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Research Article

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Bacteriocinogenic anti-listerial properties and safety assessment of *Enterococcus faecium* and *Lactococcus garvieae* strains isolated from Brazilian artisanal cheesemaking environment

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Abstract

Aims: This study aimed to prospect and isolate lactic acid bacteria (LAB) from an artisanal cheese production environment, to assess their safety, and to explore their bacteriocinogenic potential against *Listeria monocytogenes*.

Methods and results: Samples were collected from surfaces of an artisanal-cheese production facility and after rep-PCR and 16S rRNA sequencing analysis, selected strains were identified as to be belonging to *Lactococcus garvieae* (1 strain) and *Enterococcus faecium* (14 isolates, grouped into three clusters) associated with different environments (worktables, cheese mold, ripening wooden shelves). All of them presented bacteriocinogenic potential against *L. monocytogenes* ATCC 7644 and were confirmed as safe (γ-hemolytic, not presenting antibiotic resistance, no mucus degradation properties, and no proteolytic or gelatinase enzyme activity). Additionally, cell growth, acidification and bacteriocins production kinetics, bacteriocin stability in relation to different temperatures, pH, and chemicals were evaluated. According to performed PCR analysis all studied strains generated positive evidence for the presence of *ent*A and *ent*P genes (for production of enterocins A and enterocins P, respectively). However, pediocin PA-1 associated gene was recorded only in DNA obtained from *E. faecium* ST02JL and *Lc. garvieae* ST04JL.

Conclusions: It is worth considering the application of these safe LAB or their bacteriocins *in situ* as an alternative means of controlling *L. monocytogenes* in cheese production environments, either alone or in combination with other antimicrobials.

Impact Statement

Lactic acid bacteria (LAB) play an important role in the cheese-making fermentation process. Besides their biotechnological significance for the product, LAB contribute to its biopreservation by producing antimicrobial metabolites such as bacteriocins. A major hazard for artisanal cheese production is *Listeria monocytogenes*, and bacteriocins have been shown as a viable alternative to control this pathogen. Moreover, bacteriocinogenic properties can be a positive factor, improving the spread and survival of microbial cultures into the dairy production environment.

Keywords: artisanal cheeses; lactic acid bacteria; anti-listerial; food safety; bacteriocins

Introduction

Lactic acid bacteria (LAB), which are Gram-positive and catalase-negative species, have been reported as essential microbial performers in colonizing diverse ecological niches, playing beneficial, spoilage, and/or even pathogenic roles through fermented foods, gastrointestinal tract of humans and other animals, soil, surfaces, water, plants, and dairy environments (Marekova et al. 2007, Stellato et al. 2015, Colombo et al. 2018, dos Santos et al. 2020). Historically, based on empirical knowledge transferred among generations, representative LAB have been applied as starter cultures in preparations of numerous fermented food products (Foulquié Moreno et al. 2006). Since postulating the role of LAB in promoting health benefits through the consumption of some fermented dairy products by Ilia Metchnikoff and his collaborator Stamen Grigorov (Ye et al. 2023), several of these strains have been explored as effective probiotics and scientifically proven regarding their beneficial properties (Holzapfel et al. 2018). However, with the development of biomolecular research techniques, clear re-evaluation of the safety of LAB needs to be considered, and further research is emerging to systematically distinguish pathogenic strains from well-assessed beneficial ones within the LAB group. Some reports suggest that specific representatives of LAB, considered as safe based only on classical biochemical and physiological tests may carry genes associated with virulence factors, biogenic amines production, or antibiotic resistance. These strains may pose a serious health risk for the consumers of probiotics or fermented food products where such strains are applied as beneficial (starter, bioprotective) cultures (Gomes et al. 2008, Bourdichon et al. 2012).

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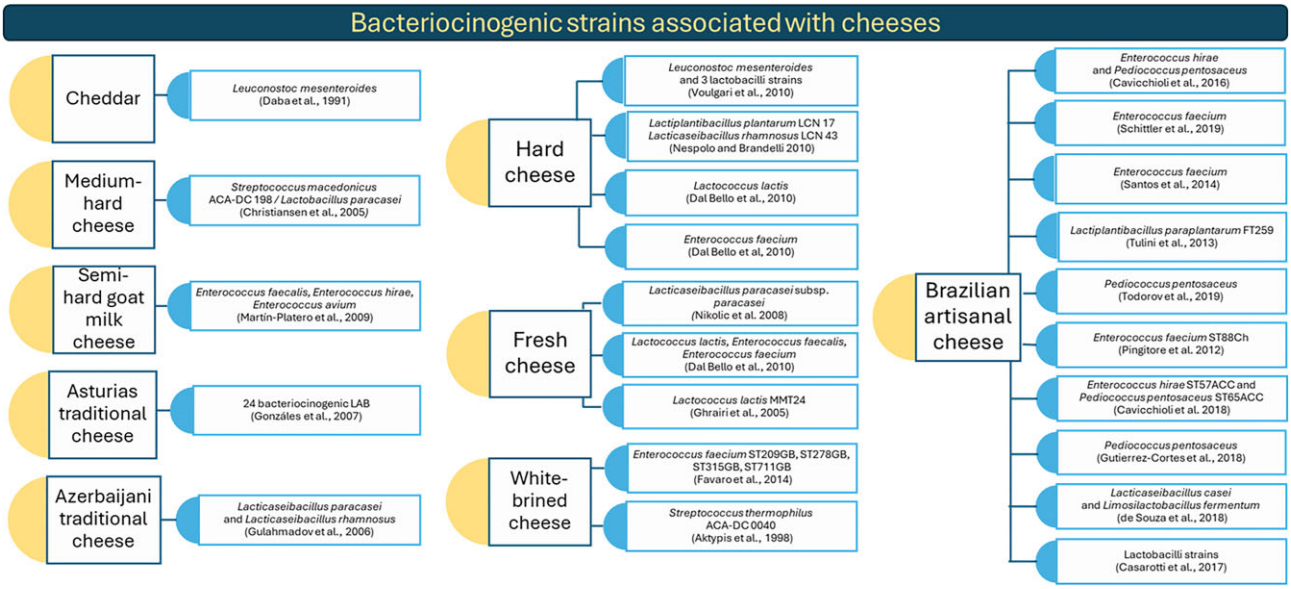

Figure 1. Some examples of bacteriocinogenic strains associated with cheese production.

LAB play an essential role in the dairy industry and can be found in milk related products where they actively participate as starters or adjunct cultures, providing technological, sensorial, and bioprotective benefits to formation of the final products. LAB may actively contribute to the safety of fermented food products by producing different metabolites, including a variety of organic acids, hydrogen peroxide, carbon dioxide, diacetyl, and bacteriocins with antimicrobial properties (Choi et al. 2023). Being a natural component of fermented dairy products, LAB can easily be found on dairy environmental surfaces, including worktables, cheese mold, and ripening wooden shelves as so called "natural contaminants" in dairy production environments.

Bacteriocins are polypeptides or small proteins, produced by the ribosomes and released extracellularly (Chikindas et al. 2018, Choi et al. 2023). Since the early years of the discovery of bacteriocins produced by LAB in the early 20th century, these compounds have been the subject of intense investigation (Favaro and Todorov 2017, Chikindas et al. 2018). The concept of applying peptides as antimicrobials is not new, as some bacteriocins have been widely used in biopreservation for many decades as part of the production of various antimicrobial compounds by LAB (Fig. 1).

