Safety profiles of beneficial lactic acid bacteria isolated from dairy systems

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Abstract

This study aimed to assess the safety aspects of 15 lactic acid bacteria (LAB) strains previously isolated from a dairy environment with relation to their beneficial features. LAB strains were assessed using phenotypic methods according to their production of virulence factors at 25 °C and 37 °C, as well as by examining their potential resistance to 15 antibiotics. Polymerase chain reaction (PCR) was also used to identify the presence of 50 genes associated with virulence factors and antibiotic resistance in the strains. None of the strains presented hemolytic activity or the production of gelatinase, lipase, deoxyribonuclease, or the tested biogenic amines. Based on the disk diffusion assay, all strains were resistant to oxacillin and sulfa/trimethoprim. Further, some were resistant to gentamicin (14), clindamycin (11), vancomycin (9), rifampicin (8), erythromycin (5), tetracycline (4), ampicillin (2), and chloramphenicol (1); no strain was resistant to imipenem. Regarding virulence- and antibiotic-resistance-related genes, 19 out of 50 tested genes were present in some strains; there was a variable association of expression. Based on the obtained data, the isolates presented relatively safe characteristics and behavior, findings that should lead to further studies to assess their potential usage as beneficial cultures in the food industry.

Keywords Lactic acid bacteria · Virulence factors · Antibiotic resistance

Introduction

Lactic acid bacteria (LAB) is a heterogeneous group of bacteria with a known history about their use in fermented products,

Highlights

- The safety aspects of LAB isolated from a dairy environment was evaluated.
- Presence of 49 virulence factors and antibiotic resistance genes was studied.
- Physiological expression of virulence factors, biogenic amine, and antibiotic resistance was tested.

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fermentation processes, and for production of antimicrobial substances, including organic acids and antimicrobial proteins, such as bacteriocins [1]. In addition, LAB are known for their ability in providing beneficial effects on consumers, being several strains described as probiotics [2]. Among LAB, *Lactobacillus* strains are the most described as probiotics, followed by *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* [3].

Scientific knowledge regarding beneficial LAB has advanced significantly in terms of selection and characterization of new useful cultures, with a particular focus on benefits to consumer health [4–6]. The available studies in this area are also focused on the safety features of the selected LAB strains. Beneficial and safety aspects must be considered in order to assess the potential use of the selected LAB strains for human consumption [7]. Despite being characterized as beneficial, LAB can express a variety of pathogenic mechanisms and cause disease in human and other animal hosts [8]. Further, several factors are reported as hazardous, especially those related to antibiotic-resistance genes, genes that facilitate genetic material exchange, possible complications in the gastrointestinal tract (GIT), and indiscriminate use of antibiotics in human and veterinary medicine practice [9–11]. These factors can pose

significant risks to public health, and they highlight the relevance of characterizing them in all bacteria that will be introduced into the food chain or recommended to be applied as probiotics [11].

A probiotic strain must resist and persist in the GIT and provide benefits to the host. It must also be safe and hazard-free for consumers [6, 7]. Based on a number of phenotypic and molecular assays, probiotic strains are generally recognized as safe (GRAS) if they possess only a minimal possibility for antibiotic-resistance gene transfer. They should be safe for human and animal food consumption and demonstrate proven health-promoting effects (e.g., non-invasive in epithelial cell line models, produce anti-inflammatory rather than pro-inflammatory cytokines) [7]. However, a GRAS status is not enough to indicate that a strain is safe, because other virulence factors are not considered in this evaluation [12, 13]. Deep research evaluation for the safety aspects of each specific strain needs to be performed in order to confirm the safety of that strain to be applied in food fermentation processes or as a beneficial culture for human or animal applications. Additionally, for these beneficial cultures to be considered safe for human health, they cannot cause disease (such as bacteremia) or be related to any toxic or metabolic effects, and they should not be able to transfer antibiotic-resistance genes [13, 14].

Thus, studies that involve bacteria with beneficial potential must characterize their virulence potential to exclude potential hazards. Here, we aimed to characterize the safety of a panel of LAB strains previously isolated from a dairy environment and characterized as possessing beneficial features [15, 16]. The examined parameters were related to virulence activity, biogenic amine production, and antibiotic resistance.

