

Article

Antimicrobial Activity and Probiotic Potential of Lactic Acid Bacteria Isolated from São Jorge Cheese

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Abstract

Six lactic acid bacteria isolated from São Jorge PDO cheese were characterised for technological, safety, antimicrobial, and probiotic properties. All isolates fermented a broad range of carbohydrates and lacked lipolytic activity, while SJC115 and SJC119 showed proteolysis. Safety profiling (γ -haemolysis, no DNase or gelatinase activity, and generally favourable antibiotic susceptibility) is promising, but tetracycline resistance warrants caution and genomic confirmation. *L. paracasei* and *L. brevis* isolates inhibited a wide range of foodborne pathogens (*Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Bacillus* spp.) and spoilage fungi (*Penicillium* and *Aspergillus* spp.). Notably, two isolates (SJC117 and SJC120) exhibited antibacterial activity in neutralized cell-free supernatants, indicating putative bacteriocin-like inhibitory substances (BLIS). The isolates survived intestinal conditions above the probiotic threshold, yet only SJC117 and SJC120 tolerated gastric acidity (pH 2.5, 1 h) with >5 log CFU/mL. Despite low hydrophobicity, strains showed good autoaggregation and pathogen coaggregation. All isolates produced exopolysaccharides (EPS) and angiotensin-converting enzyme (ACE) inhibitory peptides, whereas some exhibited moderate conjugated linoleic acid (CLA) production and glutamate decarboxylase (GAD) activity. *L. paracasei* SJC117 stood out by combining BLIS/antifungal activity, superior gastric tolerance, and an exceptional bioactive profile, making it a promising candidate for biopreservation and functional food applications that warrants further in vivo validation to confirm its efficacy and safety.

Keywords: lactic acid bacteria; LAB; GABA; EPS; cheese; probiotic; bacteriocin



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1. Introduction

Lactic acid bacteria (LAB) are essential components of traditional fermented foods, contributing to preservation, safety, flavour development, and textural properties. In raw milk cheeses, particularly those with Protected Designation of Origin (PDO) status, the indigenous LAB microbiota is uniquely shaped by geographical origin, manufacturing practices, and ripening conditions [1,2]. São Jorge cheese—a semi-hard PDO cheese produced from raw cow's milk in the Azores archipelago—harbours a rich and distinctive LAB community dominated by lactobacilli and *Leuconostoc* species [1]. However, while the technological roles of starter cultures are well established, the functional potential of autochthonous non-starter LAB remains underexplored.

This knowledge gap is particularly relevant given the growing consumer demand for clean-label products, which has intensified interest in biopreservation strategies that

reduce reliance on chemical preservatives. LAB can inhibit spoilage microorganisms and foodborne pathogens through multiple mechanisms, including organic acid production, competition for nutrients, and synthesis of bacteriocins or bacteriocin-like inhibitory substances (BLIS) [3,4]. Nevertheless, the relative contribution of each mechanism varies considerably among strains, and the discovery of novel BLIS-producing strains from underexplored ecological niches remains a priority.

Beyond antimicrobial activity, certain LAB also exhibit probiotic traits such as tolerance to gastrointestinal transit, adhesion to intestinal epithelium, and production of bioactive compounds that confer additional health benefits [5,6]. Among these bioactive metabolites, exopolysaccharides (EPS) produced by LAB have gained attention not only for their technofunctional properties—improving texture, water retention, and stability in fermented dairy products—but also for their prebiotic, immunomodulatory, and cholesterol-lowering effects [7]. Similarly, angiotensin-converting enzyme (ACE) inhibitory peptides, generated by LAB proteolysis of milk proteins, are recognized for their antihypertensive potential and have been identified in various fermented dairy products [8]. Conjugated linoleic acid (CLA), produced by LAB through biohydrogenation of linoleic acid, exhibits anticarcinogenic, anti-atherogenic, and anti-adipogenic properties, although strain-dependent production levels vary considerably [9].

Another desirable trait is glutamate decarboxylase (GAD) activity, which converts glutamate to gamma-aminobutyric acid (GABA), a compound known for its hypotensive, anxiolytic, and sleep-regulating effects [10]. Consequently, screening LAB for the simultaneous production of multiple bioactive compounds represents a promising strategy for developing functional foods with enhanced health-promoting properties. However, controversy persists regarding the safety of some LAB species, particularly concerning antibiotic resistance and biogenic amine production. Although many lactobacilli carry intrinsic resistance to certain antibiotics (e.g., vancomycin) that is not transferable, the potential for acquired resistance via mobile genetic elements raises concerns [11]. Therefore, a comprehensive safety assessment is mandatory for any strain proposed for industrial or probiotic application.

The present study aimed to characterize LAB, particularly lactobacilli strains, isolated from São Jorge cheese, with a primary focus on their antimicrobial potential against major foodborne pathogens and fungi. In addition, we evaluated their technological properties, safety profile, tolerance to simulated gastrointestinal conditions, adhesion potential, and capacity to produce EPS, ACE inhibitory peptides, CLA, and GABA (via GAD activity).

2. Materials and Methods

2.1. Bacterial Isolation

Lactic acid bacteria (LAB) were isolated from São Jorge Cheese (The Azores, Portugal), following the procedure described by Coelho et al. [12]. The 16S rDNA sequences were deposited in the GenBank under accession number *Lentilactobacillus parabuchneri* SJC114 OQ457274, *Lacticaseibacillus paracasei* SJC115 OQ457275, *L. paracasei* SJC116 PZ326616, *L. paracasei* SJC117 OQ457276, *Levilactobacillus brevis* SJC119 OQ457278 and *L. brevis* strain SJC120 OQ457279. Stock cultures were kept at -80°C in 30% (v/v) glycerol and propagated twice in MRS broth (Biokar Diagnostics, Allonne, France), with 1% (v/v) of inoculum at 30°C for 24 h.

2.2. Technological Characterization

2.2.1. Acidification Capacity

The acidification capacity of lactic acid bacteria (LAB) was evaluated in Skim milk (VWR Chemicals, Leuven, Belgium). Firstly, isolates were inoculated into MRS broth and

incubated at 30 °C for 24 h. After this incubation, a 1% inoculum was prepared in skimmed milk and incubated again at 30 °C for 24 h. During this time, the pH of the cultures was measured using a potentiometer. The pH measurements were taken after 0, 6 and 24 h.