The definition of bacteriocins has been modified in recent decades based on the construction of knowledge about these antimicrobial proteins. The ecological function of bacteriocin production is considered an omnipresent characteristic of bacteria found naturally in complex ecosystems, presumed to play a central role in stabilizing microbial populations. Different from antibiotics, they have a proteinaceous nature and after produced, generally inhibit the growth of other related bacteria (Todorov et al. 2019). Bacteriocins exert their effects through membrane disruption, targeting specific membrane receptors in sensitive bacterial species, by degradation of cellular DNA, and inhibition of peptidoglycan biosynthesis (de Vuyst 1994, Choi et al. 2023). Bacteriocins demonstrate activity across a broad pH spectrum and display tolerance to high thermal stress. Additionally, they are colorless, odorless, and tasteless, characteristics that give them an excellent recommendation for applications in food biopreservation processes. Most of the bacteriocins produced by LAB are considered as generally food-grade due to the longstanding association of LAB with food fermentation and representing generally low cytotoxicity (Perez et al. 2014). In fact, the USA Food and Drug Administration classified several LAB and their by-products, including commercially available nisin and pediocin PA-1, as generally regarded as safe for use as a food ingredient for human and other animals' consumption.

Artisanal cheese producers are actively pursuing knowledge, innovation, technology, and systems to enhance the quality and safety of their products by natural means. A significant concern for these small-scale producers is the maintenance of proper hygienic conditions, especially in areas related to transportation, the processing environment, and the workforce. These conditions are crucial to preventing the spread of pathogenic bacteria (Mounier et al. 2006, Callon et al. 2014), and alternatives for ensuring hygienic conditions have always been internal concerns for the artisanal cheese producers.

Listeria monocytogenes is a Gram-positive rod-shaped bacterium closely related to LAB. It is a pathogen commonly linked to outbreaks affecting immunocompromised persons, pregnant women, newborns, and elderly individuals (Camargo et al. 2019). This pathogen is associated with fermented and processed food products, and dairy environments and can thrive on both, biotic and abiotic surfaces (Winkelströter et al. 2013, Gungor et al. 2021, Ribeiro et al. 2023). Antagonistic interactions between antimicrobials produced by LAB, including bacteriocins, and *Listeria* spp. are a very promising approach for the control of this pathogenic species (Cleveland et al. 2001, Cotter et al. 2005, Lobos et al. 2009, Zhao et al. 2013, Pérez-Ibarreche et al. 2014, Fugaban et al. 2021a).

The aim of this study was to evaluate the safety and bacteriocinogenic potential against *L. monocytogenes* of *Enterococcus faecium* ST01JL, ST02JL, and ST03JL and *Lactococcus garvieae* ST04JL strains, isolated from surfaces in the artisanal cheese production environment of a factory located in the State of Sao Paulo, Brazil.

Materials and methods

Screening for LAB with anti-listerial properties

Microbiological swabs/samples were collected from surfaces of different locations in a dairy environment of a cheese producing facility located in Sao Paulo State, Brazil, including worktables, cheese mold, and ripening wooden shelves. The samples were collected aseptically by sterile swabs from 100 cm^2 of the surfaces and immediately placed in sterile bottles with 10 ml MRS broth (de Man, Rogosa, Sharpe—MRS, Oxoid, Basingstoke, UK) and transported to the laboratory in controlled temperature (8°C–10°C). The obtained samples were incubated for 18–24 h at 37°C with the aim to enrich potential LAB collected from the examined surfaces. In the following step, a triple layer agar method was used according to Valledor et al. (2022) with some modifications. Representatives from each enriched sample were streaked on the surface of MRS agar (Oxoid), followed with cover with a second layer of the MRS agar. After additional 18–24 h of incubation at 37°C, plates were examined for bacterial growth, and those with well-distinguished individual colonies received a third layer composed of BHI (Oxoid), supplemented with 2% agar, and seeded with *L. monocytogenes* ATCC 7644 (final concentration 10^{5-6} CFU/ml). All plates were incubated for an additional 18–24 h at 37°C, and colonies with visible inhibition zones were considered as a potential LAB with anti-listeria properties (Valledor et al. 2022). Individual colonies were isolated and purified according to standard microbiological procedures, and the obtained isolated cultures were stored in the presence of 20% glycerol at –20°C and –80°C.

Preliminary screening for LAB and safety evaluation

In preliminary evaluation, preselected isolates with antimicrobial potential against *L. monocytogenes* were observed macroscopically for colonies morphology growing on MRS agar, for Gram-staining, microscopical morphology, and catalase reaction. Experiments were performed on at least two independent occasions. Gram-positive and catalase-negatives isolates were considered for the next steps for the main safety features, which consisted of gelatin hydrolysis, proteolytic and hemolytic activity, mucin degradation tests, and antibiotic sensitivity as recommended by EFSA (EFSA 2012).

The gelatinase production was evaluated according to dos Santos et al. (2020). The studied cultures were grown in MRS broth supplemented with 5% gelatin (Oxoid) (*w/v*) for 24 h at 37°C. After the incubation, tubes were cooled for 1 h at 4°C, and the positive results were reported as test tubes retaining the liquid form after refrigeration. *Staphylococcus aureus* ATCC 29213 was applied as positive control (gelatin hydrolysis), while an untreated medium (MRS, supplemented with 5% gelatine) served as negative control (dos Santos et al. 2020). The experiment was performed on two independent occasions in duplicates.

For the evaluation of proteolytic activity, studied bacterial cultures were streaked on the surface of MRS agar supplemented with 10% skim milk powder (Molico®, Nestlé, Araçatuba, SP, Brazil). The experiment was performed on two independent occasions in duplicates. The plates were incubated for 72 h at 37°C, and the presence of proteolytic activity was confirmed by the formation of a clear zone around the colonies, following the recommendations of Domingos-Lopes et al. (2017).

For the hemolytic activity, studied cultures were streaked on the surface of blood agar plates (Blood Agar—Laborclin—TSA w/5% sheep blood) and incubated for 48 h at 37°C. Microbial cultures resulting in formation of clear transparent zones around growing colonies were considered as β-hemolytic. As evidence for α-hemolysis, the formation of green zones around growing colonies was considered, and for γ-hemolysis, the absence of halos surrounding colonies was considered as no hemolytic activity. *Listeria monocytogenes* ATCC 7644 was used as a control for α-hemolysis (Santini et al. 2010). The experiment was performed on two independent occasions.

In the evaluation of mucin digestion abilities for the studied strains, recommendations from Monteagudo-Mera et al. (2012) were followed, and experiments were performed in duplicates. MRS medium was prepared in house following the commercial product composition with exclusion of glucose as carbon source ingredient. Then the modified MRS (without glucose) and supplemented with 1.5% agar (*w/v*) received 0.3% of HGM (*w/v*) (hog gastric mucin, type III, Sigma-Aldrich, Inc., San Luis, MO, USA) or 1% of glucose (w/v) as a control. An aliquot of 10 μl of each bacterial culture was spotted in duplicate on the surface of the previously prepared plates and incubated in aerobic conditions for 72 h at 37°C. Subsequently, formed colonies were stained for 30 min with 0.1% of black starch (*w/v*) in 3.5 M of acetic acid. Plates were then washed with 1.2 M of acetic acid and observed for the mucin lysis zone around the colonies (Monteagudo-Mera et al. 2012).

The antibiotic susceptibility test was performed by a disc diffusion method, according to the guideline of EFSA (European Food Safety Authority 2012). The tested antibiotics were ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (Cefar Diagnóstica Ltda, Sao Paulo, Brazil). Nalidixic acid (antibiotic with activity versus Gram-negative species) was used as negative control. The experiment was performed on two independent occasions in duplicates.

Identification of the selected isolates

Preliminary selected as safe and putative LAB microbial cultures classified as Gram-positive and catalase-negative were further subjected to the biochemical and physiological identification according to the recommendations of Bergey's Manual of Systematic Bacteriology of Archaea and Bacteria (de Vos et al. 2009) and biomolecular identification (16S rRNA partial sequencing) for taxonomic identity. Microbial cultures of interest were grown in 10 ml MRS for 24 h at 37°C, and DNA was extracted by applying commercial Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) according to manufacturer recommendations. The quality and concentration of the obtained DNA were evaluated on nano spectrophotometer (NanoDrop—Thermo Fisher Scientific, Waltham, MA, USA). For bacterial identification, primers listed in Table 1 were used for target a conservative region of the ribosomal DNA recommended by Héquet et al. (2007). The PCR reactions were performed on Veriti 96 thermocycler (Applied Biosystems, Thermo Fischer) as follows: denaturation step at 95°C for 7 min, followed by 35 cycles at 95°C for 1 min, annealing at 58°C for 1.5 min, extension at 72°C for 2.5 min, and final DNA extension at 72°C for 5 min. The generated amplicons were separated by gel

Table 1. Primers used in the screening of safety and beneficial properties and molecular-based assay for the detection in studied strains.