Material and methods

Bacterial strains and growth conditions

In a previous study, 15 strains were selected from an LAB collection isolated from a dairy environment and characterized as beneficial by phenotypic and molecular methods [15, 16]. These strains were characterized based on their genetic profiles by repetitive palindromic sequence polymerase chain reaction (rep-PCR) and random amplified polymorphic DNA (RAPD), and identified based on physiological and biochemical features and 16S rRNA as *Lactobacillus* spp. ($n = 11$), *Pediococcus* spp. ($n = 2$), and *Weissella* spp. ($n = 2$) [15, 16]. The selected strains were named as MS11, MS15, MRUV1, and MRUV6 (*Lactobacillus casei*), MVA3 (*L. acidophilus*), MSIV4 (*L. nagelli*), MS13 and MSIV2 (*L. harbinensis*), SIVGL1 (*L. fermentum*), MLE5 and MS12 (*L. plantarum*), MLEV8 (*Pediococcus pentosaceus*), MS17 (*P. acidilactici*), and MRUV3 and MSAV5 (*Weissella paramesenteroides*). The strains were stored in de Man, Rogosa, and Sharpe (MRS) broth

(Oxoid Ltd., Basingstoke, England) supplemented with 25% (v/v) glycerol at -80°C . For use, stock cultures were streaked on MRS agar (Oxoid), incubated at 37°C for 24 h, when isolated colonies were transferred to MRS broth and incubated at 37°C for 24 h. The selected strains were subjected to a panel of phenotypic and molecular assays in order to characterize their safety profiles. Figure 1 summarizes the adopted analysis, and further details are briefly described below.

Phenotypical characterization of safety

The selected strains were subjected to phenotypical assays to characterize their safety according to Barbosa et al. [17]. For hemolytic activity, the cultures were streaked on trypticase soya agar (Oxoid) with 5% (v/v) defibrinated horse blood. After incubation at 25°C and 37°C for 24 h, the hemolytic activity of each isolate was measured and classified as total or β -hemolysis (clear halos around the colonies), partial or α -hemolysis (greenish halos around the colonies), or γ -hemolysis (absence of hemolysis). For gelatinase production, 1- μL aliquots of the examined cultures was spotted on Luria Bertani (LB) agar (BD, Franklin Lakes, NJ, USA) with 3% (w/v) gelatin (BD). After incubation at 25°C and 37°C for 48 h, the plates were maintained at 4°C for 4 h, and gelatin hydrolysis was recorded by the formation of opaque halos around the colonies. Lipase production was assessed by spotting 1 μL of each culture on LB with 0.2% (w/v) CaCl_2 (Sigma-Aldrich, Inc., St. Louis, MO, USA) and 1% (v/v) Tween 80 (Sigma-Aldrich). After incubation at 25°C and 37°C for 48 h, transparent halos around the colonies were recorded as lipase production. DNase activity was identified by spotting 1- μL aliquots of the cultures on the surface of DNase methyl green agar (BD). After incubation at 25°C and 37°C for 48 h, the formation of clear halos around the colonies was identified as a positive result. All assays were conducted in three independent repetitions.

The potential to produce biogenic amines was assessed according to Bover-Cid and Holzapfel [18] and Joosten and Northolt [19]. Aliquots (0.5 mL) from each culture were transferred five times consecutively to MRS broth supplemented with 0.005% (w/v) pyridoxal-5-phosphate (Sigma-Aldrich); 0.1% (w/v) biogenic amine precursors were added individually: lysine, tyrosine, ornithine, and histidine (Sigma-Aldrich). The incubation was performed at 25°C and 37°C for 24 h, and the last transfer performed was streaked in duplicate on a modified MRS agar that was supplemented with one of each biogenic amine precursor described as above (1% w/v). The plates were incubated at 25°C and 37°C for 4 days, and positive results were recorded when the color changed from yellow to purple.