2.2.2. Sugar Fermentation

The sugar fermentation patterns of the isolates were investigated using an array of 18 carbohydrates: glucose, fructose, galactose, ribose, arabinose, sucrose, lactose, maltose, raffinose, starch, mannitol, xylose, trehalose, rhamnose, dextrin, sorbitol, glycerol, and inulin. Briefly, a 2 McFarland suspension of each bacterial strain was dispensed, supplemented with 1% of the individual sugars, and then sealed beneath a layer of mineral oil prior to a 30 °C incubation lasting 48 h [13]. Following this, fermentation was judged by the acidification of the broth, which became evident through the distinct colour alteration of the pH-sensitive indicator present in the medium.

2.2.3. Enzymatic Activity

Enzymatic activities for a panel of 19 hydrolases, namely: alkaline phosphatase, esterase (C4), esterase-lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase—were semi-quantitatively evaluated with the APIZYM system, according to the manufacturer [14]. Colour intensity was employed as the criterion for scoring each reaction on a range from 0 to 5. Scores falling between 0 and 2 were interpreted as negative results, whereas scores from 3 to 5 were taken as indicative of a positive enzymatic response.

2.2.4. Proteolytic Activity

The assessment of extracellular proteolytic capacity was performed by inoculating the bacterial isolates onto Plate Count Agar (PCA, Biokar Diagnostics, Allonne, France) that had been enriched with 10% (*w/v*) skim milk. Incubation of the inoculated plates was carried out at 30 °C and, after 72 h, a 1% HCl solution was applied as a flood to the culture surfaces. Proteolysis was evidenced by the appearance of translucent, clear halos encircling the colonies [15]. For comparative reference, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 was used as a positive reaction control.

2.2.5. Lipolytic Activity

Lipolytic activity was evaluated using Tributyrin agar (Merck, Darmstadt, Germany), onto which the bacterial strains were deposited. Incubation of the inoculated plates was then conducted at 30 °C for a total of 72 h. Following this period, the development of a transparent, clearing zone encircling the microbial growth was recorded as a positive reaction [16]. *S. aureus* ATCC 25923 was used as a positive reaction control.

2.3. Safety Evaluation

2.3.1. Haemolytic Activity

The assessment of haemolytic properties was conducted on blood agar plates. These plates were prepared from Tryptose Blood Agar (Merck, Darmstadt, Germany), which was supplemented with ovine erythrocytes, and the bacterial isolates were streaked onto their surfaces. A 48 h incubation period at 37 °C was then applied to all inoculated plates. Following this, the culture media were examined, and the observed reactions were assigned to three distinct categories based on the zones surrounding the colonies: β -haemolysis was defined by the appearance of a transparent clearing zone; α -haemolysis was identified by a

greenish discolouration of the medium; and γ -haemolysis was recorded when no zone of altered appearance was detected [17].

2.3.2. DNase Activity

DNase activity was analysed by plating isolates on DNase-Test agar (Sigma-Aldrich, St. Louis, MO, USA), followed by incubation for 48 h at 37 °C. A positive reaction was indicated by the appearance of a pink halo around the colonies [18].

2.3.3. Gelatinase Activity

The production of the enzyme gelatinase was determined according to the methodology of Terzić-Vidojević et al. [19]. In this assay, the presence of a transparent halo around the colonies is considered an indicator of a positive reaction. *S. aureus* ATCC 25923 was used as a positive control.

2.3.4. Antibiotic Susceptibility

Antibiotic resistance was assessed using the Kirby-Bauer method (agar diffusion). Seven antibiotics were tested: Ampicillin, Oxacillin, Vancomycin, Tetracycline, Chloramphenicol, Streptomycin and Kanamycin. The discs were then incubated at 30 °C for 24–48 h. After incubation, the zones of inhibition, including the disc diameter, were measured using a ruler. The reference values from CLSI [20] were used to categorize isolates into resistant (R), intermediate (I) and sensitive (S). *S. aureus* ATCC 25923 was used as positive control.

2.4. Antimicrobial Activity

2.4.1. Antimicrobial Activity

The antimicrobial activity of the isolates was determined using the agar diffusion method as described by Ribeiro et al. [21]. Briefly, cell-free supernatants (CFS) from 48 h MRS cultures (30 °C) were obtained by centrifugation and filter-sterilized. Neutralized supernatants (pH 6.5–7.0) were also prepared (neutralized CFS). Indicator strains included *Listeria innocua* ATCC 33090, *Listeria monocytogenes* ATCC 35152, *L. monocytogenes* ATCC 13932, *Escherichia coli* ATCC 15922, *E. coli* ATCC 8739, *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *S. enterica* serovar Typhimurium ATCC 14028, *Bacillus spizizenii* ATCC 6633 and *Bacillus cereus* ATCC 11778. Prior to use, all indicator strains were propagated in Nutrient Broth at 37 °C for 18 h. These cultures were adjusted to 0.5 McFarland and inoculated into PCA (100 μ L per 200 mL medium). After solidification, 6 mm wells were filled with 60 μ L of CFS or neutralized CFS from each strain. Following overnight incubation at 37 °C, inhibition halos were measured.

2.4.2. Antifungal Activity

Antifungal activity was evaluated following the method of Cheong et al. [22], with minor modifications. Bacterial cultures were grown in MRS broth at 30 °C until abundant growth. Each isolate was then streaked in two 5 cm lines on MRS agar plates using a sterile swab and incubated at 30 °C for 48 h. After incubation, plates were overlaid with 10 mL of Potato Dextrose Agar (PDA) containing 10⁴ spores/mL of test fungi. The fungal species tested included *Aspergillus flavus* MUM 16.106 (isolated from cheese rind), *Penicillium nordicum* MUM 16.93 (from cheese ripening room), *Aspergillus chevalieri* MUM 00.07 (cheese curing chamber air), *Penicillium commune* MUM 16.56 (cheese rind), and *Penicillium brevicompactum* MUM 9906 (curing chamber surface), obtained from the University of Minho Mycotheca. Inhibition zones around LAB growth were scored as: (–) no inhibition (colonies fully overgrown by mould); (– / +) weak inhibition (inhibition on the LAB colony but no adjacent clearing); (+) low inhibition (small clearing zone); (++) moderate

inhibition (clearing zone ~50% of the plate); (+++) strong inhibition (large clearing zone covering most or all of the plate).

2.5. Probiotic Evaluation

2.5.1. Gastrointestinal Resistance

Gastrointestinal tolerance was assessed by measuring survival under conditions mimicking the stomach (pH 2.5) and the small intestine (exposure to bile acids and pancreatin), following the procedure described by Jurášková et al. [13].

