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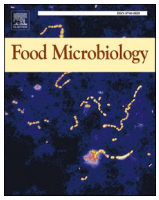

Lactic Acid Bacteria isolated from traditional and innovative *alheiras* as potential biocontrol agents

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ABSTRACT

From a selection of seven traditional and 14 innovative *alheiras*, 491 lactic acid bacteria (LAB) were isolated and tested for their antimicrobial activity against several food-borne pathogens. Among these, six strains revealed antimicrobial activity through potential bacteriocin production against 14 *Listeria monocytogenes* strains, *Enterococcus faecalis* ATCC 29212, *Clostridium sporogenes* ESB050, and *Clostridium perfringens* ESB054.

Through whole genome sequencing (WGS), these strains were identified as *Lactiplantibacillus plantarum* (2), *Leuconostoc mesenteroides* (1), and *Pediococcus acidilactici* (3). Furthermore, several orthologues of class II bacteriocins genes were identified, including Plantaricin E, Plantaricin F, Pediocin PA, Enterocin X, Leucocin A, and Coagulin A. No virulence or antibiotic resistance genes' orthologues were detected by WGS analysis. However, the selected LAB strains showed variable phenotypic patterns related to virulence genes and antibiotic resistance when assessed through classical methodologies. None of these strains demonstrated the production of biogenic amines, gelatinase or DNase. Additionally, no hemolytic activity or lipase enzyme production was observed. However, only *Lpb. plantarum* 9A3 was sensitive to all tested antibiotics and was thus chosen for further examination. The bacteriocins produced by *Lpb. plantarum* (9A3) exhibited stability across a broad range of conditions, including temperatures from 4 to 100 °C, pH values ranging from 2 to 8, exposure to surfactants and detergents (Tween 20 and 80, SDS, EDTA 0.1, 2 and 5 mM, urea and sodium deoxycholate), and enzymes (papain and catalase). Their maximum activity (AU/mL = 12,800) against four *L. monocytogenes* strains was observed between 21 and 36 h of growth of *Lbp. plantarum* 9A3, indicating a bacteriostatic mode of action. Therefore, this strain appears to be a robust candidate for potential application as a protective strain to be used in the food industry. Not only is it safe, but it also produces stable bacteriocins (harbouring genes encoding for the production of three) effectively inhibiting significant pathogens such as *L. monocytogenes* and *C. perfringens*.

1. Introduction

Sausages are a valuable part of the gastronomic legacy worldwide and have been a cherished part of gastronomy for centuries. *Alheira*, a traditional smoked and naturally fermented meat sausage originating from northern Portugal, stands as a highly appreciated product (Abrams et al., 2011; Albano et al., 2009a; Macieira et al., 2019). Traditionally, *alheiras* consist mainly of shredded pork and poultry meats, traditional wheat bread, olive oil, pork fat, and spices (Albano et al., 2009b). However, evolving consumer preferences for healthier, tastier, and higher quality products have led to the emergence of new *alheiras* crafted from ingredients such as fish, mushrooms, tofu, soy, and vegetables. These innovative variations, though produced by the same companies, lack harmful organisms often found in traditional *alheiras* (Azevedo et al., 2020). The microbiota of these fermented sausages frequently includes lactic acid bacteria (LAB), pivotal for aroma and flavour alongside the influence of the raw materials and maturation processes. Species like *Lactiplantibacillus plantarum, Leuconostoc mesenteroides* and *Pediococcus acidilactici* are commonly found in fermented meat sausages (Albano et al., 2007; Amaral et al., 2015; Azevedo et al.,

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2020; Campos et al., 2013). Given their usual presence in fermented foods, several LAB are considered "Generally Regarded As Safe" (GRAS) by the American Food and Drug Administration and "Qualified Presumption of Safety" status (QPS) granted by the European Food Safety Authority (EFSA).

Extensive research on LAB has been conducted due to their ability to produce bacteriocins - low molecular, thermally stable, antimicrobial, and ribosomal active peptides - that have proven to be active against food-borne pathogens (Barbosa et al., 2021; Martín et al., 2023; Verma et al., 2022). The emerging consumer demand for food products with fewer chemicals and more natural bio-preservation has conducted intense research on bacteriocin studies to discover new antimicrobial compounds that effectively combat pathogens in food products. In addition, the European Commission recently established new limits for nitrites and nitrates (EC, 2023), food additives commonly used in processed meats not only to enhance color and extend shelf life but also for their antimicrobial properties (e.g. preventing the germination of clostridial spores), prompting food companies to adapt within a two-year period.

Given the escalating global concerns about food safety, considerable attention is being directed toward utilizing bacteriocins to control food spoilage and/or growth of food-borne pathogens without compromising the food product itself (O'Connor et al., 2020; Soltani et al., 2021). In the study of Prpich et al. (2021), the authors highlighted the significance of indigenous microbiota in defining the uniqueness and distinctive sensory characteristics of fermented sausages. The constant need for innovation in the artisanal food production makes it essential to explore alternative tools like autochthonous cultures demonstrating technological and/or probiotic traits, or bacteriocin-producer strains, that allow for improving quality and safety.

The main objectives of this study were 1) to evaluate the antimicrobial activity of LAB isolated from both traditional and innovative *alheiras*, against several food-borne pathogens; 2) to characterize the bacteriocins produced by selected LAB, considering the absence of antibiotic resistance and virulence factors, using whole genome sequencing (WGS) analysis; and 3) to characterize *in vitro* the mode of action and stability of the produced bacteriocin(s) to ascertain the potential of the selected *Lpb. plantarum* 9A3 as a protective culture in food production.

2. Materials and Methods

2.1. Study of antimicrobial activity potential of isolated LAB

All LAB isolates were collected from both traditional and "innovative" *alheiras*, as detailed in our previous study (Azevedo et al., 2020). Target microorganisms (Supplementary Table 1) were grown on Tryptic Soy Agar supplemented with Yeast Extract (6 g/L) (TSA + YE; Biokar) and incubated at 37 °C for 24 h. Subsequently, a single colony from each isolate was inoculated into 10 mL of Tryptic Soy Broth with 6% Yeast Extract (TSB + YE; Biokar). These overnight cultures, incubated at 37 °C, were spread-plated onto TSAYE. Antimicrobial activity was assessed by spotting droplets of each LAB culture grown twice in MRS broth (Biokar), onto the bacterial lawns of each target bacterium, following the technique by Van Reenen et al. (1998). Inhibition was considered if a translucent halo was detected around the spot after overnight incubation at 37 °C.