Antibiotic resistance was characterized based on disk diffusion assay (Oxoid) and Etest® strips (bioMérieux SA, Marcy l'Etoile, France). For the disk diffusion assay, each

Fig. 1 Identification and origins of 15 lactic acid bacteria strains isolated from a dairy environment [15, 16] and the schematic description of the phenotypical and molecular assays to characterize their safety profiles. Conducted assays are indicated by filled circle, disk concentrations, and genes; open circle indicates non-conducted assays

Lactic acid bacteria strains with beneficial features				
species	id	origin		
<i>Lactobacillus casei</i>	MSI1, MSI5	silage		
	MRUV1, MRUV6	cow rumen		
<i>L. acidophilus</i>	MVA3	cow vaginal mucosa		
<i>L. nagelli</i>	MSIV4	silage		
<i>L. harbinensis</i>	MSI3, MSIV2	silage		
<i>L. fermentum</i>	SIVGL1	silage		
<i>L. plantarum</i>	MLE5	cow milk		
	MSI2	silage		
<i>Pediococcus pentosaceus</i>	MLEV8	cow milk		
<i>P. acidilactici</i>	MSI7	silage		
<i>Weissella paramesenteroides</i>	MRUV3	cow rumen		
	MSAV5	cow oral mucosa		

SAFETY PROFILE				
		Phenotypical		Molecular (genes)
virulence	hemolysis	●		<i>cyt2</i>
	gelatinase	●		<i>sprE, fsrA, fsrB, fsrC, gelE</i>
	lipase	●		○
	DNAse	●		○
	aggregation	○		<i>asa1</i>
	endocarditis	○		<i>efaA</i>
	chemotactic factors	○		<i>cob, cpd, ccf</i>
	hyaluronidase	○		<i>hyl</i>
	<i>Enterococcus</i> factors	○		<i>esp, mur-2ed, aac(6'')-li, mur-2, ddl, ace</i>
	genetic exchange	○		<i>int, int-Tn</i>
biogenic amines	lysine	●		○
	tyrosine	●		<i>tdc</i>
	ornithine	●		<i>odc</i>
	histidine	●		<i>hdc1, hdc2</i>
antibiotic resistance	aminoglycosides	gentamicin	Disk 10 µg ●	<i>aph(2'')-Ib, ant(4'')-Ia, aph(2'')-Id, aph(2'')-Ic, aph(3'')-IIIa, aac(6'')-Ie-aph(2'')-Ia</i>
	carbapenems	imipenem	10 µg ○	○
	glycopeptides	vancomycin	30 µg ●	<i>vanA, vanB, vanC, vanC1, vanC2, vanC2/C3</i>
	lincosamides	clindamycin	2 µg ○	○
	macrolides	erythromycin	15 µg ○	<i>ermA, ermB, ermC</i>
	penicillins	ampicillin	10 µg ●	○
		oxacillin	1 µg ○	○
	sulfonamides	penicillin	10 U ○	○
		trimethoprim/sulfamethoxazole	1:19, 25 µg ○	○
	tetracyclines	tetracycline	30 µg ○	<i>tet(K), tet(L), tet(M), tet(O), tet(S)</i>
				<i>catA</i>
	other	chloramphenicol	30 µg ●	○
		rifampicin	5 µg ●	○
		streptogramin	○	<i>vat(E)</i>
		streptomycin	○	<i>ant(6'')-Ia</i>
		bacitracin	○	<i>bcrB, bcrD, bcrR</i>

strain was diluted using 0.85% NaCl (w/v) until the turbidity was equivalent to 0.5 McFarland standard and swabbed onto the surface of a MRS agar plate (where the antibiotic disks were placed). A panel of 12 antibiotics from 9 classes were considered (Fig. 1). The plates were incubated at 37 °C for 18 h, when the diameter of the inhibition halos were measured and the isolates were characterized as presenting resistance (R), intermediary resistance (IR), or sensitivity (S) [20–22]. In addition, LAB strains were subjected to the antimicrobial susceptibility test based on their minimum inhibitory concentrations (MIC) against five selected antibiotics (Fig. 1), by using Etest® strips, allowing their characterization as resistant

(R) or susceptible (S) [20–22]. Breakpoint values for *Streptococcus* spp. indicated by the Clinical and Laboratory Standards Institute [22] were considered in the analysis.