2.5.2. Hydrophobicity

Bacterial hydrophobicity was evaluated using the solvent partitioning method with chloroform and ethyl acetate [23]. Isolates were grown in MRS broth at 30 °C for 24 h, harvested by centrifugation (4000× g, 20 min, 20 °C), washed twice with phosphate-buffered saline (PBS), and resuspended in PBS to an initial optical density at 600 nm (OD_{before}) of 0.5–0.6. Aliquots (3 mL) of the bacterial suspension were transferred to sealable tubes containing 1 mL of each solvent. The mixtures were vortexed for 2 min and left to stand for 10 min to allow phase separation. After removal of the organic (upper) layer, the OD₆₀₀ of the aqueous phase was measured (OD_{after}). The assay was performed in quadruplicate per isolate. Hydrophobicity (%) was calculated as:

$$\text{Hydrophobicity (\%)} = \left[1 - \left(\frac{\text{OD}_{\text{after}}}{\text{OD}_{\text{before}}} \right) \right] \times 100$$

2.5.3. Autoaggregation

Autoaggregation capacity was evaluated following the method of Todorov and Dicks [24]. Isolates were cultured in MRS broth at 30 °C for 24 h, harvested by centrifugation (4000× g, 20 min, 20 °C), washed twice with PBS, and resuspended in PBS. The initial optical density at 600 nm was recorded (OD_{before}). After 60 min of static incubation at room temperature, the bacterial suspension was gently centrifuged (300× g, 2 min, 20 °C), and the final (OD_{after}) of the supernatant was measured. Assays were performed in quadruplicate per isolate. Autoaggregation (%) was calculated as:

$$\text{Autoaggregation (\%)} = \frac{(\text{Abs}_{\text{before}} - \text{Abs}_{\text{after}})}{\text{Abs}_{\text{before}}} \times 100$$

2.5.4. Coaggregation

The coaggregation ability of LAB isolates with pathogenic bacteria was assessed according to Chen et al. [25]. The pathogens tested included *E. coli* ATCC 25922, *L. monocytogenes* ATCC 13932, *S. aureus* ATCC 25923 and *S. Typhimurium* ATCC 14028. LAB isolates and pathogen suspensions (prepared as for autoaggregation) were mixed in equal volumes (2 mL each) and left undisturbed for 60 min at room temperature. Coaggregation was calculated from OD measurements at 600 nm, using the following equation:

$$\text{Coaggregation (\%)} = \frac{[(A_x + A_y) / (2 - A_{x+y})]}{A_x + A_y} \times 100$$

where A_x and A_y designate the absorbance (600 nm) of the two bacteria cell suspensions and A_{x+y} is the absorbance of mixed bacteria cell suspensions.

2.5.5. Production of EPS

The determination of exopolysaccharide production was carried out in solid and liquid media according to the methods of Smitinont et al. [26] and Cirrincione et al. [27]. The

LAB isolates were inoculated on MRS agar plates and in liquid MRS broth containing 10% (*w/v*) of each of the following sugars: glucose, fructose, sucrose and lactose. The isolates were incubated at 30 °C for 3 days to determine which isolates could produce EPS. In solid medium, all cultures that exhibited slime colonies were considered positive for EPS. After incubation, the liquid LAB cultures were centrifuged (4000× *g*, 20 min, 4 °C) and the supernatants discarded. The ropy phenotype was assessed by inserting an inoculation loop into the pellet and visually inspecting the filament when the loop was lifted (EPS-positive—filament formation). All measurements were performed in duplicate.

2.5.6. Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

ACE inhibitory activity was measured according to Cushman and Cheung [28], based on the release of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalysed by ACE. LAB isolates were incubated in skim milk (10% *v/v*) at 30 °C for 24 h. Then, 40 µL of fermented milk were mixed with 400 µL of buffered substrate solution (5 mM HHL in 100 mM sodium borate buffer containing 300 mM NaCl, pH 8.3). Subsequently, 80 µL of ACE solution (0.1 U/mL) were added, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 250 µL of 1 M HCl. Hippuric acid released during the reaction was extracted with 1.7 mL of ethyl acetate. After centrifugation (4000× *g*, 5 min), 1 mL of the organic phase was transferred to a glass tube and evaporated in a water bath at 95 °C for 20 min. The residue was dissolved in 1 mL of distilled water, and its absorbance was measured at 228 nm.

ACE inhibition (%) was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

where *A* is the absorbance of the control without sample, *B* is the absorbance in the presence of ACE and sample, and *C* is the absorbance in the absence of ACE and sample. All measurements were performed in duplicate.

2.5.7. Conjugated Linoleic Acid (CLA) Production

CLA production was assessed using the spectrophotometric method as described by Ribeiro et al. [29]. Briefly, LAB strains were grown in MRS broth supplemented with free linoleic acid (0.5 mg/mL) and 2% (*w/v*) Tween 80 at 30 °C for 48 h. After centrifugation (20,800× *g*, 1 min), the supernatant was mixed with isopropanol (2 mL) and allowed to stand for 3 min. Fatty acids were extracted by adding hexane (1.5 mL), followed by vortexing and a further 3 min incubation. The hexane layer was transferred to a 96-well plate, and absorbance was measured at 233 nm in a microtiter reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany). Pure *cis*-9, *trans*-11 CLA was used for standard curve. All measurements were performed in duplicate.

2.5.8. GABA Production Capacity

Potential for GABA production was screened using a GAD colorimetric assay [30]. Cells from fresh cultures were harvested by centrifugation (4000× *g*, 20 min, 25 °C), washed with 0.9% NaCl, and re-centrifuged. The pellet was resuspended in a GAD solution (1 g glutamic acid, 0.3 mL Triton X-100, 90 g NaCl, 0.05 g/L bromocresol green, pH 4) and incubated anaerobically at 37 °C for 4 h. A colour change from green to blue indicated GABA production, as confirmed by the positive control *Enterococcus malodoratus* SJC62 [12].

2.6. Statistical Analysis

All experiments were performed in at least two independent biological replicates with technical triplicates. Results are expressed as mean ± standard error of the mean (SEM).

Statistical significance was evaluated using one way ANOVA followed by post hoc Tukey's test ($p < 0.05$). All statistical analysis were performed with IBM SPSS Statistics, version 30 (IBM Corporation, New York, NY, USA).

3. Results

3.1. Technological Characterization

All six LAB isolates were assessed for acidification capacity in skim milk, sugar fermentation profile, enzymatic activity, and proteolytic/lipolytic activity (Figure 1).