For LAB isolates demonstrating antimicrobial activity, the inhibition nature was determined using a qualitative agar-diffusion technique according to Tomé et al. (2006). Briefly, broth cultures of each LAB were centrifuged at 6500×*g* for 10 min at 4 °C (Hettich 108 Zentrifugen, Rotina 35 R, Germany). The clear supernatants were then sterilized by membrane filtration (CFS) (0.2 μm; Sartorius, Goettingen, Germany) and pH adjustment between 5.0 and 6.0 was performed (CFSn) using a sodium hydroxide solution (1 M NaOH, José M. Vaz Pereira, Lisbon, Portugal). To discriminate inhibition caused by hydrogen peroxide production or by proteinaceous compounds, catalase (500 IU/mL; Sigma-Aldrich) and proteinase K (0.1 mg/mL, sterile; Sigma-Aldrich), respectively, were added to neutralized cell-free supernatant (CFSn) and incubated at 37 °C for 1 h. Subsequently, the antimicrobial activity of CFS, CFSn, CFSn treated with catalase (CFSnC) and CFSn treated with proteinase K (CFSnK) was assessed whenever cultures in MRS displayed inhibition against the target organisms. *Pediococcus acidilactici* HA-6111-2 served as *anti*-listerial control strain (Albano et al., 2009a). The presence of a proteinaceous substance, potentially a bacteriocin, was indicated by a clear halo zone surrounding all spots except in the case of CFSnK.

2.2. Whole Genome Sequencing and bioinformatic analyses

The genomic DNA from the six selected LAB strains was extracted following the manufacturer's protocol for total DNA purification from Gram-positive bacteria (Grisp, Porto, Portugal). All samples underwent rigorous analysis to ensure optimal concentration, integrity, and purity for library subsequent preparation. Subsequently, 100 ng of genomic DNA from each sample was run on Illumina DNA Prep (Illumina) and sequencing parameters were obtained using NovaSeq 6000, 2 × 100 bp. For bioinformatic analysis, sequencing reads were demultiplexed using Illumina bcl2fastq 2.20 and adapters were trimmed using Skewer v0.2.2 (Jiang et al., 2014). Quality assessment of FASTQ files was conducted using FastQC v0.11.5-cegat (Andrews, 2010). Both forward and reverse fastq files were subjected to de novo assembly using SPAdes v3.13.1 (Prjibelski et al., 2020) to generate fasta DNA files, which were annotated using Prokka 1.14.6 (Seemann, 2014). Mash 2.3 software was employed to estimate the closest bacterial genome from the refseq genome database (accessed March 15, 2023 at https://gembox.cbcb.umd.edu/mash/refseq.genomes.k21s1000.msh). The closest genome for each LAB genome was downloaded from the Refseq database (accessed on May 22, 2023 at https://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria) and utilised as a reference while the genome assemblies were evaluated using QUAST v5.2.0 (Mikheenko et al., 2018) and circus 0.69–8 (Lui et al., 2021) for visual inspection of the assemblies. All programs were run in a Linux operating system environment.

To predict resistance phenotypes from the assembled genomes, the ResFinder-EFSA server was used accessed on March 22, 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 23, 2023 (sample 1A5), at https://cge.food.dtu.dk/services/ResFinder-EFSA/) (Bortolaia et al., 2020). Similarly, the presence of virulence factor determinants was assessed through a blastn alignment (accessed on March 23, 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 24, 2023 (sample 1A5)), at http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) (Liu et al., 2019), using the default parameters.

To identify bacteriocins produced by these LAB strains, the fasta files containing the generated contigs were searched for bacteriocins on the BAGEL4 web server (accessed on March 22, 2022 2022 (samples 4–8 and 10A2), November 17, 2022 (samples 9A3, 18–8 and 21–2/2) and March 23, 2023 (sample 1A5), at http://bagel4.mongenrug.nl) (Van Heel et al., 2018).

2.3. Bacteriocin-producing LAB selection and characterization

2.3.1. Selected strains

Six strains were chosen based on their demonstrated ability to produce proteinaceous compounds, potential bacteriocins, with activity against various pathogens. These stains comprised two *Lpb. plantarum* (1A5 and 9A3), one *Ln. mesenteroides* (4–8), and three *P. acidilactici* (10A2, 18–8 and 21/2-2), each isolated from distinct sources. *Lactiplantibacillus plantarum* 1A5, *P. acidilactici* 18–8 and 21/2-2 were isolated from traditional *alheiras* while the remaining strains were isolated from *alheiras* made with "innovative" ingredients such as codfish (*Ln. mesenteroides* 4–8), vegetables & mushrooms (*Lpb. plantarum* 9A3), and

shiitake mushrooms (*P. acidilactici* 10A2).

2.3.2. Antibiotic susceptibility testing

The minimum inhibitory concentrations (MIC's, μg/mL) of antibiotics ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline (all from Sigma-Aldrich) were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI, 2012; CLSI, 2017). Antibiotics were chosen based on the recommendations of the European Food Safety Authority, ensuring that concentrations covered the defined breakpoints (EFSA, 2012) for the selected LAB. To prepare each LAB inoculum, overnight cultures grown on MRS agar were used to prepare a suspension in Ringer's solution (Biokar), adjusted to a turbidity equivalent to 0.5 McFarland standards. Broth dilutions of each antibiotic were prepared in 96-well microtiter plates (Sarstedt, Sintra, Portugal) using cation-adjusted Mueller Hinton Broth (CAMHB) (Sigma-Aldrich) supplemented with 2.5% (v/v) of Lysed Horse Blood (LHB) (Thermo Fisher Scientific, Massachusetts, USA). Following 24 h incubation at 37 °C, the presence or absence of turbidity was observed in each well. The minimum inhibitory concentration was considered as the first concentration with no observed growth. Positive controls included isolates grown on CAMHB with and without lysed horse blood with no antibiotic. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 strains were used to monitor the accuracy of MIC values. The susceptibility of isolates was categorised as sensitive, intermediate, or resistant based on the criteria established by EFSA (2012). Two independent replicates were carried out for each test.

2.3.3. Virulence factors

2.3.3.1. Determination of biogenic amine-forming capacity. The screening plate method developed by Bover-Cid and Holzapfel (1999) was used to assess the potential of the six selected LAB strains in producing biogenic amines histamine, tyramine, putrescine, and cadaverine. Plates without amino acid were used as controls. A positive reaction was identified by the presence of a purple color, or the disappearance of the tyrosine precipitate surrounding the colonies. Two replicates were performed for each isolate.

2.3.3.2. Production of hydrolytic enzymes: gelatinase, lipase and DNase. The production of gelatinase and DNase by the six selected isolates was evaluated according to Tiago et al. (2004) and Ben Omar et al. (2004). Lipase production was assayed in MLB broth supplemented with 2.0 g/L of $CaCl_2$ (Sigma-Aldrich) and 10 g/L of Tween® 80 (Sigma-Aldrich). A positive reaction was denoted by the presence of a clear halo around the colonies after 7 days of incubation at 37 °C. All the experiments were conducted in duplicate, and *Staphylococcus aureus* ATCC 29213 served as a positive control.

2.3.3.3. Hemolytic activity. Hemolysis activity was determined by streaking isolates onto Columbia agar plates (Oxoid, Hampshire, United Kingdom) and incubated for 24 h at 37 °C according to the method of Semedo et al. (2003). Alfa(α)-hemolysis and beta(β)-hemolysis were denoted by greenish and translucent zones around the colonies, respectively. Gamma(γ)-hemolysis indicated the absence of hemolytic activity, characterized by the absence of clear zones around the colonies. *Enterococcus faecalis* F2 and *E. faecalis* DS16 were used as β- and α-hemolytic control strains, respectively (Oliveira et al., 2020).

