetracyclines	Tetracycline (30)	22 (S)	None	22 (S)	*tetO*
Macrolides/lincosamides	Erytromycin (15)	28 (S)	*ermB*	28 (S)	*vatE*
	Clindamycin (2)	28 (S)		30 (S)	
Bacitracin	Not tested	–	None	–	*bcrB*
Sulfonamides	Trimethoprim/sulfamethoxazole (25)	0 (R)	Not tested	0 (R)	Not tested
Rifamicin	Rifampicin (5)	25 (S)	Not tested	22 (S)	Not tested
β-Lactam	Ampicillin (10)	21 (S)	Not tested	22 (S)	Not tested
	Imipenem (10)	30 (S)	Not tested	32 (S)	Not tested
	Oxacillin (1)	0 (R)	Not tested	0 (R)	Not tested
	Penicilin G (10[b])	25 (S)	Not tested	28 (S)	Not tested

Inhibition zones in disc diffusion method are expressed in millimetres (mm), and strains were classified as susceptible (S) or resistant (R), according to CLSI (2016)

[a] Tested genes: *bcrB*, *bcrD*, *bcrR* (bacitracin resistance), *ermA*, *ermB*, *ermC* (erythromycin resistance), *tetK*, *tetL, tetM*, *tetO*, *tetS* (tetracycline resistance), *vanA*, *vanB*, *vanC1*, *vanC2* (vancomycin resistance), *catA* (chloranphenicol resistance), *vatE* (streptogramin resistance)

[b] Concentration of anti-microbial expressed as units

bacteriocinogenic strains *P. pentosaceus* ST65ACC and *E. hirae* ST57ACC, although this occurred at low MICs when compared to the common dosages indicated for humans (Table 3; Supplementary Table 1), demonstrating their poor potential for inhibiting the viability of the tested strains. Although the inhibitory doses recorded for these drugs were also lower than the daily minimum recommendations, factors such as interactions with foods and enzymes should be studied *in vivo* to clarify the interactions between these medications and potentially beneficial cultures.

As LAB can acquire and play a role in the transfer of antibiotic resistance elements to pathogenic bacteria, the food chain is considered an important route for introducing these elements into the gastrointestinal tract. There is current concern regarding the identification of LAB isolates that present antibiotic resistance genes in foods, as they can act as reservoirs and transfer these genes to pathogenic bacteria [35]. Both strains evaluated in this work were sensitive to the majority of tested antibiotics, presenting low frequencies of antibiotic resistance-related genes, and correlation with phenotypic results was rarely observed (Table 4).

Vancomycin-encoding genes were detected in both strains. This is important as vancomycin is considered an antibiotic of last resort when most other antibiotics fail in treating infections caused by Gram-positive bacteria. Resistance to this antibiotic is recognised as an intrinsic trait of *Pediococcus* spp., and is commonly reported [36, 37]. In contrast, vancomycin-resistant enterococci represent the main source of infections in humans and carriers of transferable vancomycin resistance markers [38]. Whilst *vanA* and *vanB* are normally associated with plasmid DNA, *vanC*, *vanD*, *vanE* and *vanG* are encoded on the bacterial chromosome [39]. *Pediococcus* spp. is described as intrinsically resistant to several groups of antibiotics, including β-lactams, cephalosporins, aminoglycosides, glycopeptides, streptomycin, kanamycin, tetracyclines, doxycycline and sulfa (with or without trimethoprim) [36, 37], and phenotypic resistance to some of these antibiotics was observed in the present study (Table 4). However, studies related to genetic markers in *Pediococcus* spp. are scarce, and this appears to be the first report of the presence of *bcrB*, *tetO* and *vatE* genes in *P. pentosaceus* isolated from raw milk cheese. Investigations into the role of these genes in resistance and their contribution to acquired resistance need to be studied further, as well as the presence of other genes related to antibiotic resistance that could explain the differences between results of phenotypic and genotypic assays.

Virulence factors are usually surveyed in enterococcal species, and virulent activity has previously been reported in dairy isolates [25, 40]. In this study, phenotypic tests were negative for both tested strains, and only the presence of the *fsrB* gene was detected in *E. hirae* ST57ACC. The *fsr* operon has previously been identified in *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae* and *E. dispar*, indicating that these genes may be widespread amongst species of the *Enterococcus* genus [40, 41]. Genes belonging to this operon (*fsrA*, *fsrB* and *fsrC*) in association with *gelE* are responsible for gelatinase activity [40]. The expression of *gelE* is dependent on cell density and is positively regulated by the *fsr* operon. This

means that the expression of all *fsr* genes is required in order to regulate *gelE* and its consequent positive phenotype [40]. Concerning sex pheromones, these have been well characterised in *Enterococcus* spp., known to play a role in the exchange of genetic information by conjugation. However, although sex pheromones are not considered to be virulence factors *per se*, their production in enterococci may favour the dissemination of virulence determinants and promote the acquisition of antibiotic resistance and other linked traits from other enterococci, thereby leading to increased virulence [42]. Whilst the presence of sex pheromones has not yet been reported in *Pediococcus* spp., and its role in this genus is currently unknown, *P. pentosaceus* ST65ACC was found to harbour *ccf* in this study. Further characterisation of this activity and its possible contribution to virulence or participation in horizontal gene transfer needs to be investigated. The production of biogenic amines by LAB are variable, and are usually associated with the presence of genes that are generally strain-specific [20, 43] and to specific growth conditions that promote decarboxylase synthesis [25]. Ladero et al. [44] suggested that in the *Enterococcus* genus, the production of tyramine is specific to *E. faecalis*, *E. faecium* and *E. durans*; putrescine is specific to *E. faecalis*; and no biogenic amine is linked to *E. hirae*. The absence of biogenic amine production and target genes related to this characteristic further corroborates the safety of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC and supports their use as beneficial or biopreservative strains.

E. hirae ST57ACC and *P. pentosaceus* ST65ACC both presented interesting beneficial properties, especially due to their ability to survive gastrointestinal conditions and aggregate with *L. monocytogenes*, which can help in the control of this pathogen. Also, the tested strains presented very low frequencies of virulence markers and showed no production of biogenic amines, indicating that they could be considered suitable for industrial applications. However, the tested strains presented some antibiotic resistance characteristics, which represent a current concern worldwide. Further studies should investigate how to minimise the hazards linked to antibiotic resistance in *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, as well as provide a deeper understanding of the interactions between these strains and *L. monocytogenes* to assess the use of these bacteria or their bacteriocins as alternatives to control this foodborne pathogen.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval None animal was used in any of the described experiments.

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