Molecular characterization of safety

LAB strains were subjected to DNA extraction using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). After measuring their concentration and quality (NanoDrop 2000, Thermo Scientific Inc., Waltham, MA, USA), DNA samples were subjected to PCR assays to detect the presence of 50 genes related to virulence, biogenic amines,

Table 1 Primers and references for the PCR protocols conducted in the present study to assess the presence of virulence, biogenic amines, and antibiotic-resistance genes in 15 lactic acid bacteria strains with beneficial

features obtained from a dairy processing environment [15, 16]. Functions of target genes are presented in Fig. 1

Target gene	Primers	Reference
<i>cyt2</i>	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	Vankerckhoven et al. [23]
<i>sprE</i>	F: TTGAGCTCCGTTCTGCCGAAAGTCATTC R: TTGGTACCGATTGGGGAACCAGATTGACC	Nakayama et al. [24]
<i>fsrA</i>	F: ATGAGTGAACAAATGGCTATTTA R: CTAAGTAAGAAATAGTGCCTTGA	Nakayama et al. [24]
<i>fsrB</i>	F: GGGAGCTCTGGACAAAGTATTATCTAACCG R: TTGGTACCCACACCATCACTGACTTTTGC	Nakayama et al. [24]
<i>fsrC</i>	F: ATGATTTTGTGCTTATTAGCTACT R: CATCGTTAACAACCTTTTTTACTG	Nakayama et al. [24]
<i>gelE</i>	F: TATGACAATGCTTTTTGGGAT R: AGATGCACCCGAAATAATATA	Vankerckhoven et al. [23]
<i>asa1</i>	F: GCACGCTATTACGAACATATGA R: TAAGAAAGAACATCACACGA	Vankerckhoven et al. [23]
<i>efaA</i>	F: GCCAATTGGGACAGACCCTC R: CGCCTTCTGTTCTTCTTTGGC	Martin-Platero et al. [25]
<i>cob</i>	F: AACATTGAGCAAAACAAGC R: TTGTCATAAAGAGTGGTCAT	Eaton and Gasson [26]
<i>cpd</i>	F: TGGTGGGTTATTTTCAATTC R: TACGGCTCTGGCTTACTA	Eaton and Gasson [26]
<i>ccf</i>	F: GGGAAATTGAGTAGTGAAGAAG R: AGCCGCTAAAATCGGTAAAAT	Eaton and Gasson [26]
<i>hyl</i>	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	Vankerckhoven et al. [23]
<i>esp</i>	F: AGATTTTCATCTTTGATTCTTG R: AATTGATTCTTTAGCATCTGG	Vankerckhoven et al. [23]
<i>mur-2_{ed}</i>	F: AACAGCTTACTTGACTGGACGC R: GTATTGGCGCTACTACCCGTATC	Robredo et al. [27]
<i>aac(6)-li</i>	F: GCGGTAGCAGCGGTAGACCAAG R: GCATTTGGTAAGACACCTACG	Costa et al. [28]
<i>mur-2</i>	F: CGTCAGTACCCTTCTTTTGCAGAGTC R: GCATTATTACCAAGTGTAGTGTTG	Chu et al. [29]
<i>ddl</i>	F: ATCAAGTACAGTTAGTCT R: ACGATTCAAAGCTAACTG	Dutka-Malen et al. [30]
<i>ace</i>	F: GAATTGAGCAAAAGTTCAATCG R: GTCTGTCTTTTCACTTGTTTC	Martin-Platero et al. [25]
<i>int</i>	F: GCGTGATTGTATCTCACT R: GACGCTCCTGTTGCTTCT	Gevers et al. [31]
<i>int-Tn</i>	F: TGACACTCTGCCAGCTTTAC R: CCATAGGAACTTGACGTTTCG	Barbeyrac et al. [32]
<i>tdc</i>	F: GAYATNATNGGNATNGGNYTNGAYCARG R: CCRTARTCNNGNATAGCRAARTCNTRTG	Rivas et al. [33]
<i>odc</i>	F: GTNTTYAAYGCNGAYAARCANTAYTTYGT R: ATNGARTTNAGTTTCRCAYTTYTCNGG	Rivas et al. [33]
<i>hdc1</i>	F: AGATGGTATTGTTTCTTATG R: AGACCATAACCCATAACCTT	Favaro et al. [34]
<i>hdc2</i>	F: AAYTCNTTYGAYTTYGARAARGARG R: ATNGGNGANCCDATCATYTTTGNCC	Favaro et al. [34]
<i>aph(2'')-Ib</i>	F: TATGGATCCATGGTTAACTTGGACGCTGAGAT R: TAAGCTTCTGTCTAAATATAAACATCTCTGCT	Kao et al. [35]
<i>ant(4')-Ia</i>	F: CAAACTGCTAAATCGGTAGAAGCC R: GGAAAGTTGACCAGACATTACGAACT	Vakulenko and Mobashery [36]
<i>aph(2'')-Id</i>	F: GTGGTTTTTACAGGAATGCCATC R: CCCTCTTCATACCAATCCATATAACC	Fortina et al. [5]
<i>aph(2'')-Ic</i>	F: CCACAATGATAATGACTCAGTTCCC R: CCACAGCTTCCGATAGCAAGAG	Fortina et al. [5]
<i>aph(3')-IIIa</i>	F: GCCGATGTGGATTGCGAAAA R: GCTTGATCCCCAGTAAGTCA	Fortina et al. [5]
<i>aac(6')-Ie-aph(2'')-Ia</i>	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	Van de Klundert and Vliegenthart [37]
<i>vanA</i>	F: TCTGCAATAGAGATAGCCGC R: GGAGTAGCTATCCAGCAIT	Martin-Platero et al. [25]