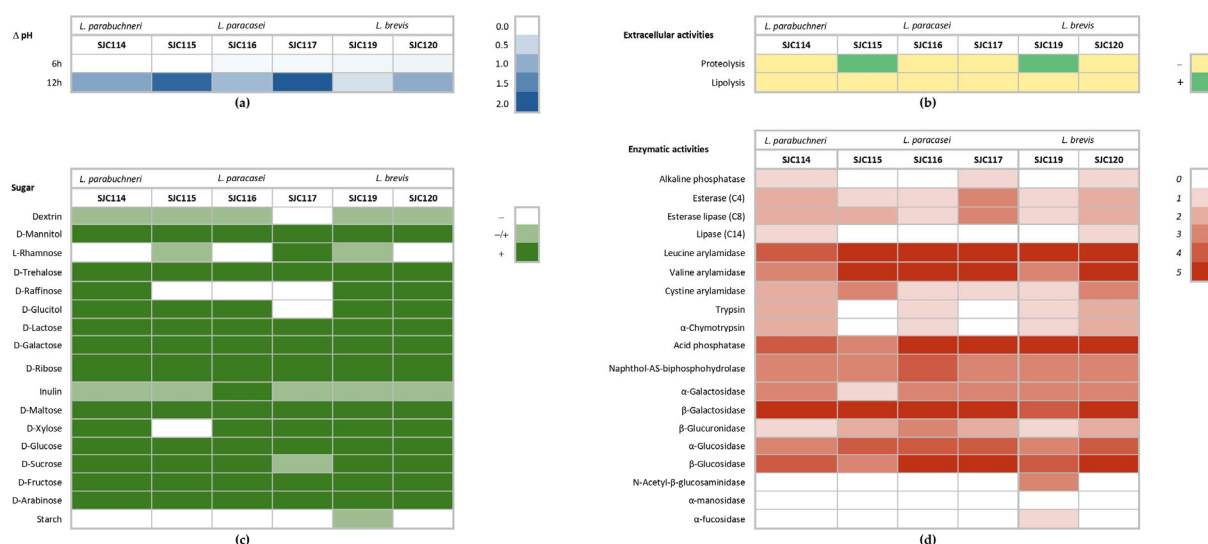


Figure 1. Heatmap of LAB isolates showing (a) acidification (ΔpH) in milk after 6 and 24 h, (b) proteolytic and lipolytic activities (negative −, and positive +), (c) carbohydrate fermentation (classified as negative −, slight positive −/+, and positive +); (d) enzymatic activities: ranging from 0 (no activity) to 5 (40 nmol hydrolysed substrate).

After 24 h of incubation, the highest milk acidification (ΔpH) was observed for *Lactobacillus paracasei* SJC115 ($\Delta pH = 1.8$), followed by SJC117 ($\Delta pH = 1.5$) and SJC116 ($\Delta pH = 1.3$). *Lentilactobacillus parabuchneri* SJC114 and both *Levilactobacillus brevis* strains (SJC119 and SJC120) showed weak acidification ($\Delta pH < 0.8$) (Figure 1a).

Proteolytic activity (clear zones on milk-PCA) was positive for SJC115 and SJC119, while the remaining isolates showed no proteolysis (Figure 1b).

Concerning carbohydrate fermentation (Figure 1c), all isolates fermented glucose, fructose, galactose, sucrose, lactose, maltose, trehalose, and mannitol. Strain-dependent variability was observed for ribose, arabinose, raffinose, xylose, rhamnose, sorbitol, and glycerol. Starch, dextrin, and inulin were not fermented by any isolate.

Enzymatic profiling (Figure 1d) revealed that all isolates produced high levels of β -galactosidase, leucine arylamidase, valine arylamidase, and acid phosphatase. Moderate to high α - and β -glucosidase activities were also present. Undesirable enzyme activities such as trypsin and α -chymotrypsin were absent in isolates, whereas low levels of N-acetyl- β -glucosaminidase was detected in *L. brevis* SJC119, and low activity of β -glucuronidase was detected in *L. paracasei* SJC116. Alkaline phosphatase, lipase (C14), α -mannosidase and α -fucosidase activities were absent in all isolates.

3.2. Safety Evaluation

Haemolytic activity on blood agar was γ -haemolysis (no halo) for all isolates, indicating absence of β -haemolysis. In addition, no DNase or gelatinase activity was detected for any strain.

Antibiotic susceptibility test (Table 1) showed that all isolates were sensitive to ampicillin and chloramphenicol, except for *L. brevis* SJC119. In contrast, all isolates were resistant to oxacillin. Resistance to vancomycin was observed for SJC116, SJC119 (resistant) and SJC117, SJC120 (intermediate). Tetracycline resistance was found in all *L. paracasei* and *L. brevis* isolates, whereas *L. parabuchneri* SJC114 was sensitive. For aminoglycosides, streptomycin resistance was observed for SJC115 and intermediate susceptibility for SJC116 and SJC119; kanamycin resistance was present in SJC115, SJC116, and intermediate for SJC117, SJC119, SJC120 (Table 1).

Table 1. Results of resistance/sensitivity tests of LAB isolates toward seven antibiotics.

Group	Antibiotic *	<i>L. parabuchneri</i>	<i>L. paracasei</i>			<i>L. brevis</i>	
		SJC114	SJC115	SJC116	SJC117	SJC119	SJC120
Penicillins	Ampicillin	S	S	S	S	R	S
	Oxacillin	R	R	R	R	R	R
Glycopeptides	Vancomycin	S	S	R	I	R	I
Tetracyclines	Tetracycline	S	R	R	R	R	R
Phenicals	Chloramphenicol	S	S	S	S	R	S
Aminoglycosides	Streptomycin	S	R	I	S	I	S
	Kanamycin	S	R	R	I	I	I

* S—Sensitive, I—Intermediate sensitivity, R—Resistant.

3.3. Antimicrobial Activity

The antimicrobial activity was evaluated against key foodborne pathogens and spoilage fungi. The target bacteria included several Gram-positive strains: *L. innocua*, *L. monocytogenes*, *S. aureus*, *B. spizizenii* and *B. cereus*; and Gram negative: *E. coli*, *P. aeruginosa*, and *S. enterica* serovars Enteritidis and Typhimurium (Table 2). None of the crude supernatants (CFS) from isolate SJC114 inhibited any indicator strain. For the remaining isolates, CFS exhibited variable inhibition zones against *L. innocua*, *L. monocytogenes* (both strains), *E. coli* strains, *S. aureus*, *P. aeruginosa*, *Salmonella* serovars, *B. spizizenii*, and *B. cereus*.

Notably, neutralized supernatants (NS, pH 6.5–7.0) retained inhibitory activity only for isolates SJC117 and SJC120. The NS of SJC117 inhibited *L. monocytogenes* ATCC 13932 (5.2 ± 4.2 mm), *E. coli* ATCC 25922 (6.3 ± 8.9 mm), and *B. spizizenii* (9.1 ± 0.2 mm). SJC120 NS showed activity only against *L. monocytogenes* ATCC 13932 (5.6 ± 7.9 mm). All other neutralized supernatants showed no inhibition, indicating that the antimicrobial activity was mostly due to organic acids, except for SJC117 and SJC120 where BLIS production is suggested.