2.3.3.4. Presence of virulence genes. The six LAB isolates were examined for the presence of fifteen virulence genes encoding for the various virulence factors and amino acid decarboxylating enzymes: *ace* (collagen adhesion), *hyl* (hyaluronidase gene), *asa1* (aggregation substance precursor), *agg* (aggregation substance), *esp* (enterococcal surface protein), *gelE* (gelatinase), *efaAfs* and *efaAfm* (cell wall adhesins), *cylA*, *cylB cylM, cylLL* and *cylLS* (cytolytic activity), and *hdc1*, *tdc* and *odc* (histidine, tyrosine and ornithine decarboxylase activity, respectively).

PCR amplifications were conducted in 0.2 mL reaction tubes using a ThermoCycler (Bio-Rad, Hercules, California, USA), each containing 25 μL of mixtures. Details regarding PCR target genes and primers used, respective sequence, and product size of each virulence gene tested are listed in Supplementary Table 2. Electrophoretic separation and controls were carried out according to Oliveira et al. (2020).

2.4. Characterization of bacteriocin(s) produced by Lpb. plantarum 9A3

As a result of the preliminary studies, *Lpb. plantarum* 9A3 was selected for further testing.

2.4.1. Maximum bacteriocin production (AU/mL) during Lpb. plantarum growth

To ascertain the maximum bacteriocin production during its growth, 1% (v/v) of an overnight *Lpb. plantarum* 9A3 culture was inoculated into 100 mL of MRS broth and incubated at 37 °C. At regular intervals over 36 h of incubation, aliquots were taken. Changes in pH, viable counts (Colony Forming Units (CFU)/mL) and bacteriocin activity (AU/mL) against four *L. monocytogenes* serovars (NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a)) were recorded every hour until 24 h, as described by Van Reenen et al. (1998). Three independent replicates were performed.

Bacteriocin activity was determined by successive dilutions of 9A3 CFSn in phosphate buffer (pH 6.5), and 10 μL aliquots from each dilution were spotted onto soft agar plates (BHI with 0.7% w/v agar) inoculated with approximately 10^6 CFU/mL of each target *L. monocytogenes* strain. Plates were then incubated at 37 °C for 24 h. *Pediococcus acidilactici* HA 6111–2 was used as a control. Antimicrobial activity was expressed as Arbitrary Units per mL (AU/mL), calculated as a^b x 100, where "a" represents the dilution factor and "b" is the last dilution that resulted in an inhibition zone of at least 2 mm in diameter. Activity was expressed per mL by multiplying by 100.

2.4.2. Effect of enzymes, surfactants/detergents, pH, and temperature on bacteriocin activity

Lactiplantibacillus plantarum 9A3 was grown in MRS broth overnight at 37 °C followed by cell harvesting through centrifugation (8000×*g* for 10 min at 4 °C). The resulting supernatant (CFS) was pH-adjusted to the range of 5–6 using 1 M NaOH and incubated at 80 °C for 10 min (CFSn). Subsequently, 1 mL of this CFSn was incubated at 37 °C (or at the specific temperature to be studied) under various conditions of pH and studied compound, as outlined in Albano et al. (2007). Antimicrobial activity determination against all four *L. monocytogenes* strains, was carried out according to Van Reenen et al. (1998).

2.4.3. Cell lysis

The mode of action of the bacteriocin(s) produced by *Lpb. plantarum* 9A3 was assessed according to Van Reenen et al. (1998). A volume of 20 mL of filter-sterilized supernatant containing bacteriocin (12,800 AU/mL, pH ≈ 6.0) was added to 100 mL of early exponential phase cultures (6 h old) of each target *L. monocytogenes* serovars. Samples were taken every hour for 12 h and at 24 h and 30 h for enumeration of *L. monocytogenes*. Each *L. monocytogenes* culture without added bacteriocin was used as control and two independent replicates were accomplished.

2.4.4. Adsorption studies, partial purification, and determination of Lpb. plantarum bacteriocin molecular size

Adsorption of *Lpb. plantarum* 9A3 bacteriocin was conducted according to Yang et al. (1992). Succinctly, bacteriocin-producing cells, cultured for 24 h at 37 °C and adjusted to pH 6.0, were harvested by centrifugation (8000×*g*, 10 min, 4 °C) and washed using sterile 0.1 M phosphate buffer (pH 6.5). Pellets were resuspended in 10 mL of 100

Table 1
Distribution of MICs of tested antibiotics for all six LAB selected.

Origin	Sample	Strain	Antibiotics Minimum Inhibitory Concentration (MICs) (mg/L)							
			AMP	CHL	CLI	ERY	GEN	KAN	STR	TET
			Microbiological cut-off values (mg/L) proposed by EFSA for *Lactiplantibacillus plantarum/ pentosus*							
			2	8	2	1	16	64	n.r.	32
Traditional *Alheira*	1A5	*Lactiplantibacillus plantarum*	0.125	4	0.06	**4**	0.5	8	1	8
Vegetables & Mushrooms *Alheira*	9A3		0.125	2	0.125	1	2	32	4	2
			Microbiological cut-off values (mg/L) proposed by EFSA for *Leuconostoc*							
			2	4	1	1	16	16	64	8
Codfish *Alheira*	4–8	*Leuconostoc mesenteroides*	**16**	**128**	**2**	**64**	0.25	16	1	**128**
			Microbiological cut-off values (mg/L) proposed by EFSA for *Pediococcus*							
			4	4	1	1	16	64	64	8
Shiitake mushrooms *Alheira*	10A2	*Pediococcus acidilactici*	**128**	**128**	0.5	**64**	4	64	64	**128**
Traditional *Alheira*	18–8		0.125	2	0.25	**8**	4	8	0.5	4
Traditional *Alheira*	21/2-2		0.06	2	0.06	0.5	0.125	1	0.5	8

MICs higher than EFSA cut-off values are in bold and indicate resistance to the corresponding antibiotic.
AMP – Ampicillin; CHL – Chloramphenicol; CLI – Clindamycin; ERY – Erythromycin; GEN – Gentamycin; KAN – Kanamycin; STR – Streptomycin; TET – Tetracycline. n.r. – not required.

mM NaCl (pH 2.0) for 12 h at 4 °C to facilitate bacteriocin detachment from cells. After cell collection, the cell-free supernatant was neutralized and assessed for bacteriocin activity following Van Reenen et al. (1998). For partial purification, the initial supernatant was refrigerated at 4 °C after which ammonium sulphate was gradually added to reach 60% of saturation and kept at low stirring for 4 h at 4 °C.

The precipitated proteins, in the pellet and floating on the surface, were dissolved in 25 mM ammonium acetate buffer (pH 6.5), after collection by centrifugation (18000×*g*, 20 min, 4 °C), according to the method of Sambrook et al. (1989). All samples were stored at −20 °C. To determine the molecular size of *Lpb. plantarum* 9A3 bacteriocin(s), saturated samples were separated via tricine-SDS-PAGE as previously described by Schägger and von Jagow (1987). A low molecular weight marker ranging from 6.5 kDa to 270 kDa (GRS Protein Marker PLUs; Grisp) was used. Samples were loaded in duplicate onto the acrylamide gel, followed by gel division after run completion. One portion was fixed with 20% isopropanol and 10% acetic acid. Coomassie Brilliant Blue R250 (Bio-Rad) was used to stain the other half to visualise the peptide band position. After unstained and extensively pre-washed with the sterile distilled water, the other half was overlaid with *L. monocytogenes* CECT 911 and CECT 936 cells (10^6 CFU/mL), initially incorporated in BHI soft agar (0.7% agar w/v; Biokar), to determine the position of the active bacteriocin.