Gene		Sequence	Product size	Reference	Results			
					Enterococcus faecium ST01JL	*Enterococcus faecium* ST02JL	*Enterococcus faecium* ST03JL	*Lc. garvieae* ST04JL
		Vancomycin resistance associated genes						
vanA	F	5′-GTA GGC TGC GAT ATT CAA AGC-3′	231	Fugaban et al. (2021b)	–	–	–	–
	R	5′-CGA TTC AAT TGC GTA GTC CAA-3′						
vanB	F	5′ -GTA GGC TGC GAT ATT CAA AGC-3′	330		–	–	–	–
	R	5′- GCC GAC AAT CAA ATC ATC CTC-3′						
vanC	F	5′-ATC CAA GCT ATT GAC CCG CT-3′	402		–	–	–	–
	R	5′-TGT GGC AGG ATC GTT TTC AT-3′						
vanD	F	5′- TGT GGG ATG CGA TAT TCA A-3′	500		–	–	–	–
	R	5′- TGC AGC CAA GTA TCC GGT AA-3′						
vanE	F	5′- TGT GGT ATC GGA GCT GCA G-3′	513		–	–	–	–
	R	5′- GTC GAT TCT CGC TAA TCC-3′						
vanG	F	5′- GAA GAT GGT ACT TTG CAG GGC A-3′	519		–	–	–	–
	R	5′-AGC CGC TTC TTG TAT CCG TTT T-3′						
		Virulence genes						
is16	F	5′- CAT GTT CCA CGA ACC AGA G-3′	547	Werner et al. (2011)	–	–	–	–
	R	5′- TCA AAA AGT GGG CTT GGC-3′						
ace	F	5′-GAA TTG AGC AAA AGT TCA ATC G-3′	1008	Martín-Platero et al. (2009)	–	–	–	+
	R	5′-GTC TGT CTT TTC ACT TGT TTC-3′						
efa	F	5′-GCC AAT TGG GAC AGA CCC TC-3′	688		–	–	–	–
	R	5′-CGC CTT CTG TTC CTT CTT TGG C-3′						
esp	F	5′-AGA TTT CAT CTT TGA TTC TTG G-3′	510	Vankerckhoven et al. (2004)	–	–	–	–
	R	5′-AAT TGA TTC TTT AGC ATC TGG-3′						
asa	F	5′-GCA CGC TAT TAC GAA CTA TGA-3′	375		–	–	–	–
	R	5′-TAA GAA AGA ACA TCA CCA CGA-3′						
hyl	F	5′-ACA GAA GAG CTG CAG GAA ATG-3′	276		–	–	–	–
	R	5′-GAC TGA CGT CCA AGT TTC CAA-3′						
hdc	F	5′-AGA TGG TAT TGT TTC TTA TG-3′	367	de Las Rivas et al. (2005)	–	–	–	–
	R	5′-AGA CCA TAC ACC ATA ACC TT-3′						
tdc	F	5′-GAY ATN ATN GGN ATN GGN YTN GAY CAR G-3′	924		+	–	+	–
	R	5′-CCR TAR TCN GGN ATA GCR AAR TCN GTR TG-3′						
odc	F	5′-GTN TTY AAY GCN GAY AAR CAN TAY TTY GT-3′	1446		–	–	–	–
	R	5′-ATN GAR TTN AGT TCR CAY TTY TCN GG-3′						
cylA	F	5′-ACT CGG GGA TTG ATA GGC-3′	688	Vankerckhoven et al. (2004)	–	–	–	+
	R	5′-GCT GCT AAA GCT GCG CTT-3′						
gel	F	5′-TAT GAC AAT GCT TTT TGG GAT-3′	213		–	–	–	–
	R	5′-AGA TGC ACC CGA AAT AAT ATA-3′						

Table 1. Continued

Gene		Sequence	Product size	Reference	Results			
					Enterococcus faecium ST01JL	*Enterococcus faecium* ST02JL	*Enterococcus faecium* ST03JL	*Lc. garvieae* ST04JL
		Bacteriocin genes						
nis	F	5′-ATG AGT ACA AAA GAT TTCAAC TT-3′	203		–	–	–	–
	R	5′-TTA TTT GCT TAC GTG AAC GC-3′						
				Fugaban et al. (2021a)				
entA	F	5′-GAG ATT TAT CTC CAT AAT CT-3′	452		+	+	+	+
	R	5′-GTA CCA CTC ATA GTG GAA-3′						
entB	F	5′-GAA AAT GAT CAC AGA ATG CCT A-3′	159		–	–	–	–
	R	5′-GTT GCA TTT AGA GTA TAC ATT TG-3′						
ent-l50b	F	5′-ATG GGA GCA ATC GCA AAA TTA-3′	135		–	–	–	–
	R	5′- TAG CCA TTT TTC AAT TTG ATC-3′						
entP	F	5′-ATG AGA AAA AAA TTA TTT AGT TT-3′	216		+	+	+	+
	R	5′-TTA ATG TCC CAT ACC TGC CAA ACC-3′						
ped	F	5′-CAA GAT CGT TAA CCA GTT T-3′	1043		–	+	–	+
	R	5′-CCG TTG TTC CCA TAG TCT AA-3′						
lgnA	F	5′-ATT TAA TAC GGA CGG TAT TGA T-3′	205	Maldonado Barragán et al. (2013)	–	–	–	–
	R	5′-GGA GTA AAA AGA TGG AAA ACA A-3′						
lgaI	F	5′-AGA AAA TGG GCT AAC TCC GG-3′	261		–	–	–	–
	R	5′-ATG AAT AAA ACA GAA ATA ATG ACT-3′						
lcn972	F	5′-TTG TAG CTC CTG CAG AAG GAA CAT GG-3′	232	Mirkovic et al. (2015)	–	–	–	–
	R	5′-GCC TTA GCT TTG AAT TCT TAC CAA AAG-3′						
gak	F	5′-CGT AAT TCG AGC TCC ACC TCT GCT GTT TTT C-3′	341	Telke et al. (2019)	–	–	–	–
	R	5′-AGA CTT TGC AAG CTT GCA ATA TTA CGT TTG TGG G-3′						
lact-gq	F	5′-GAA AGA ATT ATC AGA AAA AG-3′	382	Mirkovic et al. (2015)	–	–	–	–
	R	5′-CCA CTT ATC TTT ATT TCC CTC T-3′						
		Differentiation						
GTG_5		5′-GTG GTG GTG GTG GTG -3′		Castilho et al. (2019)				
		Identification						
BSF8/20	F	5′-AGA GTT TGA TCC TGG CTC AG-3′	1518	Héquet et al. (2007)				
BSR 1541/20	R	5′-AAG GAG GTG ATC CAG CCG CA-3′						

electrophoresis on 1% (w/v) agarose stained with SYBR® Safe DNA Gel Stain (Thermo Scientific) on running conditions of 100 V for 45 min and visualized with Molecular Imager® Gel-Doc™ XR (Bio-Rad). Obtained amplicons were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil, through Sanger DNA sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit) and identified by Sequencing Analysis 7.0 (Base Caller KB™). The generated sequences were analyzed using Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA) for identification.

Selected isolates were differentiated by repPCR, with primer $(GTG)_5$, listed in Table 1 (Castilho et al. 2019) on Veriti 96 thermocycler as follows: denaturation step at 95°C for 5 min, 95°C for 30 s, annealing at 40°C for 30 s, and extension at 65°C for 8 min for the next 30 cycles, and final DNA extension at 65°C for 16 min concluded the amplification. The generated fingerprints amplicons were separated by gel electrophoresis on 1.5% (w/v) agarose, stained, and visualized as described before.