LAB's antifungal activity is an important parameter for their technological use. Various filamentous fungi from the *Aspergillus* and *Penicillium* genera isolated from cheese rind and cheese-making environment were evaluated (Table 3). Strong inhibition (large clearing zone covering most or all the plate) against *P. commune*, *P. brevicompactum*, *P. nordicum*, *A. chevalieri*, and *A. flavus* was observed for isolates SJC116, SJC117, SJC119, and SJC120. *L. parabuchneri* SJC114 showed strong inhibition against *P. brevicompactum*, *P. nordicum*, and *A. chevalieri*, but weak inhibition ($\pm$) against *P. commune* and *A. flavus*. SJC115 exhibited moderate to strong inhibition against most moulds, except *P. commune* (low inhibition, +) and *A. flavus* (low, +).

Table 2. Antibacterial activity of LAB isolates. Data are presented as mean $\pm$ SEM of inhibition zone diameters (mm) obtained with crude cell-free supernatant (CFS) and neutralized supernatant (NS).

Target Strains ¹	<i>L. parabuchneri</i>		<i>L. paracasei</i>				<i>L. brevis</i>					
	SJC114		SJC115		SJC116		SJC117		SJC119		SJC120	
	CFS	CFS	NS	CFS	NS	CFS	NS	CFS	NS	CFS	NS	
<i>L. innocua</i> ATCC 33090	0.0 ± 0.0 ^a	10.1 ± 0.3 _b	0.0 ± 0.0 _A	10.4 ± 0.1 _b	0.0 ± 0.0 _A	10.3 ± 0.3 _b	0.0 ± 0.0 ^A	9.6 ± 0.7 _b	0.0 ± 0.0 _A	9.2 ± 0.1 ^b	0.0 ± 0.0 _A	
<i>L. monocytogenes</i> ATCC 35152	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	6.4 ± 1.2 ^b	0.0 ± 0.0 ^A	0.0 ± 0.0 _a	0.0 ± 0.0 _A	9.3 ± 0.3 ^c	0.0 ± 0.0 _A	
<i>L. monocytogenes</i> ATCC 13932	0.0 ± 0.0 ^a	11.2 ± 0.8 _b	0.0 ± 0.0 _A	10.2 ± 0.4 _b	0.0 ± 0.0 _A	10.5 ± 0.5 _b	10.7 ± 0.9 _B	9.5 ± 0.5 _b	0.0 ± 0.0 _A	10.5 ± 0.1 _b	5.6 ± 7.9 _B	
<i>E. coli</i> ATCC 15922	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	10.7 ± 0.3 _b	0.0 ± 0.0 _A	9.1 ± 0.8 ^b	0.0 ± 0.0 ^A	0.0 ± 0.0 _a	0.0 ± 0.0 _A	10.2 ± 0.3 _b	0.0 ± 0.0 _A	
<i>E. coli</i> ATCC 8739	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	9.9 ± 0.8 ^b	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 ^A	0.0 ± 0.0 _a	0.0 ± 0.0 _A	9.2 ± 0.6 ^b	0.0 ± 0.0 _A	
<i>E. coli</i> ATCC 25922	0.0 ± 0.0 ^a	10.3 ± 0.3 _{bc}	0.0 ± 0.0 _A	10.6 ± 0.1 _c	0.0 ± 0.0 _A	10.3 ± 0.3 _{bc}	11.3 ± 1.3 _B	8.6 ± 0.4 _b	0.0 ± 0.0 _A	10.4 ± 0.6 _{bc}	0.0 ± 0.0 _A	
<i>S. aureus</i> ATCC 25923	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	10.9 ± 0.9 _b	0.0 ± 0.0 ^A	0.0 ± 0.0 _a	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	
<i>P. aeruginosa</i> ATCC 27853	0.0 ± 0.0 ^a	7.8 ± 0.1 ^b	0.0 ± 0.0 _A	9.3 ± 0.2 ^b	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 ^A	8.4 ± 0.4 _b	0.0 ± 0.0 _A	7.9 ± 0.2 ^b	0.0 ± 0.0 _A	
<i>S. Enteritidis</i> ATCC 13076	0.0 ± 0.0 ^a	10.5 ± 0.1 _b	0.0 ± 0.0 _A	11.2 ± 0.6 _b	0.0 ± 0.0 _A	9.6 ± 0.4 ^b	0.0 ± 0.0 ^A	8.9 ± 0.2 _b	0.0 ± 0.0 _A	10.2 ± 0.1 _b	0.0 ± 0.0 _A	
<i>S. Typhimurium</i> ATCC 14028	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	11.3 ± 0.6 _b	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 ^A	9.7 ± 0.3 _b	0.0 ± 0.0 _A	10.7 ± 0.1 _b	0.0 ± 0.0 _A	
<i>B. spizizenii</i> ATCC 6333	0.0 ± 0.0 ^a	10.5 ± 0.2 _b	0.0 ± 0.0 _A	11.8 ± 0.2 _b	0.0 ± 0.0 _A	10.4 ± 0.4 _b	9.1 ± 0.2 ^B	10.8 ± 0.4 ^b	0.0 ± 0.0 _A	10.9 ± 0.1 _b	0.0 ± 0.0 _A	
<i>B. cereus</i> ATCC 11778	0.0 ± 0.0 ^a	0.0 ± 0.0 ^a	0.0 ± 0.0 _A	9.6 ± 0.1 ^b	0.0 ± 0.0 _A	0.0 ± 0.0 ^a	0.0 ± 0.0 ^A	0.0 ± 0.0 _a	0.0 ± 0.0 _A	9.2 ± 0.1 ^b	0.0 ± 0.0 _A	

¹ Within each row (i.e., for each target strain), significant differences ($p < 0.05$) between CFS are indicated by different lowercase letters (a–c), whereas significant differences between NS are indicated by different capital letters (A,B).

Table 3. Antifungal activity of LAB isolates. The following filamentous fungi were tested: *Penicillium commune*, *P. brevicompactum*, *P. nordicum*, *Aspergillus chevalieri*, and *A. flavus*.

LAB Isolates	<i>Penicillium</i> ¹			<i>Aspergillus</i> ¹	
	<i>P. commune</i>	<i>P. brevicompactum</i>	<i>P. nordicum</i>	<i>A. chevalieri</i>	<i>A. flavus</i>
<i>L. parabuchneri</i>					
SJC114	+ / –	+++	+++	+++	+ / –
<i>L. paracasei</i>					
SJC115	+	+++	+++	+++	+
SJC116	++	+++	+++	+++	+++
SJC117	+++	+++	+++	+++	+++
<i>L. brevis</i>					
SJC119	+++	+++	+++	+++	+++
SJC120	+++	+++	+++	+++	+++

¹ Very weak inhibition (+ / –), low inhibition (+), moderate inhibition (++), strong inhibition (+++).