3. Results and discussion

A total of 491 isolates, comprising 299 from vegetarian and 192 from traditional *alheiras*, previously isolated and classified as LAB, were selected for assessment regarding potential bacteriocinogenic activity against food-borne pathogens.

3.1. Study of antimicrobial activity potential of isolated LAB

A first screening against 49 target microorganisms (Supplementary Table 1) was performed with 491 LAB isolates, of which 98 were selected due to antimicrobial activity against *E. faecalis* ATCC 29212, various strains of *L. monocytogenes*, *C. sporogenes,* and *C. perfringens*. Furthermore, one among the 98 LAB isolates exhibited inhibition of *S. aureus* ATCC 29213. Subsequently, antimicrobial activity due to bacteriocinogenic activity was investigated using cell-free extracts (CFS) subjected to neutralization (CFSn), addition of catalase (CFSnC), and digestion with proteinase K (CFSnK). Ten LAB strains were selected based on CFS activity, and eight of these maintained their antimicrobial activity even after CFSn and CFSnC treatments against the aforementioned food-borne pathogens. Ultimately, only six isolates demonstrated potential bacteriocin production, demonstrating antimicrobial activity against all strains of *L. monocytogenes*, *E. faecalis* ATCC 29212, one strain of *C. sporogenes* and one strain of *C. perfringens*. Lactic acid bacteria have been widely recognized and described by several authors for its effectiveness in inhibiting Gram-positive bacteria (Abrams et al., 2011; Albano et al., 2007, 2009a; Peng et al., 2017).

3.2. Characterization of lactic acid bacteria bacteriocin producers by whole-genome sequencing

No orthologues corresponding to antibiotic resistance genes were identified using the ResFinder EFSA software. Similarly, no significant blastn alignments were detected between the assembled contigs and databases containing virulence pathogens of pathogenic bacteria, such as proteins involved in secretion systems and their effectors, toxins, and iron acquisition, adhesion and invasion by the bacterial cell. Previous studies have also found LAB isolates lacking virulence factors (Behera et al., 2018; Muñoz-Atienza et al., 2013).

On the other hand, the BAGEL4 software showed that these LAB encode multiple bacteriocins, which may explain their producer phenotype. Supplementary Figs. 1–6 show the genetic organisation maps of the bacteriocin-encoding regions.

Supplementary Fig. 1A display a contig in the assembled bacterial genome that encodes a two-peptide protein. This protein, exhibiting 100% identity with class II bacteriocins, Plantaricin E and Plantaricin F from *Lpb. plantarum*, is adjacent to two *lanT* gene homologues involved in synthesizing lantibiotic compounds, which are synthesised antimicrobial peptides (Singh and Sareen, 2014), and genes encoding the bacteriocin ABC transporter, the ATP-binding protein, and the permease protein PlnG. Another sequence contig in Supplementary Fig. 1B showed 100% identity with pediocin proteins from *P. acidilactici*. Downstream is a *lanT* gene encoding the pediocin PA-1 transport/processing ATP-binding protein PedD and a gene encoding pediocin PA-1 biosynthetic protein PedC.

A contig in strain 4-8 encoding Enterocin X, a 100% identical class II bacteriocin chain beta identical to that found in *Ln. mesenteroides* was revealed in Supplementary Fig. 2. Originally discovered in *E. faecium*, Enterocin X is a two-peptide bacteriocin (Xα and Xβ) with a narrow spectrum of weak to moderate antibacterial activity (Hu et al., 2010). This bacteriocin was later identified in *Leuconostoc* species isolated from a traditional Korean fermented vegetable - kimchi (Mun et al., 2021).

Supplementary Fig. 3A shows a contig encoding a two-peptide protein that is 100% identical to the class II bacteriocins, Plantaricin E and Plantaricin F from *Lpb. plantarum*. Upstream of these plantaricin-encoding genes and in the same operon, two *lanT* gene homologues encode the bacteriocin ABC transporter, the ATP-binding and permease protein PlnG. Similarly to what is shown in Suppl. Fig. 1B, Suppl. Fig. 3B

displays a sequence contig 100% identical with pediocin proteins, as well as a LanT gene and a gene encoding the pediocin PA-1 biosynthetic protein. Moreover, this operon also contains a gene encoding a leucocin A homologue. These class II bacteriocins have been reported in several other studies (Barbosa et al., 2021; Holo et al., 2001; Loessner et al., 2003; Wang et al., 2018a,b).

In Supplementary Fig. 4, the genetic organisation map of *P. acidilactici* strain 10A2 shows the coagulin A and pediocin gene clusters. Coagulin A, a pediocin-like inhibitory substance produced by *Bacillus coagulans*, was first reported in a *Pediococcus* strain by Zommiti et al. (2018), but several other authors have found this bacteriocin in *Pediococcus* strains (Jiang et al., 2021; Rodrigues Blanco et al., 2022; Todorov et al., 2023). Downstream of this gene encoding Coagulin A, and in the same operon, is a *LanT* gene encoding the Pediocin PA-1 transport/processing ATP-binding protein PedD and a gene encoding the pediocin PA-1 biosynthetic protein PedC.

Supplementary Figs. 5 and 6 for *P. acidilactici* 18–8 and 21/2-2, respectively, show a similar genetic organisation map. In both, a gene encoding Enterolysin A can be identified, which has a high prevalence in *Enterococcus* and *Pediococcus* strains, but also in *Lactococcus lactis* (Mileriene et al., 2023; Ormaasen et al., 2023).

3.3. Bacteriocin-producing LAB selection and characterization

3.3.1. Antibiotic susceptibility testing

Table 1 presents the antibiotic susceptibility results for all six LAB isolates. According to EFSA guidelines, evaluating vancomycin susceptibility is not required for obligate and facultative heterofermentative lactobacilli, *Pediococcus* spp. and *Leuconostoc* spp. As these strains are inherently resistant (Keter et al., 2022; Swenson et al., 1990).

In a recent study, Colautti et al. (2022) emphasized lactobacilli as a reservoir of antibiotic resistance (AR) genes. While lactobacilli are generally considered more resistant to vancomycin and aminoglycosides like gentamicin, kanamycin, and streptomycin, and susceptible to erythromycin, β-lactam antibiotics (ampicillin), chloramphenicol, and tetracycline. Despite that, in our study, all isolates demonstrated sensitivity to all aminoglycoside antibiotics tested. Despite the general susceptibility to erythromycin, *Lpb. plantarum* (1A5) and both *P. acidilactici* (10A2 and 18–8) exhibited resistance to this antibiotic, consistent with prior reports (Čanžek Majhenič et al., 2007; Holmes et al., 2016).