Table 1 (continued)

Target gene	Primers	Reference
<i>vanB</i>	F: GCTCCGCAGCCTGCATGGACA R: ACGATGCCGCCATCCTCCTGC	Paulsen et al. [38]
<i>vanC</i>	F: GGTATCAAGGAAACCTC R: CTTCCGCCATCATAGCT	Dutka-Malen et al. [30]
<i>vanC1</i>	F: GCTGAAATATGAAGTAATGACCA R: CGGCATGGTGTGATTTTCGTT	Miele et al. [39]
<i>vanC2</i>	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	Dutka-Malen et al. [30]
<i>vanC2/C3</i>	F: CTCCTACGATTCTCTTG R: CGAGCAAGACCTTTAAG	Dutka-Malen et al. [30]
<i>ermA</i>	F: TCTAAAAAGCATGTAAAAGAA R: CTTTCGATAGTTTATTAATATTAG	Sutcliffe et al. [40]
<i>ermB</i>	F: CATTTAACGACGAACTGGC R: GGAACATCTGTGGTATGGCG	Jensen et al. [41]
<i>ermC</i>	F: ATCTTTGAAATCGGCTCAGG R: CAAACCCGTATTCCACGATT	Jensen et al. [41]
<i>tet(K)</i>	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACCTCATTCCAGAAGCA	Aarestrup et al. [42]
<i>tet(L)</i>	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCGATTTTCGG	Aarestrup et al. [42]
<i>tet(M)</i>	F: GTTAAATAGTGTTCTTGAGAG R: CTAAGATATGGCTCTAACAA	Aarestrup et al. [42]
<i>tet(O)</i>	F: GATGGCATAACAGGCACAGAC R: CAATATCACCAGAGCAGGCT	Aarestrup et al. [42]
<i>tet(S)</i>	F: TGGAACGCCAGAGAGGTATT R: ACATAGACAAGCCGTTGACC	Aarestrup et al. [42]
<i>catA</i>	F: GGATATGAAATTTATCCCTC R: CAATCATCTACCCTATGAAT	Aarestrup et al. [42]
<i>vat(E)</i>	F: ACGTTACCCATCACTATG R: GCTCCGATAATGGCACCAGC	Duh et al. [43]
<i>ant(6)-Ia</i>	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	Fortina et al. [5]
<i>bcrB</i>	F: AAAGAAACCGACTGCTGATA R: GCTTACTTGTATAGCAGAGA	Manson et al. [44]
<i>bcrD</i>	F: AGGATTGGGCCGAATGGCACTTGATTTTAT R: GTTTCTTCGCGAAATTGCCGTTATAAGTAA	Manson et al. [44]
<i>bcrR</i>	F: AACAAACAGGGAGCGCCGCATGGAATTTA R: TGATGTTGCGGATTTTCATCCCATCTGCTT	Manson et al. [44]