3.4. Probiotic Potential

3.4.1. Gastrointestinal Tolerance

To be considered probiotic, bacteria must be able to survive at low pH in the stomach and to tolerate the conditions of the intestine (presence of bile acids and pancreatin). Thus, viable colony counts of LAB were performed over 3 h (simulating digestion time) under gastrointestinal conditions. Resistance to gastrointestinal conditions was evaluated based on two parameters: pH 2.5 (acidic stomach conditions) and 0.3% bile acids with 0.1% pancreatin (intestinal conditions). The results, expressed in colony-forming units (CFU/mL), are presented in Figure 2a,b.

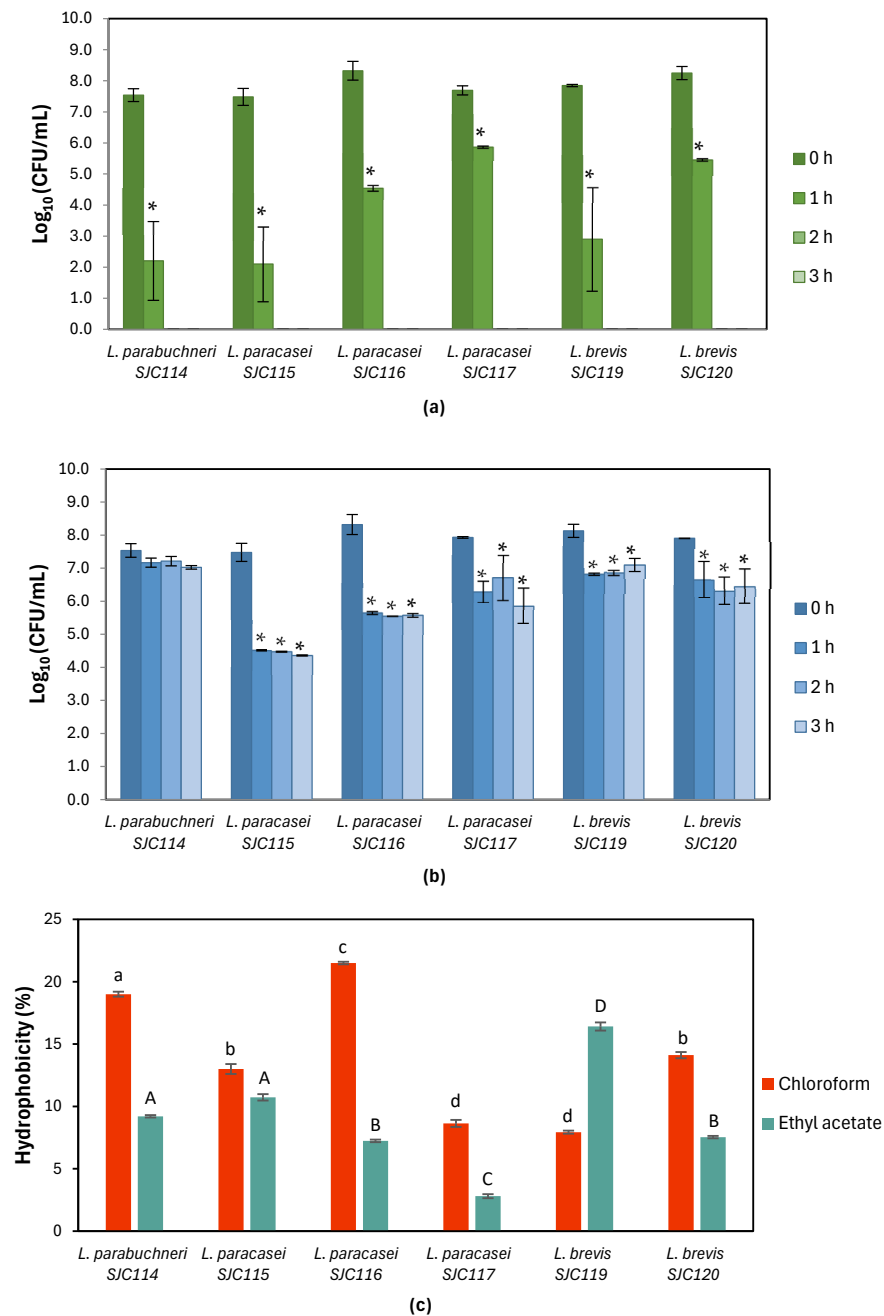


Figure 2. (a) Tolerance of LAB isolates (log CFU/mL) to acidic conditions (pH 2.5) over 3 h. (b) Tolerance to bile acids (0.3%) and pancreatin (0.1%) over 3 h. Results are expressed as mean $\pm$ SEM ($n = 2$ independent experiments). * $p < 0.05$ versus 0 h (within the same isolate). (c) Hydrophobicity (%) of LAB isolates toward chloroform (red bars) and ethyl acetate (blue bars). Values are mean $\pm$ SEM ($n = 4$). For each solvent, different lowercase letters (a–d) indicate significant differences ($p < 0.05$) between strains for chloroform adhesion, and different capital letters (A–D) indicate significant differences ($p < 0.05$) for ethyl acetate adhesion.

All isolates showed greater tolerance to bile acids and pancreatin than to an acidic pH (2.5). Under acidic conditions, a time-dependent reduction in viability was observed for all isolates, with no detectable viable counts after 2 h. Nevertheless, all isolates maintained countable levels during the first hour, with *L. paracasei* SJC117 exhibiting the highest count (5.9 log CFU/mL).

Regarding survival in the presence of bile acids and pancreatin, all isolates remained viable after 3 h of exposure (Figure 2b). Although a slight reduction in counts occurred for some isolates after the first hour, viability remained stable thereafter until 3 h.

3.4.2. Hydrophobicity

The hydrophobicity of the isolates was tested with chloroform (electron acceptor, indicative of basic surface properties) and ethyl acetate (electron donor). The results are presented in Figure 2c.

Adhesion to chloroform ranged from 8% (SJC119) to 22% (SJC116), while adhesion to ethyl acetate was lower, varying between 3% (SJC117) and 16% (SJC119). SJC115 was the isolate that showed the most comparable hydrophobicity values between the two solvents.

3.4.3. Autoaggregation and Coaggregation

Autoaggregation after 60 min was highest for *L. paracasei* SJC115 ($32.8 \pm 0.08\%$), followed by SJC116 ($10.2 \pm 0.02\%$). SJC117 and SJC119 showed no autoaggregation (0%), while SJC114 ($7.3 \pm 0.06\%$) and SJC120 ($2.4 \pm 0.05\%$) exhibited low values (Table 4).