Partial characterization of produced bacteriocins

To produce bacteriocin containing cell-free supernatant (CFS), selected bacterial cultures were incubated in MRS broth for 18–24 h at 37°C. CFSs were collected by centrifugation (7000 × *g*, 15 min), pH adjusted to 5.5 to 6.5 using a sterile 3 M NaOH, then heat-treated for 10 min at 80°C, and finally filtered using 0.2 μm syringe filters (Kasvi®, São José dos Pinhais, SP, Brazil) (Valledor et al. 2022). The bacteriocin activity test was performed according to the spot on-the-lawn method (dos Santos et al. 2020) against *L. monocytogenes* ATCC 7644. In the test, 10 μl aliquots were spotted onto the surface of BHI supplemented with 2% agar and seeded with *L. monocytogenes* ATCC 7644 (final concentration 10^{5-6} CFU/ml). Plates were left to dry for 30 min, and followed incubation for 18–24 h at 37°C. The diameters of the inhibition halos were measured in millimeters, wherein zones of at least 2 mm were considered as positive evidence for the presence of antibacterial (bacteriocinogenic) activity. In an additional experiment, activity of the produced bacteriocins was evaluated against selected pathogens and spoilage bacteria listed in Table 2.

For the evaluation of the levels of activity of expressed bacteriocins, two-fold serial dilutions from the obtained CFS were prepared with sterile 100 mM potassium phosphate buffer (pH 6.5). An aliquot of 10 μl from each dilution was spotted on the surface of plates with test microorganism *L. monocytogenes* ATCC 7644 as previously described. Plates were rested and incubated, and results were observed as described before. The level of bacteriocin activity was expressed as AU/ml taking into account the type of serial dilution (two-fold), the volume in ml (0.01 ml) applied in the test, and the highest dilution still presenting inhibition zone of minimum 2 mm in diameter (Valledor et al. 2022).

CFS from the strains of interest were prepared as described earlier, and the proteinaceous nature of the expressed antimicrobials was also confirmed according to a previous work (Valledor et al. 2022). The obtained CFS was treated with 0.1 mg/ml Proteinase K (final concentration), incubated for 1 h at 37°C, and the enzymatic reaction was stopped by heat treatment for 3–5 min at 98°C–100°C. The residual antimicrobial activity (AU/ml) was evaluated against *L. monocytogenes* ATCC 7644 as described before. CFS not treated with Proteinase K served as inhibition control.

The stability of the bacteriocins was evaluated by exposing the previously obtained CFS to the effect of different temperatures, pH levels, and some selected chemicals applied in analytical and food processing practices (Valledor et al. 2022). For the evaluation of the effect of pH, aliquots of CFS prepared as described before had their pH adjusted with sterile 1 M NaOH or 1 M HCl to levels of 2.0, 4.0, 6.0, 8.0, or 10.0. Samples were incubated for 1 h at 37°C and pH readjusted back to 5.0–7.0 with 1 M NaOH or 1 M HCl as necessary. For the effect of the temperature, aliquots of CFS were incubated for 1 h at 8°C, 30°C, 37°C, 45°C, 60°C, 80°C, and for 15 min at 121°C. For the effect of selected chemicals, CFS from the selected strains was exposed to 1% NaCl, SDS, or Tween 80 for 1 h at 37°C. After incubation, all experimental setups were tested for residual bacteriocin activity versus *L. monocytogenes* ATCC7644, and AU/ml was determined as described before (Valledor et al. 2022). The experiment was performed on two independent occasions.

Bacterial growth kinetic, acidification, and production of bacteriocins were evaluated for the selected strains by incubating them at 5% (v/v) levels from 18 h-old previous activated cultures and growing them in 250 ml of MRS broth for 24 h at 37°C. At regular intervals, every 1 h for determination of bacterial population growth and level of pH and every 3 h for estimation of bacteriocin activity expressed in AU/ml versus *L. monocytogenes* ATCC7644, samples were withdrawn. Bacterial population growth was estimated from the changes in the optical density (OD), evaluated spectrophotometrically at 600 nm on Ultrospec 2000 (Pharmacia Biotech, England), while changes in pH were recorded on pH meter (Láctea Aparelhos Científicos e Eletrônicos LTDA, Sao Paulo, Brazil).

For the evaluation of the effect of the studied bacteriocins, CFS prepared as described before was added to the exponentially growing cultures of *L. monocytogenes* ATCC7644 (in early exponential growth phase). In the experimental procedure, *L. monocytogenes* ATCC7644 was inoculated to 200 ml of BHI broth 1% (v/v) inoculum and incubated at 37°C. Previously prepared CFS of the studied bacteriocinogenic strains as described before was filtered via 0.22 μm (Kasvi®) and added to the exponentially growing culture of *L. monocytogenes* ATCC7544 (after 3 h from the inoculation), and changes in OD 600 nm were followed for the next 12 h on Ultrospec 2000 spectrophotometer (Pharmacia Biotech, England). The AU/ml for the added bacteriocins was estimated as described before. *Listeria monocytogenes* ATCC7644 without supplementation with bacteriocins served as growth control (Todorov and Dicks 2009). The experiment was performed on two independent occasions.

Screening for presence of bacteriocins genes in the DNA from studied strains

The DNA extracted from the studied strains was used to screen for the presence of known bacteriocin genes. This was performed using primers designed to target nisin, pediocin PA-1, enetrocin A, enterocin B, enterocin P, enterocin 50, lactococcin, lacticin, and garvicin genes (Table 1). The PCR reactions were performed on Veriti 96 thermocycler according to the recommendations of Barbosa et al. (2016). The obtained

Table 2. Antibacterial activity spectrum of bacteriocins produced by the investigated strains.

Strain evaluated	*Enterococcus faecium* ST01JL	*Enterococcus faecium* ST02JL	*Enterococcus faecium* ST03JL	*Lc. garvieae* ST04JL
Bacillus cereus ATCC 11778	–	–	–	–
Escherichia coli ATCC 8739	–	–	–	–
Enterobacter aerogenes ATCC 13048	–	–	–	–
Clostridium perfringens ATCC 13124	–	–	–	–
Salmonella Enteritidis ATCC 13076	–	–	–	–
Staphylococcus aureus ATCC 29213	–	–	–	–
Listeria monocytogenes ATCC 7644	+	+	+	+
Listeria monocytogenes 211 serovar 4b	+	+	+	+
Listeria monocytogenes 724 serovar 4b	+	+	+	+
Listeria monocytogenes 712 serovar 1/2c	+	+	+	+
Listeria monocytogenes 101 serovar 4b	+	+	+	+
Listeria monocytogenes 506 serovar 1/2a	+	+	+	+

amplicons were separated on agarose gel electrophoresis (1–2%, *w/v*) taking in consideration the size of the targeted amplicons, stained, and visualized as described before.

Screening for virulence genes

For the screening of the presence different genes associated with safety (virulence, antibiotic resistance, and biogenic amines production genes), the total DNA isolated from the studied strains was subjected to the PCR amplification with primers listed in Table 1. The PCR reactions were performed on Veriti 96 thermocycler, and each condition was made according to the references listed in Table 1. The generated amplicons were separated on agarose gel electrophoresis (1–2%, *w/v*, taking in consideration size of the targeted amplicons), stained, and visualized as described before.

Results

Sampling and screening of LAB with anti-listerial potential

In the initial screening, 10 environmental swabs taken from the production surfaces of a local artisanal cheese producer in Sao Paulo, Brazil, resulted in the identification of 21 colonies exhibiting visible inhibition zones. These colonies were considered as potential anti-listerial bacterial cultures. The colonies were all identified as catalase-negative, Gram-positive, with coccus morphology. Each of the 21 evaluated microbial cultures was described as gelatinase negative, with no α or β-hemolytic activity (all of them were characterized as γ-hemolytic) and with no evidence for proteolytic activity. Out of the 21 investigated isolates, the CFS derived from 15 showed inhibitory activity against the primary test organism, *L. monocytogenes* ATCC7644, during the screening process. Following the antibiotic resistance/susceptibility test recommended by EFSA, it was observed that none of the selected microbial cultures showed resistance to more than one antibiotic indicating that none of them are multidrug resistant. The studied strains, *E. faecium* ST01JL, ST02JL, ST03JL, and *Lc. garvieae* ST04JL were shown to be sensitive to the antimicrobial action of ampicillin, vancomycin, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. However, drug resistance was only seen against streptomycin for the studied strains.