and antibiotic resistance (Fig. 1). The primers and references for PCR conditions are described in Table 1. PCR products were separated on 0.8 to 2.0% (w/v) agarose gels in 0.5× Tris-borate-ethylene diamine tetra acetic acid (TBE) and stained with Tris-acetic acid-EDTA (TAE) buffer that contained GelRed (Biotium Inc., Hayward, CA, USA) at 0.5 µg/mL.

Results and discussion

Here, we considered as much as we were able of different safety features usually assessed to characterize beneficial and probiotic strains before use for potential human consumption [45]. Based on the adopted panel (Fig. 1, Table 1), Table 2 summarizes the results for the detected safety features in the 15 LAB strains. None of the investigated strains presented hemolysis, gelatinase or lipase production, and DNase activity in the in vitro tests at either 25 °C or 37 °C. Similarly, there

was no in vitro detection of biogenic amines production. All investigated strains showed negative results for the presence of genes related to the production of lysine, histidine, and ornithine biogenic amines (Table 2), as expected for safe cultures [46, 47]. Only some strains presented positive results for *tdc* (*L. casei* MS11, from silage, MRUV6, from cow rumen, and *L. acidophilus* MVA3, from cow vaginal mucosa), related to production of tyrosine [18, 33].

The absence of hemolytic activity and biogenic amines production in *Lactobacillus* was already reported and it is to be expected. Indeed, this characteristic is essential for beneficial and probiotic candidate strains [45, 48, 49]. However, some *Lactobacillus* spp. are described as biogenic amine producers, and they may be present in microbiota related to a dairy environment, perhaps introduced through contamination at some steps in the dairy production processes [46]. The production of extracellular enzymes and hemolytic activity are not exhibited by *Pediococcus* spp., as shown by Borges

Table 2 Phenotypic and genotypic antibiotic resistance and resistance and virulence genes detected by PCR of lactic acid bacteria

Species	Strain	Phenotypic antibiotic resistance		Resistance and virulence genes detected by PCR
		Disk diffusion assay	MIC (µg/mL)	
<i>L. casei</i>	MSI1	GEN, CLI, ERY, OXA, TRS, RIF	VAN (A), AMP (1.0)	<i>vanC2, bcrB, mur-2ed, tdc</i>
	MSI5	GEN, VAN, CLI, OXA, TRS	VAN (A),	<i>vanC2, tet(S), bcrR, cpd</i>
	MRUV1	GEN, VAN, AMP, OXA, TRS, TET	VAN (A), AMP (1.0)	<i>vanA, ant(4')-Ia, int</i>
	MRUV6	GEN, VAN, OXA, TRS	VAN (A), AMP (1.0)	<i>ermA, ant(4')-Ia, tdc</i>
<i>L. acidophilus</i>	MVA3	GEN, VAN, CLI, ERY, OXA, TRS, RIF	VAN (A), AMP (1.0), RIF (A)	<i>tet(K), tet(S), ermA, ant(4')-Ia, bcrR, asa1, ccf, tdc</i>
<i>L. nagelli</i>	MSIV4	GEN, CLI, OXA, TRS, RIF	VAN (A), AMP (50.0)	<i>ccf, int</i>
<i>L. harbinensis</i>	MSI3	GEN, VAN, CLI, OXA, TRS, TET	GEN (A), VAN (A), AMP (1.0), CHL (A)	<i>asa1</i>
	MSIV2	VAN, CLI, OXA, TRS	VAN (A), RIF (32.0)	<i>vanC2, cpd</i>
<i>L. fermentum</i>	SIVGL1	GEN, VAN, AMP, OXA, TRS	VAN (A), AMP (1.5)	--
<i>L. plantarum</i>	MLE5	GEN, CLI, ERY, OXA, TRS, RIF	VAN (A), AMP (0.64)	<i>vanC1, aph(3')-IIIa</i>
	MSI2	GEN, VAN, CLI, OXA, TRS, CHL, RIF	VAN (A)	--
<i>P. pentosaceus</i>	MLEV8	GEN, CLI, ERY, OXA, TRS, RIF	VAN (A), AMP (50)	<i>ermB, aac(6')-Ie-aph(2'')-Ia</i>
<i>P. acidilactici</i>	MSI7	GEN, VAN, OXA, TRS, TET	VAN (A)	<i>tet(K), int</i>
<i>W. paramesenteroides</i>	MRUV3	GEN, VAN, CLI, OXA, TRS, TET, RIF	VAN (A), AMP (50), CHL (50.0), RIF (4.0)	<i>vanA, ant(4')-Ia, int</i>
	MSAV5	GEN, CLI, ERY, OXA, TRS, RIF	--	<i>mur-2ed, cpd, hyl, int</i>