Table 4. Autoaggregation (%) of LAB isolates and coaggregation (%) with pathogenic bacteria: *E. coli* ATCC 25922, *L. monocytogenes* ATCC 13932, *S. aureus* ATCC 25923, and *S. Typhimurium* ATCC 14028. Results are expressed as mean $\pm$ SEM of four independent experiments.

LAB Isolates ¹	Auto-Aggregation (%)	Coaggregation (%)			
		<i>E. coli</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. Typhimurium</i>
<i>L. parabuchneri</i> SJC114	6.93 ± 1.46 ^{bc}	38.29 ± 0.36 ^b	28.27 ± 0.44 ^a	27.25 ± 0.43 ^{ab}	39.69 ± 0.43 ^a
<i>L. paracasei</i> SJC115	31.71 ± 0.98 ^d	31.68 ± 0.30 ^a	31.78 ± 0.65 ^{abc}	30.74 ± 0.22 ^{bc}	31.75 ± 0.62 ^b
SJC116	10.24 ± 0.32 ^c	40.95 ± 0.48 ^c	34.24 ± 0.50 ^{bc}	33.29 ± 0.73 ^c	39.28 ± 0.95 ^a
SJC117	0.0 ± 0.0 ^a	33.60 ± 0.91 ^a	31.47 ± 0.34 ^{ab}	29.02 ± 0.29 ^{ab}	31.97 ± 0.54 ^b
<i>L. brevis</i> SJC119	0.0 ± 0.0 ^a	38.05 ± 0.05 ^b	26.65 ± 0.60 ^a	25.59 ± 1.37 ^a	39.68 ± 0.17 ^a
SJC120	2.89 ± 1.63 ^{ab}	41.02 ± 0.38 ^c	37.46 ± 2.91 ^c	33.29 ± 1.13 ^c	40.19 ± 0.65 ^a

¹ For each column, values with different lowercase superscript letters are significantly different ($p < 0.05$).

Coaggregation percentages with pathogenic bacteria ranged from 26.7% to 40.7% (Table 4). The highest values were observed for SJC116 with *E. coli* ($40.7 \pm 0.01\%$) and *S. aureus* ($35.1 \pm 0.02\%$), and for SJC120 with *L. monocytogenes* ($36.7 \pm 0.07\%$) and *Salmonella* Typhimurium ($40.5 \pm 0.01\%$). All isolates exhibited moderate to high coaggregation ($\geq 27\%$) against every pathogen tested.

3.4.4. EPS Production

Figure 3 presents the EPS-producing ability of the LAB isolates from various sugars. All isolates produced EPS from at least one tested sugar.

Isolates SJC114, SJC115, and SJC120 produced EPS only in liquid medium. Isolates SJC115 and SJC117 produced EPS with all tested sugars (sucrose, fructose, glucose, and lactose), whereas other isolates were more selective. For example, *L. paracasei* SJC116 used only fructose for EPS production (in either solid or liquid medium), and the *L. brevis* isolates used only glucose (*L. brevis* SJC119 in solid medium and SJC120 in liquid medium). EPS production in solid medium was observed only with fructose (for *L. paracasei* SJC116 and SJC117) and with glucose (for SJC120).

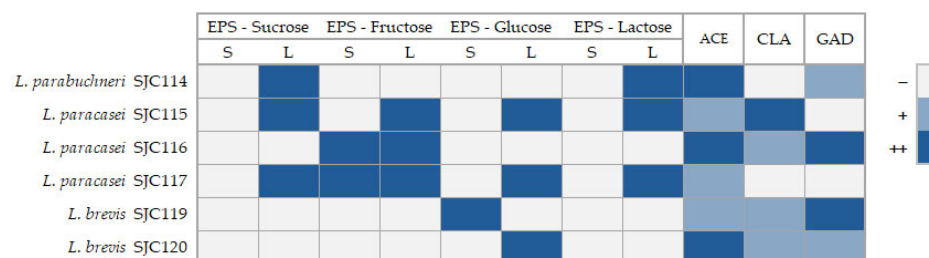


Figure 3. Screening of LAB isolates for the production of EPS, ACE-inhibitory peptides, CLA, and GAD activity. EPS production was assessed with sucrose, fructose, glucose, and lactose in solid (S) and liquid (L) media: (–) no production; (++) production. ACE inhibition: (–) none; (+) high (80–85%); (++) very high (>85%). CLA production: (–) none; (+) low (<2 µg/mL); (++) high (~3.5 µg/mL). GAD activity: (–) none; (+) low; (++) high.

3.4.5. ACE Inhibitory Activity

All LAB isolates were found to possess the ability to produce ACE-inhibitory peptides (Figure 3). Isolates SJC114, SJC116, and SJC120 showed the highest ACE inhibition by the fermented milk peptides (>85%).

3.4.6. CLA Production

Conjugated linoleic acid (CLA) production varied among LAB isolates (Figure 3). Although several isolates could produce CLA from linoleic acid, the achieved CLA concentrations remained relatively low. The highest CLA production (3.54 µg/mL) was observed for *L. paracasei* SJC115. Low production (0.6–2 µg/mL) was observed for SJC116, SJC119, and SJC120, while SJC114 and SJC117 did not produce CLA.

3.4.7. GAD Activity

The ability of the isolates to convert monosodium glutamate (MSG) into γ -aminobutyric acid (GABA)—i.e., glutamate decarboxylase (GAD) enzyme activity—was also evaluated. The isolates *L. paracasei* SJC116 and *L. brevis* SJC119 exhibited high GAD activity (Figure 3).

4. Discussion

In this study, six LAB isolates (QPS status) from São Jorge PDO cheese were evaluated for technological properties, safety, antimicrobial activity, and probiotic potential. The results revealed strain-specific differences, with *L. paracasei* SJC117 consistently standing out as the most promising candidate for biopreservation and functional food applications.

All isolates acidified milk only moderately ($\Delta\text{pH} < 1.8$ after 24 h), which is typical for non-starter LAB (NSLAB) that are not selected for rapid fermentation [5]. Nevertheless, their ability to ferment a broad range of carbohydrates, including lactose and galactose, indicates good adaptation to the dairy environment. The absence of lipolytic activity is desirable because it prevents off-flavour development during cheese ripening, whereas the proteolytic activity observed in SJC115 and SJC119 may contribute to texture and flavour formation [31,32]. These results agree with those reported by other studies, which refer to a weak lipolytic capacity of LAB, although some strains exhibit proteolytic activity [33,34].