Identification of the isolates

In the following step, the 15 remaining isolates showing clear inhibitory properties against *L. monocytogenes* ATCC7644 were confirmed to be non-producers of CO_2 when cultivated on glucose or glycogen as carbohydrate sources. Additionally, these cultures were able to grow at 15°C and 45°C. Considering the results obtained from biochemical, physiological, and biomolecular tests, including the partial 16S rRNA gene sequencing followed by BLAST analysis, it was possible to identify the isolates as *Lc. garvieae* (1 strain, with 98% of identity compared to the *Lc. garvieae* type strains in GenBank) and *E. faecium* (14 strains, with 98% or greater identity compared to the *E. faecium* type strains in GenBank). Based on the performed differentiation by repPCR, the 14 *E. faecium* strains were clustered into three different groups as shown in Fig. 2.

Integrity and stability of bacteriocins present in the CFS

The putative bacteriocins produced by the studied strains were stable at temperature treatments between 8°C and 100°C for 60 min as well as to 121°C for 15 min and to the presence of 1% NaCl, SDS, and Tween 80. When the tested CFS were exposed to the pH treatment in the range of 2.0, 4.0, and 6.0, no decrease in the antimicrobial activity against *L. monocytogenes* ATCC7644 was observed. However, when the pH was adjusted to 8.0 and 10.0, there was great reduction in the antimicrobial activity, which dropped from 25 600 AU/ml to 3200 and 1600, respectively, for all studied strains. The proteinaceous nature of the produced antimicrobials was confirmed after treatment with Proteinase K, when all tested CFS lost their antimicrobial activity against *L. monocytogenes* ATCC 7644.

Bacterial growth kinetics, changes in the pH, and bacteriocin production

The changes in culture turbidity were considered as evidence for the bacterial growth of *Lc. gavieae* ST04JL and *E. faecium* ST01JL, ST02JL, and ST03JL in MRS both at 37°C, as shown in Fig. 3. The maximum OD 600 nm increased to 2.278, 2.354, 2.364, and 2.116 (presented OD was calculated taking in consideration levels of dilutions and recorded values for the evaluated samples), respectively, recorded at 24 h from the fermentation process, showing a robust growth in the tested conditions. Furthermore, the bacteriocin activity evaluated against *L. monocytogenes* ATCC7644, yielded up to

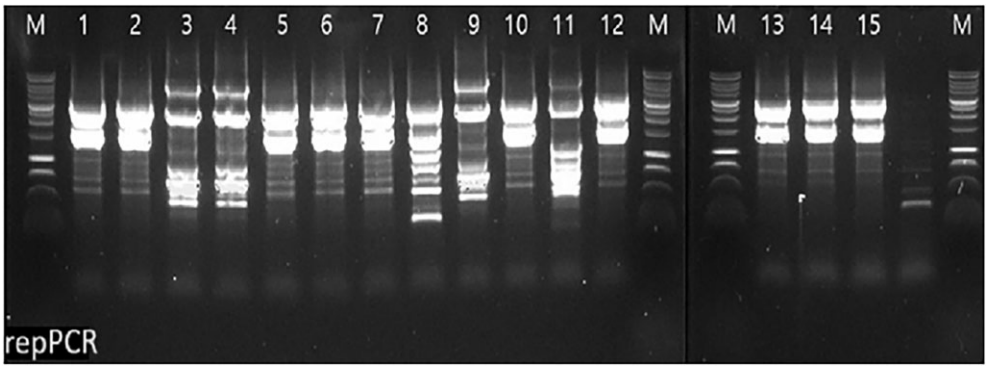

Figure 2. Electrophoresis fingerprint differentiation of the isolates based on repPCR. A cluster represented in this study by *E. faecium* ST01JL is widely represented in different environment swab samples—Samples: 1, 2, 5, 6, 7, 10, 12, 13, 14, and 15; M: 1 kb marker.

25 600 AU/ml from each *E. faecium* strain after 15 h and after 18 h for *Lc. garvieae*. After 24 h of incubation, the pH decreased from 6.3 to 4.4 for *E. faecium* ST01JL, to 4.5 for *E. faecium* ST02JL, to 4.39 for *E. faecium* ST03JL, and to 4.4 for *Lc. garvieae* ST04JL.

Inhibition of metabolically active *L. monocytogenes* and spectrum of activity

The growth inhibition was recorded when an exponential culture of *L. monocytogenes* ATCC7644 was exposed to the presence of bacteriocin containing CFS from the evaluated strains. During early logarithmic phase (3 h after inoculation) 25 600 AU/ml of the selected bacteriocins, corresponding to 2560 AU/ml of final concentration, were added and bacterial growth of *L. monocytogenes* ATCC 7644 was recorded for following 9 h. The addition of all bacteriocin containing CFSs showed a significant inhibition in the growth of *L. monocytogenes* ATCC7644 in comparison with the control without CFS (Fig. 4).

The evaluation of the spectrum of activity of the four bacteriocins against microorganisms listed in Table 2 showed that only *L. monocytogenes* ATCC 7644 and five other *Listeria* species isolated from foods were inhibited when exposed to bacteriocinogenic CFSs (Table 2). No inhibition was observed for the other spoilage species analyzed.

Screening for virulence and bacteriocin production associated genes

Based on the performed PCR screening for the presence of genes associated with safety, the detection technique showed that both *E. faecium* ST01JL and ST03JL harbor the gene *tdc*, and *Lc. garvieae* ST04JL harbors the genes *ace* and *cyt* (Table 1). Concerning the presence of genes associated with the bacteriocin production, positive results were recorded for the harboring of *entA* and *entP* for all studied strains and *ped* for *E. faecium* ST02JL and *Lc. garvieae* ST04JL (Table 1).

Discussion

The application of LAB and their antimicrobials has been the subject of numerous studies in the last decades aiming to formulate effective tools for improving food safety and effectively controlling food borne pathogens and spoilage microorganisms. The dairy industry is the sector in which this approach was effectively explored with the use of nisin, a bacteriocin produced by *Lactococcus lactis* strains (Rollema et al. 1995, Kruger et al. 2013, Furtado et al. 2019).

The isolation of LAB strains with bacteriocinogenic properties obtained in the present study underlines the persistence of this group of bacteria at different production surfaces in an artisanal cheese making facility. LAB play an essential technological role in the production of different dairy products (including cheeses) in addition to being associated to the preservation and safety of different dairy food commodities. As part of the milk natural microbiota (or should we say bacteriobiota, since according to Shanahan and Hill (2019), this can be more appropriate term), LAB can be isolated from different dairy environmental surfaces, where they can contribute to the safety and reduction of spoilage and/or pathogenic bacterial species.

The presence of *L. monocytogenes*, a well-known dairy food borne pathogen, was previously reported to be associated with dairy environment surfaces due to the ability to form biofilms, making this pathogen a serious hazard for the industry and a health threat to consumers (Zhao et al. 2013, Pérez-Ibarreche et al. 2014, Ribeiro et al. 2023). Moreover, some LAB, including beneficial ones, have been reported to be isolated from surfaces of dairy processing facilities (Irlinger et al. 2009, Bokulich and Mills 2013, Ksontini et al. 2013, Stellato et al. 2015), from milk (Martín-Platero et al. 2009, Furtado et al. 2019), from animals' gut (Van Tyne and Gilmore 2014), and from animal feed (Colombo et al. 2018). However, not all LAB may be beneficial. In fact, some of them are characterized as foodborne pathogens and/or spoilage organisms and can be associated with several clinical cases (Fiore et al. 2019). A few illustrative examples are different *Streptococcus* spp., where only a few are considered beneficial, such as *Streptococcus thermophilus*, *Streptococcus salivarius*, and *Streptococcus gallolyticus*, with the remaining species being classified as pathogens (dos Santos et al. 2020, Bourdichon et al. 2022). The genus *Enterococcus* can be an additional example, where representatives can be considered as pathogens associated with nosocomial infections and antibiotic resistance carriers (Vankerckhoven et al. 2004, Gomes et al. 2008, Werner et al. 2011, Arias and Murray 2012, Kim et al. 2019, Liese et al. 2019) or effective probiotics and multitasking starter cultures with biopreservative functions (Marekova et al. 2007, Todorov et al. 2010, Favaro et al. 2014, Cavicchioli et al. 2017, Holzapfel et al. 2018, Iseppi et al. 2019, Valledor et al. 2022).