GEN gentamicin, IMI imipenem, VAN vancomycin, CLI clindamycin, ERY erythromycin, AMP ampicillin, OXA oxacillin, PEN penicillin, TRS trimethoprim/sulfamethoxazole, TET tetracycline, CHL chloramphenicol, RIF rifampicin, STG streptogramin, STM streptomycin, BAC bacitracin, A absence of inhibition zone

Breaking points have been determined according to manufacturer instructions (bioMérieux, France), EUCAST, and CLSI standard [20–22]

et al. [50]. To the best of our knowledge, there is no study about hemolytic activity or extracellular enzymes for *W. paramesenteroides*. Jeong and Lee [51] described a *W. paramesenteroides* strain that produces histamine and tyramine. Only two strains, *L. fermentum* SIVGL1 (silage) and *L. plantarum* MSI2 (silage), did not present any positive result for any of the assayed virulence related genes; *L. harbinensis* MSI3 (silage) presented only positive result for *asa1*. Asa1 is responsible for aggregation substance capacity, and this protein increases bacterial adherence to renal tubular cells and heart endocardial cells, enhances internalization in intestinal epithelial cells, and increases the valvular vegetation mass in an animal model of endocarditis [23]. However, Asa1 can be an important adhering feature to help the persistence of a beneficial strain in the GIT [15]. The presence of *Enterococcus* spp.-related genes indicates the ability of the strains in acquiring mobile elements from other bacteria, what can explain the presence of *cpd*, *ccf*, *int*, and *hyl* in some of the strains (Table 2), and considered a special concern related to antibiotic-resistance spread in GIT [26].

Most of the evaluated strains were susceptible to the majority of the tested antibiotics. Based on the results obtained by the disk diffusion, all strains were resistant to oxacillin and sulfa/trimethoprim, and just one strain (*L. harbinensis*

MSIV2, silage) was susceptible to gentamicin (all other 14 strains were resistant). Most of the strains were also resistant to clindamycin, vancomycin, and rifampicin (Table 2). No strain was resistant to more than 7 of the 12 evaluated antibiotics. *L. acidophilus* MVA3 (cow vaginal mucosa), *L. plantarum* MSI2 (silage), and *W. paramesenteroides* MRUV3 (cow rumen) were the only three strains that exhibited more variable resistance to the tested antibiotics; they showed resistance to seven antibiotics. As strains obtained from similar samples did not show equivalent resistance patterns (Fig. 1, Table 2), apparently, the origin of the strains is not related to this feature, as indicated by Duar et al. [52]. The results obtained in this study are in accordance with the results obtained by other authors who investigated antibiotic resistance in beneficial LAB [10, 53]. Based on MIC, *L. harbinensis* MSI3 and *W. paramesenteroides* MRUV3 were the only two strains that presented high levels of resistance, to four antibiotics and susceptible only to only rifampicin and gentamicin, respectively (Table 2). Ampicillin and vancomycin were the two antibiotics to which most strains showed resistance, a potential concern as vancomycin is considered a key indicator in the evaluation of LAB safety [10, 45]. However, several reports demonstrate that LAB resistance to vancomycin can be intrinsic, and more dedicated