Pediococcus acidilactici (10A2) showed resistance to four out of eight antibiotics tested, namely ampicillin, chloramphenicol, erythromycin, and tetracycline. While some studies found *Pediococcus* to be susceptible to ampicillin (Barathikannan et al., 2022; Silva et al., 2019; Singla et al., 2018), all pediococci tested in the study of Federici et al. (2014) were resistant to ampicillin which corroborates our findings related to *P. acidilactici* 10A2. Similarly, while most *Pediococcus* are considered sensitive to chloramphenicol and erythromycin, exceptions have been observed (Basbülbül et al., 2015; Shi et al., 2019; Temmerman et al., 2003) consistent with our study. Some studies reported intrinsic resistance of pediococci to tetracyclines (Danielsen et al., 2007; Federici et al., 2014; Lüdin et al., 2018; Rojo-Bezares et al., 2006), while others found no resistance in isolates from food products (Abbasiliasi et al., 2012; de Sant'Anna et al., 2017; Fguiri et al., 2016; Morandi et al., 2015). Contrary to vancomycin resistance, there is evidence that the *tet (M)* gene can be conjugatively transferred among microorganisms *in vitro* and that resistance to tetracycline is acquired (Gevers et al., 2003), which is a matter of concern.

Regarding *Ln. mesenteroides* (4–8), this LAB exhibited resistance to the same antibiotics as *P. acidilactici* 10A2, plus clindamycin. Regarding ampicillin resistance, *Leuconostoc* species are typically sensitive, but some strains show resistance to other β-lactams (Morandi et al., 2013; Rodríguez-Alonso et al., 2009). While *Leuconostoc* strains are usually susceptible to erythromycin, chloramphenicol, clindamycin, and tetracyclines, cases of resistance have been reported by several authors (Akpinar and Yerlikaya, 2021; Basbülbül et al., 2015; Flórez et al., 2005;

Table 2
Presence of virulence genes.

Origin	Sample	Strain	Virulence Genes															
			agg	esp	gelE	efaAfm	efaAfs	cylA	cylB	cylM	cylLL	cylLS	ace	hyl	*asa1*	*hdc1*	tcd	odc
Traditional *Alheira*	1A5	*Lactiplantibacillus plantarum*	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	-
Codfish *Alheira*	4–8	*Leuconostoc mesenteroides*	+	-	+	+	+	-	-	+	+	-	+	-	+	-	-	-
Vegetables & Mushrooms *Alheira*	9A3	*Lactiplantibacillus plantarum*	+	-	+	+	+	-	-	-	-	-	+	-	+	-	-	-
Shiitake mushrooms *Alheira*	10A2	*Pediococcus acidilactici*	+	-	+	-	+	-	-	+	+	-	-	-	+	-	-	-
Traditional *Alheira*	18–8	*Pediococcus acidilactici*	+	-	+	+	+	-	-	-	+	-	+	-	+	-	-	-
Traditional *Alheira*	21/2-2	*Pediococcus acidilactici*	+	-	+	+	+	-	-	+	+	-	+	-	+	-	+	-

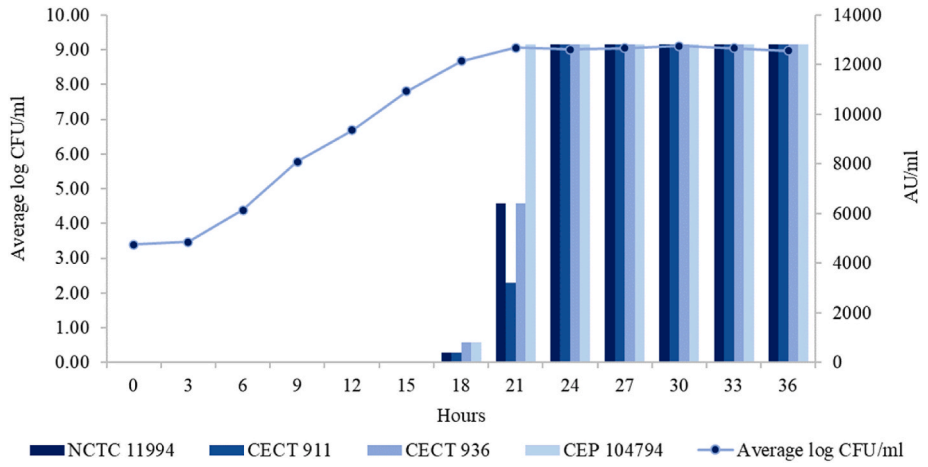

Fig. 1. Bacteriocin production by *Lpb. plantarum* 9A3 in MRS broth. The antimicrobial activity of cell-free supernatant is presented as AU/mL (bars) for *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a). Viable cell counts are also presented as log CFU/mL (line).

Morandi et al., 2013) consistent with our findings. As previously stated by others (Basbülbül et al., 2015; Flórez et al., 2016; Morandi et al., 2013), *Leuconostoc* strains are intrinsically resistant to vancomycin.

3.3.2. Virulence factors

3.3.2.1. Determination of biogenic amine-forming capacity. Lactic acid bacteria are recognized for their decarboxylase activity, potentially leading to the generation of biogenic amines from available amino acids (Alfaia et al., 2018; Özogul and Hamed, 2018). Notably, none of the tested LAB isolates produced cadaverine, histamine, putrescine, or tyramine. Several studies suggest the use of "amine-negative" starter cultures to prevent amine formation in fermented foods (Grujović et al., 2022; Héquet et al., 2007; Lim, 2022). Certain bacteriocin-producing LAB strains possess the ability to reduce biogenic amines levels in fermented foods by producing amine oxidase production or catalysing the oxidative deamination of amines. Consequently, the application of particular strains capable of degrading and/or inhibiting biogenic amines formation has been suggested as a safe approach in the preservation of fermented foods and beverages within the food industry (Joosten and Nunez, 1996; Lim and Choi, 2018).

3.3.2.2. Production of hydrolytic enzymes: gelatinase, lipase and DNase. Gelatinase activity is regarded as harmful as it has the potential to break down collagen, initiating an inflammatory response (Leonardo and Pennypacker, 2009). While lipases are vital enzymes with wide industrial applications, growing evidence suggests their role as significant microbial virulence factors (Dinçer and Kıvanç, 2018; Stehr et al., 2003). DNase, capable of degrading DNA by cleaving phosphodiester linkages in the DNA backbone, may be involved in bacterial growth, biofilm maturation, and evasion of the immune system, thereby acting as a virulence factor (Varela-Ramirez et al., 2017). Interestingly, none of the studied LAB strains produced gelatinase, lipase or DNAse. Similar findings have been reported in several studies indicating the absence of gelatinase and DNase-producing LAB strains (Javed et al., 2022; Keter et al., 2022; Pinto et al., 2020; Silva et al., 2019). However, it is noteworthy that lipase-producing strains are commonly found among lactobacilli, *Pediococcus* and *Leuconostoc* strains (García-Cano et al., 2019).