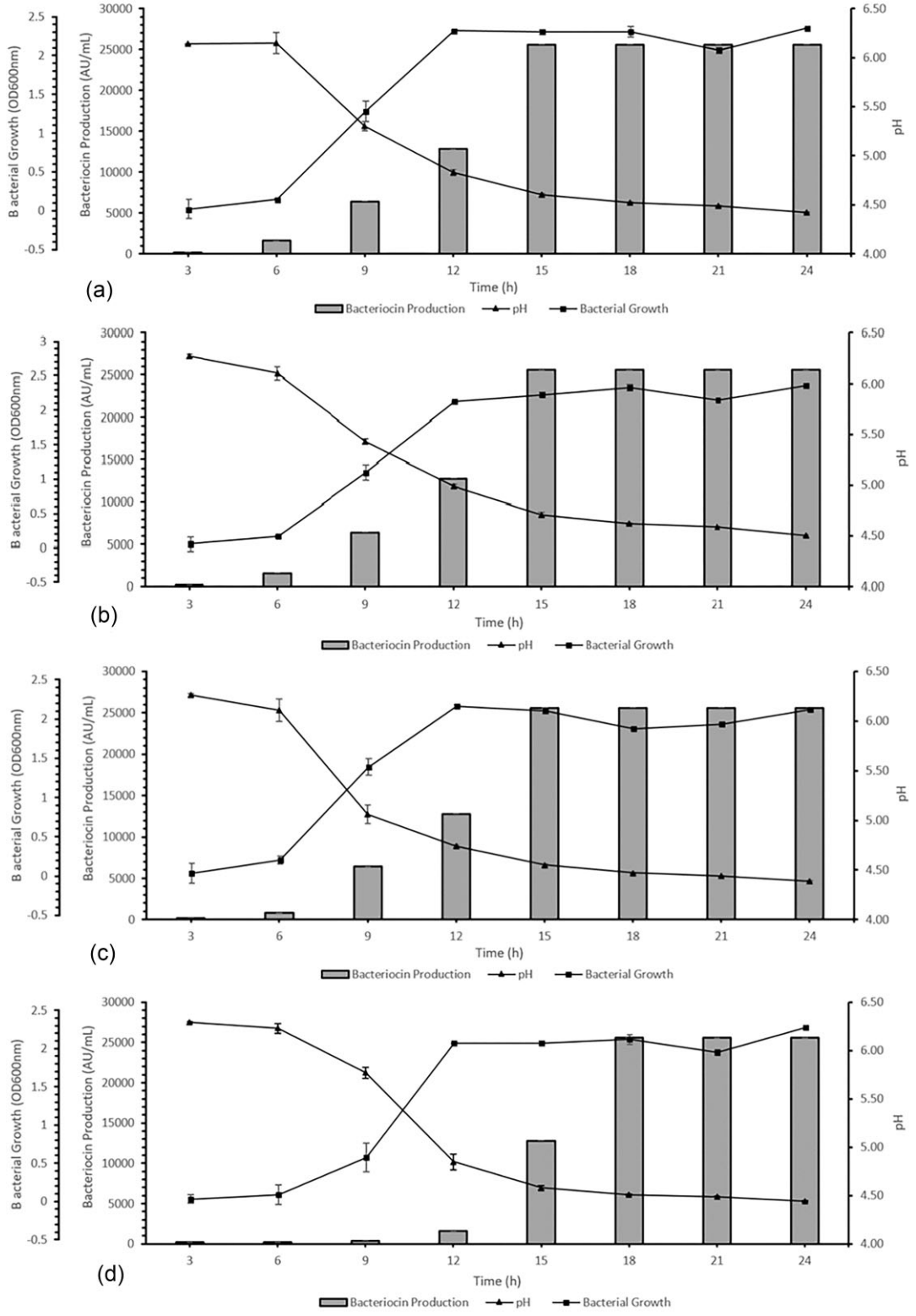

Figure 3. Bacteriocin production, acidification profile, and bacterial growth of strains: (a) *E. faecium* ST01JL, (b) *E. faecium* ST02JL, (c) *E. faecium* ST03JL, and (d) *Lc. garvieae* ST04JL.

In the present study, after preliminary screening for potential producers of bacteriocins, we preselected 14 LAB isolates, identified to be part of the *E. faecium* species and one as *Lc. garvieae*. According to the performed tests reported in Table 1 and a preliminary screening, all of them were confirmed to be producers of bacteriocins with anti-listerial activity and to present a safe profile. The presence of *E. faecium* strains in the dairy environment has been reported as

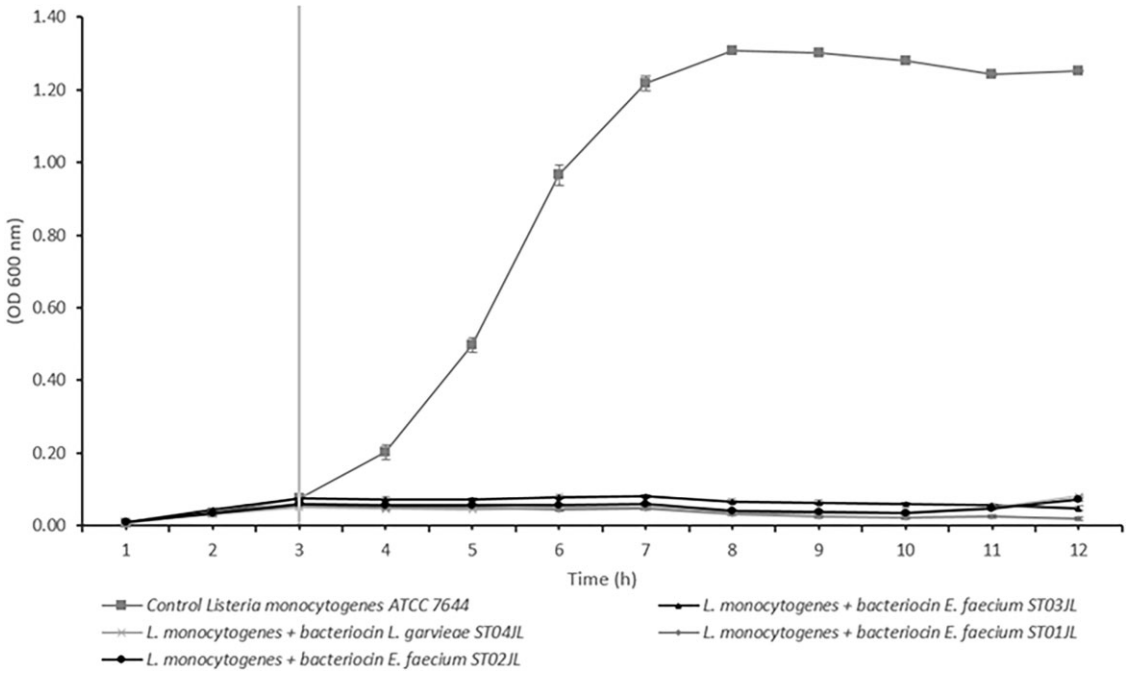

Figure 4. Bacterial growth inhibition of *L. monocytogenes* ATCC7644. The vertical line represents bacteriocinogenic CFS addition at 3 h of incubation except for the control.

common (Marekova et al. 2007, Stellato et al. 2015, Furtado et al. 2019). Different representatives of this species were reported, including those showing beneficial and technological properties in cheeses (Cavicchioli et al. 2019, Furtado et al. 2019), fermented milks (Lianou et al. 2017), fermented fruits and vegetables (Onda et al. 2002, Linares-Morales et al. 2020), and meat and fish products (Todorov et al. 2012). Schirru et al. (2014) and Favaro et al. (2015) reported the presence of different *E. faecium* strains isolated form Italian and Bulgarian cheeses, respectively, with potential biopreservation beneficial properties for the production of South-European cheeses.