studies need to be performed in order to draw conclusions about safety related to sensitivity to this specific antibiotic. Gentamicin, chloramphenicol, and rifampicin were the antibiotics to which the 15 evaluated LAB strains were most susceptible (Table 2). The results obtained in this study agree with those reported by other authors related to the susceptibility of the lactobacilli strains to the selected antibiotics [10, 12]. In addition to the genus *Lactobacillus*, Munoz-Atienza et al. [54] described antibiotic resistance in the *Weissella* and *Pediococcus* genera. Although lactobacilli and other LAB are considered safe by several regulatory agencies, they should still be characterized on a strain-by-strain basis as safe in order to be applied as probiotic cultures [55]. Beneficial bacteria are considered alternatives for antibiotic therapy, once the spread of antibiotic resistance is a worldwide concern in a context of One Health [56], leading to a careful analysis of potential probiotic candidates as resistant or resistance-related gene carriers [57]. As many of the probiotic carriers are fermented foods, it is also important to verify the potential transfer of resistance genetic elements to starter cultures, what would spread this feature in a food industry and be potentially transferred to pathogenic and spoilage bacteria, jeopardizing the safety and quality of end products [58].

Based on the One Health approach, beneficial and probiotic strains must be checked for the presence of antibiotic-resistance genes, independently of presenting or not phenotypic resistance [57]. Based on the obtained results (Table 2), it was possible to characterize a diversity pattern of antibiotic-related genes in the assessed strains, and many of them can be located in plasmids, facilitating their transference to other bacteria [26]. Considering the molecular panel included in our study (Fig. 1), the most frequent antibiotic-resistance genes were related to vancomycin and gentamicin, as observed by the disk diffusion assay and MIC (Table 2). However, direct correspondence between phenotypic and molecular results for antibiotic resistance was not observed; this lack of correspondence can be considered expected in some situations, as the presence of a genetic element related to resistance is not necessarily an indicative of expression and thus resistance [59]. Bacteria usually adopt a number of resistance mechanisms for different antibiotics, resulting in alternative pathways to express this feature; because of this, it is currently well accepted the need for a wide approach, based on phenotypic and molecular assays, to characterize the antibiotic resistance in bacteria, mainly when they are supposed to be included in foods for human consumption, as considered for beneficial and probiotic strains [13, 57, 59].

None of the 15 tested strains were positive for the following genes: *vanB*, *vanC-1*, *vanC2/C3*, *tet(L)*, *tet(M)*, *tet(O)*, *int-Tn*, *ermC*, *catA*, *aph(2'')-Ib*, *aph(2'')-Id*, *aph(2'')-Ic*, *aph(3')-IIIa*, *vat(E)*, *bcrD*, *ant(6)-la*, *mur-2*, *ddl*, *ace*, *cyt2*, *esp*, *efaA*, *cob*, *sprE*, *fsrA*, *fsrB*, *fsrC*, *gelE*, *odc*, *hdc1*, and *hdc2*. The results

obtained in this study agree with those obtained by other authors who investigated LAB probiotic candidates [10, 53, 54] and highlight the safety profiles of the selected strains.

Based on the obtained results, the selected LAB strains can be considered relatively safe to be used as beneficial cultures in the food industry. None strain presented phenotypic production of the assayed virulence features and biogenic amines, and resistance was limited to a few antibiotics. In addition, only a few genetic markers related to virulence, biogenic amines, and antibiotic resistance were detected. Based on the characterized beneficial features [15, 16], and the safety profile identified in this study, the LAB strains can be considered candidates for specific studies to characterize their probiotic potential and industrial use.

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Compliance with ethical standards

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

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