3.3.2.3. Hemolytic activity. Hemolysis is a virulence factor that supports microorganisms in accessing iron and can lead to host anemia (Keter et al., 2022). In this study, all studied isolates exhibited no hemolytic activity. Lactobacilli are commonly recognized as non-hemolytic (Chen et al., 2022; Cizeikiene and Jagelaviciute, 2021; Halder et al., 2017). Similar observations were made in the studies of Jeong and Lee (2015) and Wang et al. (2018a,b), where *Leuconostoc* strains also showed an absence of hemolytic activity. Recent studies have further confirmed the lack of hemolytic activity in *Pediococcus* species (Barathikannan et al., 2022; Shazadi and Arshad, 2022).

3.3.2.4. Presence of virulence genes. The absence of biogenic amine production as well as hemolytic, gelatinase and DNase activities by our isolates does not necessarily mean that they are not potentially pathogenic or express virulence. In the present study, all isolates lacked *esp*, *cylA*, *cylB*, *hyl*, *hdc1* and *odc* genes (Table 2). Virulence factors have been reported in LAB isolates although they are more commonly associated with enterococci strains. Gelatinase gene (*gelE*) (Suppl. Fig. 7), for example, is commonly found in *Enterococcus* strains and was previously described by Eaton and Gasson (2001) as highly influenced by culture conditions and laboratory manipulation. This might lead to the loss of the structural genes and can explain why, although all tested isolates harboured the *gelE* gene, none presented gelatinase activity during *in vitro* testing.

Aggregation substance, encoded by *agg* and *asa1* genes (Suppl. Fig. 7), was detected in all isolates except *Lpb. plantarum* 1A5. This is a plasmid-carried gene that facilitates the conjugative transfer of sex pheromone gene-containing plasmids through the clattering of one bacterium to another (Galli et al., 1990). *Leuconostoc* and lactobacilli strains tested in the study of Jeronymo-Ceneviva et al. (2014) harboured *asa1* gene yet, from what we know thus far, this gene has never been reported in *P. acidilactici* strains.

3.4. Characterization of bacteriocin produced by Lpb. plantarum 9A3

Following the phenotypic characterization, *Lpb. plantarum* 9A3 was selected due to its susceptibility to all tested antibiotics and absence of virulence factors. The crude supernatant served as the primary material for all experiments conducted to characterize 9A3 bacteriocin(s). WGS confirmed that strain 9A3 has the capability to produce more than one bacteriocin, and therefore, all reported outcomes can be attributed to one or more bacteriocins.

3.4.1. Growth of Lpb. plantarum 9A3 and bacteriocin production

Fig. 1 illustrates the viable counts and antimicrobial activity (AU/mL) of *Lpb. plantarum* 9A3 cell-free supernatant against *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a) over time. The maximum bacteriocin activity (12,800 AU/mL) against all serovars was achieved after 24 h except for *L. monocytogenes* CEP 104794 (1/2a), where it was observed at 21 h. Viable cell counts increased by approximately 3 log CFU/mL after 12 h

Table 3
Temperature effect on antimicrobial activity reduction of *Lactiplantibacillus plantarum* 9A3 bacteriocin, expressed in percentage values, against four different *Listeria monocytogenes* serovars.

Listeria monocytogenes		NCTC 11994		CECT 911		CECT 936		CEP 104794	
		1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h
T (°C)	4	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	0.00%
	25	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	0.00%
	30	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	37	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	60	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
	80	0.00%	0.00%	50.00%	50.00%	0.00%	0.00%	0.00%	50.00%
	100	75.00%	87.50%	87.50%	87.50%	50.00%	75.00%	75.00%	87.50%
	121	98.44%		98.44%		100.00%		98.44%	

Note: With no treatment, maximum activity of 9A3 bacteriocin (s) for each *L. monocytogenes* strain was 12.800 AU/mL. Percentage values of inhibition are represented according to this maximum activity.

Table 4
Antimicrobial activity reduction of *Lactiplantibacillus plantarum* 9A3 bacteriocin, expressed in percentage values, against four different *Listeria monocytogenes* serovars, under pH, detergents, surfactants and protease inhibitors effect.

Listeria monocytogenes		NCTC 11994	CECT 911	CECT 936	CEP 104794
pH	2	0.00%	0.00%	0.00%	0.00%
	4	0.00%	0.00%	0.00%	0.00%
	6	0.00%	0.00%	0.00%	0.00%
	8	0.00%	0.00%	0.00%	0.00%
	10	50.00%	50.00%	75.00%	50.00%
	12	99.22%	99.22%	100.00%	99.22%
Detergents	Tween 20	0.00%	0.00%	75.00%	0.00%
	Tween 80	0.00%	0.00%	75.00%	0.00%
	Triton X-100	50.00%	50.00%	87.50%	0.00%
	SDS	0.00%	0.00%	50.00%	0.00%
	EDTA 0.1 mM	0.00%	0.00%	75.00%	0.00%
	EDTA 2 mM	0.00%	0.00%	75.00%	0.00%
	EDTA 5 mM	0.00%	0.00%	75.00%	0.00%
	Urea	0.00%	0.00%	75.00%	0.00%
	NaCl	0.00%	50.00%	87.50%	50.00%
	Sodium carbonate	87.50%	87.50%	96.88%	87.50%
	Sodium deoxycholate	0.00%	0.00%	75.00%	0.00%
Enzymes (mg/ml)	Proteinase K 1.0	100.00%	100.00%	100.00%	100.00%
	Proteinase K 0.1	100.00%	100.00%	100.00%	100.00%
	Papain 1.0	0.00%	50.00%	87.50%	50.00%
	Papain 0.1	0.00%	50.00%	75.00%	0.00%
	Pepsin 1.0	100.00%	100.00%	100.00%	100.00%
	Pepsin 0.1	100.00%	100.00%	100.00%	100.00%
	Catalase 1.0	0.00%	75.00%	87.50%	50.00%
	Catalase 0.1	0.00%	0.00%	75.00%	0.00%

Note: With no treatment, maximum activity of 9A3 bacteriocin (s) for each *L. monocytogenes* strain was 12.800 AU/mL. Percentage values of inhibition are represented according to this maximum activity.

of incubation, reaching the maximum cell growth around 21 h of incubation.

Considering the characterization of bacteriocin-producing *Lpb. plantarum*-producing strains, various authors have highlighted their robust bacteriocin production, particularly showing potent activity against diverse *L. monocytogenes* strains (Barbosa et al., 2021; García-Reyes et al., 2023; Zareie et al., 2023). Based on the observed maximum cell growth and achievement of maximum bacteriocin activity (12,800 AU/mL), 24 h was selected for all subsequent assays.