The addition of selected chemicals (1% NaCl, SDS, and Tween 80) to the CFS of studied strains did not influence the bacteriocin activity. Among the range of temperature exposure (1 h at 8°C, 30°C, 37°C, 45°C, 60°C, 80°C, and for 15 min at 121°C) no differences were found in activity. Regarding the pH ranging from 2.0 to 10.0, we observed that the bacteriocinogenic activity dropped in alkaline conditions. These results are similar to those reported by Cavicchioli et al. (2017) and Iseppi et al. (2019). Also, Rollema et al. (1995) pointed out that nisin is stable at low pH and progressively loses its activity with higher pH levels. Similarly, bacteriocin LacB23 produced by *Lactiplantibacillus plantarum* J23 (Zhang et al. 2018) loses its activity only when exposed to 121°C for 30 min. Thermostability is a typical characteristic for most of the bacteriocins, and even 15 min at 121°C does not influence their structure and biological activity, most probably associated with the fact that they are small peptides with low molecular weight, generally below 10 kDa (for class II bacteriocins), but other can be a bit bigger, as it is the case of Class III bacteriocins, according to the classification of Klaenhammer (1988). This specificity in their molecular size can be a reason for the stability of the majority of the described bacteriocins in the literature. Moreover, most of the bacteriocins are single-molecule polypeptides or small proteins, and this is an additional argument in explaining their stability to thermal treatments.

Stability of bacteriocins to different chemical agents can provide relevant information related to the practical application of antimicrobials. In this context, stability to the presence of NaCl or milk proteins is an argument for the potential applications in biopreservation of dairy products. However, it is well known that bacteriocins can lose their activity in meat products associated with the interactions between them and lipids, resulting in complete or partial deactivation (Todorov et al. 2022, Gu 2023). It is worth to underline that several bacteriocins, especially those belonging to the class IIa, recognize lipid II on the cell surface of the target microorganisms, and in some cases, the presence of a high lipid content in the food can result in the formation of complexes in the environment and as consequence, reduced bacteriocin effectiveness. From a practical standpoint, understanding the tolerance of the studied bacteriocins to various conditions examined in the current study is crucial since it can lead the different technological applications of these potential antimicrobials in different biopreservation processes.

Based on biochemical and physiological tests, followed by 16S rRNA partial gene sequencing, only one of the tested isolates, the ST04JL, was identified to be part of the *Lc. garvieae* with identity score of 98% (NR_113268.1 accession number from the GenBank), and remaining 14 isolates were identified as part of the species *E. faecium* with identity score equal or more than 98% for isolates ST01JL, ST02JL, and ST03JL (accession numbers from the GenBank: NR_112039.1, NR_042054.1, and NR_114742.1, respectively), representing highest and lowest obtained identity values. At this stage, the question remained, are these 14 isolates identified as *E. faecium* different strains or are they replica of the same strain isolated from multiple sites? Based on the performed repPCR analysis according to the recommendations of de Moraes et al. (2000), the 14 *E. faecium* isolates can be

grouped into three clusters, taking into consideration the generated fingerprint profile. It is interesting to mention that cluster one, grouping 10 isolates, was associated with different abiotic spots from the investigated environment. The combination of the bacteriocinogenic properties with such a high presence of the mentioned strain in different surfaces of the dairy processing plant can be an argument in agreement with the hypothesis that bacteriocinogenic properties may improve the survival of bacterial cultures and their spread in an ecological niche. Previously, it was reported that *S. thermophilus*, thermophilic lactobacilli, mesophilic lactobacilli, lactococci, and enterococci, commonly bacteriocinogenic strains, were predominant in the production facilities (Lortal et al. 2009). In a previous study, Favaro et al. (2023) pointed to the isolation of the same bacteriocinogenic strain of *Pediococcus pentosaceus* ST58 from the oral cavity of the same volunteer over a 24-week period. Bacteriocin production enhances the competitiveness of microbial cultures. It is even suggested that the primary physiological role of bacteriocins may not be their killing properties. Instead, they may play a crucial role in microbial interactions and facilitate the competitiveness of their producers in the competition for living resources (Chikindas et al. 2018).

Representatives from the *Lactococcus* genus have been previously isolated from dairy environments, as reported by Casalta and Montel (2008) and Cleveland et al. (2001). Their role in dairy fermentations is controversial, as they are considered more as agents of spoilage rather than as starters or beneficial cultures. Furthermore, this species is predominantly classified as an opportunistic pathogen for fish (Abdelfatah and Mahboub 2018, Martinovic et al. 2021). However, some studies have mentioned this species as naturally present in milk and dairy products (Cleveland et al. 2001, Villani et al. 2001).

Regarding *E. faecium*, it can be stated as one of the most controversial species within the LAB group. On one hand, *E. faecium* is associated with fermentation process of different food products, especially those from the Mediterranean region (Terzić-Vidojević et al. 2015), also associated with production of dairy (Schirru et al. 2014, Terzić-Vidojević et al. 2014, Favaro et al. 2015), meat (Lauková et al. 2020), fruit, and vegetables (Linares-Morales et al. 2020), where it plays an essential role in formation of organoleptic properties (Foulquié-Moreno et al. 2006). The species is part of the gastrointestinal tracts of humans and other animals (Sánchez et al. 2007, Borrero et al. 2011) with some strains even being described as probiotics and applied for a few decades as safe beneficial cultures (Holzapfel et al. 2018, Shi et al. 2019). On the other side, *E. faecium* cultures can be characterized as nosocomial pathogens, posing serious health concerns in hospital settings (Liese et al. 2019), including several cases of vancomycin resistant enterococci and even some multidrug resistance strains (Veljović et al. 2014). Thus, a thorough safety evaluation was conducted for the isolated LAB strains selected in this study.

The selected strains were characterized as γ-hemolytic, not presenting mucin degradation properties, with a very moderate profile of antibiotic resistance. Ensuring safety for the strains with potential to be applied in food industry is an essential milestone. Antibiotic resistance is crucial in the evaluation of safety profile for new isolates. The spread of the antibiotic resistance in the last decade between different starter and other microbial cultures with technological and/or beneficial properties is linked to serious health problems (Kim et al. 2019, Duche et al. 2023). As previously mentioned, enterococci can be carriers of vancomycin resistance genes, and some can be even characterized as multidrug resistance microbial cultures (Arias and Murray 2012). For this reason, physiological tests applying antibiotic discs and screening for the presence of vancomycin resistance genes has become a compulsory test in the evaluation of enterococci as safe cultures (Humphries et al. 2021). The four currently investigated strains in this project were identified as not carrying vancomycin resistance genes, neither presenting resistance based on the applied physiological test with antibiotic disks. However, ST01JL, ST02JL, ST03JL, and ST04JL only showed resistance to streptomycin among the nine tested antibiotics. Suvorov (2020) and Favaro et al. (2023) suggested that moderate antibiotic resistance in beneficial microbial cultures can be viewed positively. This is particularly relevant for cultures intended for use as probiotics, as their coexistence with antibiotics may enhance therapeutic benefits when used together (Favaro et al. 2023).