3.4.2. Effects of enzymes, temperature, pH, and surfactants on bacteriocin activity

A variety of detergents, enzymes, pH, and temperature that have an impact on bacteriocin(s) activity against *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b), CEP 104794 (1/2a) are presented in Tables 3 and 4. The bacteriocin activity of *Lpb. plantarum* 9A3 remained stable within a temperature range of 4 °C to 60 °C against most *L. monocytogenes* serovars (Table 3). However, for serovar 1/2c, residual activity was observed below 30 °C, while activity persisted above 80 °C for all serovars tested. Similar thermal stability has been noted in bacteriocins produced by other lactobacilli strains (Barbosa et al., 2021; Zhao et al., 2022). It was also observed that bacteriocin(s) from *Lpb. plantarum* 9A3 were quite resistant to a wide pH range (from 2 to 10), indicating sensitivity to alkaline conditions (Table 4). Similar decreases in antimicrobial activity have been reported for other bacteriocins such as plantaricins and pediocins under altered pH conditions (Barbosa et al., 2021; Heredia-Castro et al., 2015; Ramos et al., 2016). Regarding surfactants commonly used in the food industry, *Lpb. plantarum* 9A3 bacteriocin(s) showed resistance (Table 4), with exceptions noted to treatments with sodium carbonate, NaCl and Triton X-100, which promoted the loss of bacteriocin (s) activity. Among the enzymes tested, only papain and catalase treatments did not reduce bacteriocin(s) activity, while proteinase K and pepsin drastically reduced bacteriocinogenic activity by 100%. Furthermore, a lower reduction in activity was noted following treatment with papain and the antioxidant enzyme (catalase). This has been reported in several other studies not only when bacteriocins were treated with proteinase K (Barbosa et al., 2021; Oliveira et al., 2020) but also when exposed to pepsin, papain, and catalase (Ramos et al., 2016; Todorov et al., 2013).

3.4.3. Cell lysis

The impact of *Lpb. plantarum* 9A3 bacteriocin(s) on the growth of *L. monocytogenes* serovars NCTC 11994 (1/4 b), CECT 911 (1/2c), CECT 936 (1/2 b) and CEP 104794 (1/2a) is exhibited in Fig. 2. Upon the addition of *Lpb. plantarum* 9A3 bacteriocin(s) to mid-log *L. monocytogenes* cultures (6 h-old), a suppression of cell growth was evident, particularly notable for *L. monocytogenes* CEP 104794 (serovar 1/2a) with a reduction of 3.57 log CFU/mL (Fig. 2D). Conversely, no noticeable change in cell counts was observed for untreated samples (control). Across all studied *L. monocytogenes* serovars, the treated cell-free supernatant of *Lpb. plantarum* 9A3 significantly impacted their viability, effectively controlling their growth for at least 12 h. These findings parallel those reported by Martín et al. (2023), where the numbers of *L. monocytogenes* and *E. faecalis* decreased following the addition of a bacteriocin produced by *Latilactobacillus sakei* 205 (3200 and 6400 AU/mL, respectively). Similarly, Barbosa et al. (2021) documented a decrease of approximately 2 log cycles in *L. monocytogenes* 7947 upon the addition of the cell-free supernatant containing *Lpb. plantarum* R23 bacteriocin (12,800 AU/mL). Notably, observations at 24 and 30 h indicated that despite growth suppression, *L. monocytogenes* were able to recover after 24 h across all cases. Previous studies typically assessed the behaviour of *L. monocytogenes* over a 12-h growth period (Barbosa et al., 2021; Martín et al., 2023).

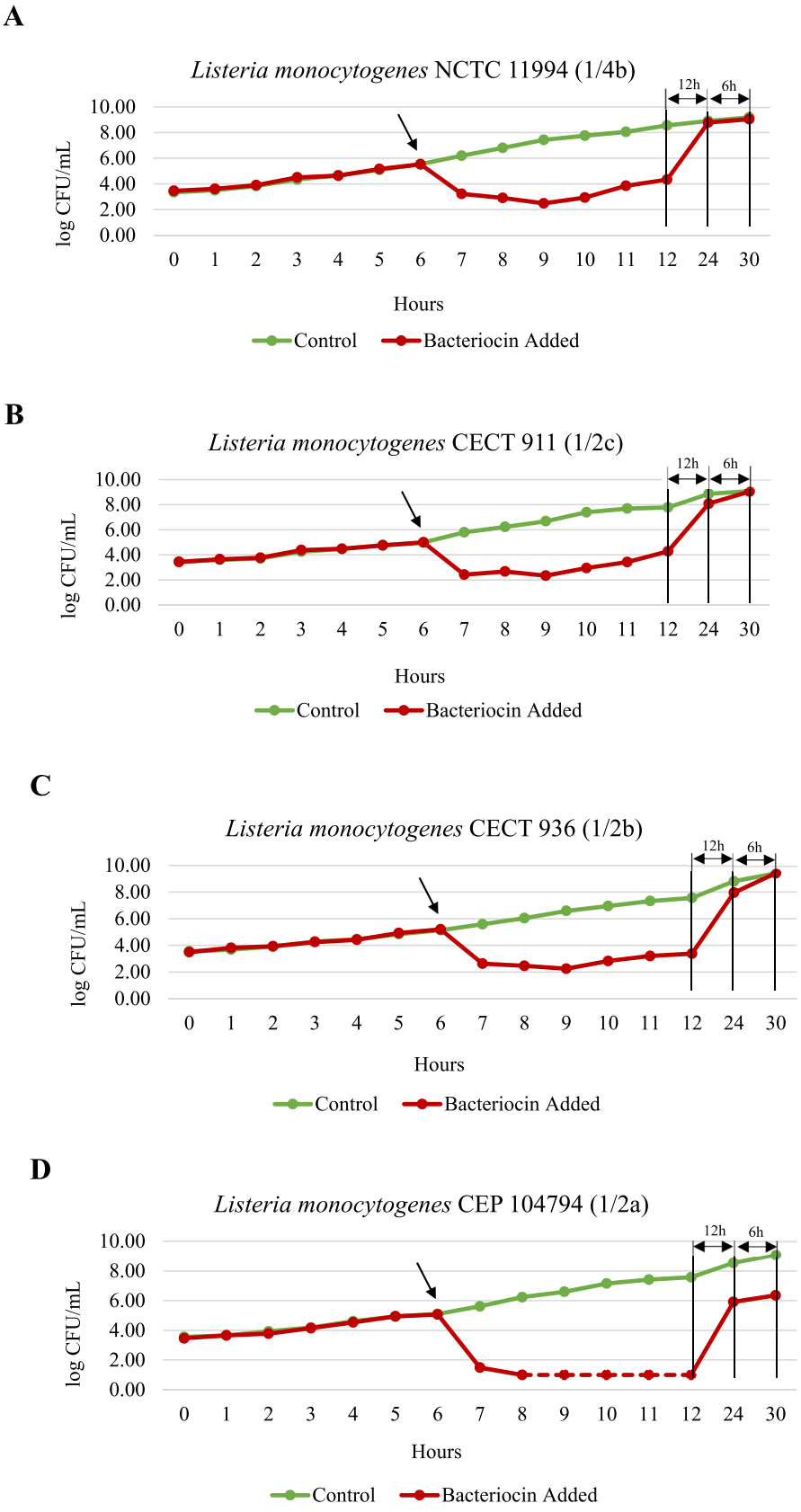

Fig. 2. Effect of *Lpb. plantarum* 9A3 bacteriocin (s) on the growth of *L. monocytogenes* NCTC 11994 (**A**), CECT 911 (**B**), CECT 936 (**C**) and CEP 104794 (**D**) presented as log CFU/mL. Green lines represent target cultures without added bacteriocin. The arrow indicates the point at which the bacteriocin was added. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

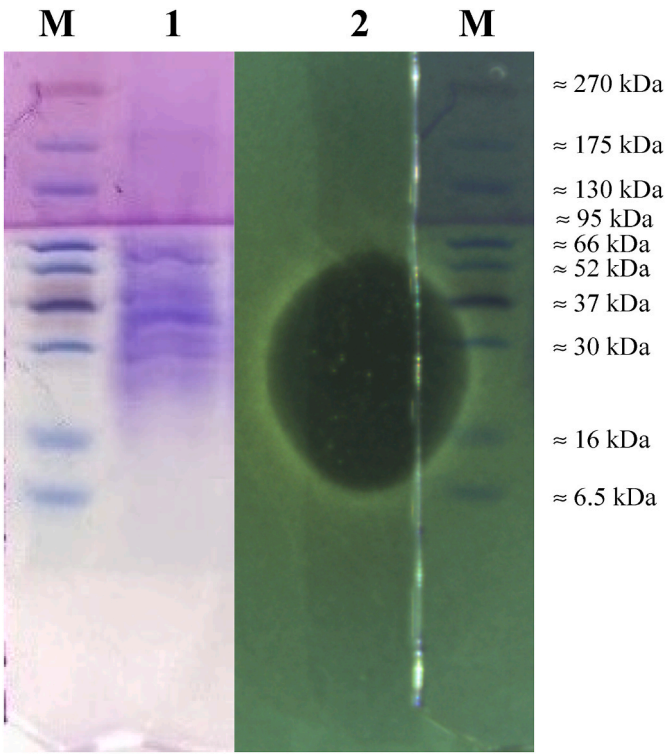

Fig. 3. Tricine/SDS-PAGE of *Lpb. plantarum* 9A3 bacteriocin: Lanes 1 and 4: Molecular mass marker (M); Lane 2: peptide bands in the stained gel with Coomassie Blue R250; Lane 3: growth inhibition zone, corresponding to the position of the peptide band in lane 2 (the gel was covered with viable cells of *L. monocytogenes* CECT 911 (10^6 CFU/mL), embedded in BHI soft agar and incubated at 37 °C for 24 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4.4. Adsorption studies and molecular size determination

Bacteriocin adherence to the surface of producer cells was not observed upon treating *Lpb. plantarum* 9A3 with 100 mM NaCl at pH 2.0, aligning with findings reported for other bacteriocins produced by different lactobacilli (Barbosa et al., 2021; Martín et al., 2023; Martinez et al., 2013).

Tricine/SDS-PAGE analysis estimated the molecular size of *Lpb. plantarum* 9A3 bacteriocin(s) to range between 37 and 52 kDa, deduced from the correlation between the position of the peptide band and the growth inhibition clear zone of *L. monocytogenes* CECT 911 (Fig. 3). A comparable outcome for *L. monocytogenes* CECT 936 is presented in Supplementary Fig. 8. Surprisingly, the molecular weight estimated by SDS-PAGE exceeded the anticipated range, considering the bacteriocins suggested by WGS. Class II bacteriocins like Plantaricin E, Plantaricin F, Pediocin PA, Enterocin X, Leucocin A and Coagulin A generally exhibit a molecular weight of less than 10 kDa (Drider et al., 2006), consistent with most bacteriocins produced by *Lpb. plantarum* strains (Han et al., 2023; Sidhu and Nehra, 2021; Wang et al., 2023). However, without further isolation and characterization studies of the purified bacteriocins, any explanation would remain speculative. This leaves us with hypotheses requiring validation: the observed halo on the gel could result from the activity of multiple bacteriocins, and therefore, the value determined may be an artefact of the technique; the molecular weight standards may not be sufficiently accurate (Neris et al., 2020); or we may even be in the presence of new bacteriocin(s). Other authors have reported high molecular weights for bacteriocins produced by other lactobacilli: Noroozi et al. (2019) reported a novel large molecular weight bacteriocin (68 kDa) produced by a *Lacticaseibacillus casei* strain and Islam et al. (2020) identified lactobacilli producing 30 and 40 kDa bacteriocins. Moreover, the SwissProt database lists only three bacteriocins from *Lpb. plantarum*: plantaricin ASM1 (https://www.uniprot.org/uniprotkb/C7G1H4, accessed on October 19, 2023), plantaricin-A (https://www.uniprot.org/uniprotkb/P80214, accessed on October 19, 2023) and plantaricin KL-1Y (https://www.uniprot.org/uniprotkb/C0HJC0, accessed on October 19, 2023) with molecular weights of 6891 KDa, 5458 KDa and 3498 KDa respectively. The bacteriocin Plantaricin ASM1 produced by *Lpb. plantarum* A-1 has been isolated and characterized, and its molecular mass of 5045.72 Da was determined by MALDI mass spectrometry (Hata et al., 2010), suggesting that the protein is processed into a mature form. Bacteriocin KL-1Y from *Lpb. plantarum* was also successfully purified and its molecular mass, determined by electrospray mass spectrometry, was 3498 Da (Rumjuankiat et al., 20150), which is consistent with the prediction. Concerning Leucocin A, identified as a homologous sequence in the genome of *Lpb. plantarum* 9A3, a putative Leucocin A (https://www.uniprot.org/uniprotkb/A0A2S3U2A4, accessed on October 19, 2023) with a predictive molecular weight of 13,375 KDa, have been deposited in the UniProtKB unreviewed TrEMBL database. These data suggest that the bacteriocins from *Lpb. plantarum* remains inadequately understood, posing challenges in confirming their molecular weight via SDS-PAGE.

4. Conclusions

Lactiplantibacillus plantarum 9A3, isolated from vegetables & mushrooms *alheira*, was chosen due to its high bacteriocin activity spectrum against not only several *L. monocytogenes* strains but also against *E. faecalis* ATCC 29212, *C. sporogenes* and *C. perfringens*. Both phenotypic and WGS analysis confirm *Lpb. plantarum* 9A3 as a safe culture for potential use in the food industry, as it lacks virulence and antibiotic resistance genes. Additionally, this bacterium produces safe and stable bacteriocin(s), suggesting its potential as a bio-preservative culture. Notably, it not only inhibits *L. monocytogenes* but also *Clostridium* spp. Commonly present in fermented meat products. This capacity might extend food shelf life and mitigate microbiological risk associated with *alheiras* and similar processed meats. With a two-year window approaching for companies to comply with new regulations requiring a reduction in the use of nitrites, exploring viable alternatives for this preservative, especially those effective against clostridial species, becomes crucial. The presence of genes encoding Plantaricin E, Plantaricin F, Pediocin and Leucocin A in *Lpb. plantarum* 9A3 prompts the need to establish conditions conducive to the production ofone or more bacteriocins. Understanding these conditions is crucial to enhance our understanding of about its applicability. This strain possesses promising characteristics that warrant further investigation to assess its effect on the flavour and quality of fermented sausages. This exploration could pave the way for its use as a protective culture in the food industry.

CRediT authorship contribution statement

Inês Azevedo: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Joana Barbosa:** Conceptualization, Formal analysis, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Helena Albano:** Supervision, Writing - review & editing. **Teresa Nogueira:** Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Paula Teixeira:** Supervision, Validation, Writing - review & editing.

Declaration of competing interest

None

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Appendix A. Supplementary data

Supplementary data to this article can be found online at.https://doi.org/10.1016/j.fm.2023.104450.

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