One requirement imposed by regulatory authorities is that potential probiotics (and other beneficial microbes) need to be free of virulence factors, γ-hemolytic activity, not presenting mucin degradation properties, and not producing biogenic amines. These characteristics were verified for the studied strains based on performed physiological tests and screening for the selected genetic determinants (Table 1). According to the results listed in Table 1, the strain of *E. faecium* ST02JL should be considered the most promising culture once that was the only one that did not have any virulence gene while expressing three different bacteriocin genes. On the other hand, the *tdc* gene (described to produce tyramine) (de Las Rivas et al. 2005) is potentially present in the strains ST01JL and ST03JL. Consequently, a thorough investigation should be conducted to confirm if these strains are able to produce tyramine. It is worth noting that production of tyramine is commonly observed in the strains within the enterococci group (Bover-Cid and Holzapfel 1999).

The cytolysis gene *cyl* related to adherence to eukaryotic cells and *ace* related to adhesin of collagen protein, both found in the screening of ST04JL strain, can be considered important virulence genes. However, among enterococci, the frequency of the *cyl* gene is very variable, and it has not been clearly associated to every clinical isolate (Semedo et al. 2003, Creti et al. 2004). Additionally, *ace* gene displays allelic sequence variations that can influence the ability for adherence to collagen protein (Creti et al. 2004). Therefore, there is not enough evidence to rule out the strains as promising biotechnological or probiotic agents.

Based on the performed tests for interaction between produced antimicrobials by *Lc. garvieae* ST04JL, and *E. faecium* ST01JL, ST02JL, and ST03JL and applied Proteinase K, the proteinaceous nature of the expressed bacteriocins has been confirmed. In assessing the spectrum of activity of the expressed bacteriocins, by investigating CFS from the studied strains, robust antimicrobial activity was observed against *L. monocytogenes* ATCC 7644, 101, 211, 506, 712, and 724 (food isolates from collection of the Faculty of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil). No inhibitory activity was observed against *Bacillus cereus* ATCC11778, *Escherichia coli* ATCC8739, *Enterobacter aerogenes* ATCC13048, *Clostridium perfringens* ATCC13124, *Salmonella* serotype Enteritidis ATCC13076, and *S. aureus* ATCC29213. One of the main differences

between bacteriocins and antibiotics lies in the specificity of their spectrum of activity. In general, antibiotics have a broad spectrum of activity, while bacteriocins are much more selective and specific in their inhibitory spectrum, even in some cases down to some specific species (Perez et al. 2014). This characteristic is significant as the application of bacteriocins ensures high selectivity, avoiding the inhibition of other beneficial microbial cultures. Thus, it is important that in the evaluation of the spectrum of activity, several beneficial microbial cultures are included to guarantee that they will not be inhibited by the studied bacteriocins.

Addition of the CFS from the studied bacterial cultures to the exponentially growing *L. monocytogenes* ATCC7644 resulted in complete inhibition over 12 h incubation period (Fig. 4). However, in following additional 12 h (up to 24 h time experiment), *L. monocytogenes* recovered growth and reached levels of OD similar to that of the control, which did not receive CFS. When samples from 12 h experimental setups were taken and checked for the presence of viable cells of *L. monocytogenes* by plating on Palcam agar plates, a recovery of individual colonies was observed. All of this is an argument that the studied bacteriocins present a bacteriostatic mode of action and in a 12 h period, they were able to suppress the bacterial growth of *L. monocytogenes*. The absence of changes in the turbidity of the experimental setups receiving filter-sterilized CFS containing the studied bacteriocins provided a strong indication of inhibition against the tested bacteria. However, low levels or even no changes in the OD of the experimental setups do not always conclusively demonstrate the complete eradication of the tested microorganisms. Similar observations have already been reported in the evaluation of different bacteriocins, including enterocins and lactocins (Kruger et al. 2013, Furtado et al. 2019, Fugaban et al. 2021a, Valledor et al. 2022).

When cultured in MRS broth at 37°C, all four bacteriocinogenic strains showed similar behavior regarding bacterial growth, acidification, and production of bacteriocin/s tested against *L. monocytogenes* ATCC7644 (Fig. 3). All strains reached the stationary phase after 12 h of incubation (Fig. 3). Acidification resulted in changes from 6.26 to 4.42 for ST01JL, from 6.27 to 4.50 for ST02JL, from 6.24 to 4.39 for ST03JL, and from 6.26 to 4.44 for ST04JL. This pattern was previously reported for other enterococci grown in similar conditions (Fugaban et al. 2021a, Valledor et al. 2022). Moreover, the main objective of the experiment was to follow the production of bacteriocins. For *E. faecium* ST01JL, *E. faecium* ST02JL, and *E. faecium* ST03JL 25 600 AU/ml was recorded at 15 h and for *Lc. garvieae* ST04JL it was 26 600 AU/ml at 18 h during the fermentation process. None of the studied bacteriocins showed a decrease in the activity over the studied period. Most of the reported bacteriocins in the literature have been reported to be stable during the fermentation period of 24–48 h (Fugaban et al. 2021a, Valledor et al. 2022). However, in some cases, a decrease in bacteriocin activity has been observed in late stationary phase, and it has been suggested that this can be associated with autodegradation of the bacteriocins, or inhibitory processes associated with the environmental pH, or with formation of the aggregation sub-molecular structures where bacteriocin activity may be compromised (Perez et al. 2014, Chikindas et al. 2018).

Screening of bacteriocin production genes showed that *entA* and *entP* were collectively identified for all the evaluated strains. These results are expected and similar to those found in other studies (Fugaban et al. 2021a, Valledor et al. 2022). The gene *ped* was also found for *E. faecium* ST02JL and *Lc. garvieae* ST04JL. In this study, the presence of the identified genes reinforces the bacteriocinogenic properties observed in the previous assays. The enterocin genes identified in our strains belong to class II, "pediocin-like" bacteriocins, with a general mechanism of action that causes cell lysis. They are usually small and heat-stable peptides and have specific docking region that keep them in their active (open) conformation, leading to the leakage of extracellular matrix components, ultimately resulting in cell death (Ness et al. 2014, Perez et al. 2014).

The Brazilian Ministry of Health has established safety standards for foods, including artisanal cheeses, to ensure consumer health (Brazilian Ministry of Health 2022a, 2022b). In order to meet these safety standards, the current isolates and their respective bacteriocins can be good candidates to be considered as alternative bio-preservation tools aligned with current trends in society, industry, and academia, which seek to replace traditional preservation methods that employ common chemical agents.

Conclusion

The findings from this study revealed *E. faecium* strains as highly prevalent on dairy production surfaces, demonstrating strong inhibitory activity against *L. monocytogenes*. From initial 21 isolates with safe profile, 15 were selected for further studies associated with their bacteriocinogenic activity, and 14 of them were identified as *E. faecium*. Moreover, they were clustered into three groups, where *E. faecium* ST01JL generated the same repPCR profile as nine other isolates obtained from different collecting spots in the same dairy facility. In contrast, *E. faecium* ST02JL was a duplication of two other isolates; and *E. faecium* ST03JL was a unique strain in the screening procedure. The studied strains *E. faecium* ST01JL, ST02JL and ST03JL and *Lc. garvieae* ST04JL generated amplicons for the presence of genes *ent*A and *ent*P, associated with production of two types of enterocins (A and P); moreover, *E. faecium* ST02JL and *Lc. garvieae* ST04JL presented evidence for carrying a gene associated with production of pediocin PA1. These LAB strains, along with their bacteriocins, hold potential for effectively controlling the growth of *L. monocytogenes* on abiotic surfaces associated with artisanal cheese production. Most importantly, these strains and their bacteriocins showed satisfactory phenotypic and genotypic safety profiles. Further studies on application, including dosage, frequency, and combinations with other antimicrobials will need to be performed to suggest effective strategies for controling *L. monocytogenes*.

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

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Author contributions

João Marcos Scafuro Lima (Formal analysis, Investigation, Methodology, Writing – original draft), Kayque Ordonho Carneiro (Formal analysis), Uelinton Manoel Pinto (Funding acquisition, Project administration, Writing – review & editing, Resources, Supervision), and Svetoslav Dimitrov Todorov (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing)

Data availability

All data generated or analyzed during this study are included in this published article and comply with research standards.

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