The high levels of β -galactosidase, leucine arylamidase, valine arylamidase, and acid phosphatase observed in the LAB isolates, together with moderate to high α - and β -glucosidase activities, are technologically advantageous for dairy applications. The presence of high β galactosidase activity in all strains is technologically relevant for lactose hydrolysis, enabling LAB to ferment milk efficiently and produce lactic acid, which drives curd acidification and prevents spoilage [5]. Moreover, strains with high β -galactosidase

activity are valuable for producing lactose-free or low-lactose dairy products, addressing the needs of lactose-intolerant consumers [35].

Leucine arylamidase and valine arylamidase are aminopeptidases that play a key role in cheese ripening [36]. They hydrolyse bitter-tasting peptides derived from casein breakdown, thereby reducing bitterness and contributing to the development of typical cheese flavour and texture [33]. High aminopeptidase activity is considered a desirable trait for adjunct cultures [37]. In addition, acid phosphatase is involved in the dephosphorylation of phosphoproteins and phosphopeptides, which can affect the mineral balance and texture of fermented dairy products [38]. Its presence has been linked to improved cheese ripening and enhanced bioavailability of phosphorus [39].

The α - and β -glucosidase activities detected at moderate to high levels further expand the metabolic versatility of these strains. β -Glucosidase can hydrolyse glucosides present in plant-based ingredients, potentially increasing the availability of bioactive aglycones (e.g., isoflavones) in functional dairy products [40]. α -Glucosidase activity may also contribute to the breakdown of α -linked oligosaccharides, although some studies suggest that probiotics with low α -glucosidase activity could be beneficial for diabetic individuals by reducing postprandial hyperglycaemia [41]. Nevertheless, in the context of dairy fermentation, moderate to high α -glucosidase activity is generally considered neutral or positive [42].

Undesirable enzymes (e.g., α -chymotrypsin, β -glucuronidase, and N-acetyl- β -glucosaminidase), which are associated with intestinal diseases and potential colonic harm, were either absent or present at low levels in some isolates. These enzymes can exert negative effects in the colon and have been associated with gastrointestinal diseases [43]. β -Glucuronidase can hydrolyse various glucuronides (or glucuronosides), leading to the release of carcinogenic compounds in the colon, including polycyclic aromatic hydrocarbons [44]. N-Acetyl- β -glucosaminidase reduces N-nitro compounds (e.g., nitrobenzenes) to amines, which are often mutagenic and carcinogenic [45]. In the present study, low activity of N-acetyl- β -glucosaminidase was observed in isolate SJC119, and β -glucuronidase in isolate SJC116. Although the detected intensities were not high, the presence of these enzymatic activities in these isolates may preclude their use as probiotics.

Regarding safety, all isolates were negative for DNase, gelatinase, and haemolysis—key safety criteria for potential probiotic or starter cultures [46]. Antibiotic susceptibility testing revealed a generally favourable profile, with most isolates sensitive to clinically relevant antibiotics such as ampicillin and chloramphenicol. The observed resistance patterns must be interpreted with caution, given the limitations of disk diffusion for LAB; confirmatory minimum inhibitory concentrations (MIC) testing and whole-genome sequencing for resistance genes are necessary before reliable classifications can be made. Antibiotic resistance in LAB is not considered a safety concern unless actual transfer of resistance genes occurs. Resistance to oxacillin (all isolates), vancomycin (several *L. paracasei* and *L. brevis* strains), and aminoglycosides (including kanamycin and streptomycin) is commonly reported as intrinsic in many lactobacilli and is not considered a safety concern due to lack of transferability [47]. However, resistance to other antibiotics, such as tetracycline (observed in all *L. paracasei* and *L. brevis* isolates), is often acquired—although in some groups it may also be intrinsic [47]. The absence of β -haemolysis and gelatinase activity further supports the non-pathogenic nature of these strains. Nevertheless, a full genomic assessment for mobile genetic elements, biogenic amine gene clusters, and acquired resistance genes is advisable before industrial application.

Evaluating the antimicrobial activity of LAB isolates is essential for identifying strains that can enhance food safety by inhibiting foodborne pathogens and spoilage microorganisms, serve as natural biopreservatives (including bacteriocin producers), and provide probiotic benefits through competition against enteric pathogens. With the exception of

L. parabuchneri SJC114, all other isolates (*L. paracasei* and *L. brevis*) exhibited antibacterial activity in their crude supernatants against a broad range of foodborne pathogens, including *L. monocytogenes*, *E. coli*, *S. enterica*, *Pseudomonas*, and *Bacillus*. For most isolates, this activity was largely lost after neutralisation, indicating that organic acids are the primary inhibitory mechanism. Nevertheless, because acidic foods such as fruit juices are particularly susceptible to contamination by spoilage bacteria [48], these strains show potential as natural preservatives for food applications.

Remarkably, neutralised CFS from *L. paracasei* SJC117 retained activity against *L. monocytogenes*, *E. coli*, and *B. spizizenii*, suggesting the production of putative bacteriocin-like inhibitory substances (BLIS), although the contribution of other metabolites cannot be excluded. Likewise, neutralised CFS from *L. brevis* SJC120 remained active against *L. monocytogenes*. This is a notable finding, as BLIS-producing lactic acid bacteria can serve as biopreservatives in foods without adversely affecting sensory properties [49].

The antifungal activity against major spoilage moulds (*Penicillium* and *Aspergillus* spp.) was remarkably strong for most isolates, especially SJC116, SJC117, SJC119, and SJC120, which showed the highest inhibition against all test fungi. Such a broad antifungal spectrum, rarely reported for dairy-derived lactic acid bacteria, suggests that these strains could effectively control mould contamination in cheese ripening rooms and packaged products [22]. Compared to other cheese derived LAB, the simultaneous production of putative BLIS, strong antifungal activity, and high levels of ACE inhibitory peptides, CLA and GABA in SJC117 is uncommon. For instance, *L. paracasei* strains from other PDO cheeses typically exhibit narrower antifungal spectra (e.g., only against *Penicillium* spp.) and lower GABA yields [9,10]. Given the harmful effects of fungal contamination, strategies to mitigate mycotoxin production are of growing interest [50]. The antifungal activity of LAB has been shown to result from a range of metabolites, including phenyllactic acid (PLA), cyclic dipeptides, fatty acids, and organic acids [51]; however, further research is necessary to identify the specific compounds responsible in these strains.

To be considered probiotic, it is important that bacteria have the ability to survive low pH in order to tolerate the initial stress in the stomach and the conditions of the intestine (presence of bile acids and digestive enzymes) [52]. The gastrointestinal tolerance assays revealed a clear differentiation among the isolates. While all strains survived the simulated intestinal conditions with viable counts above 6 log CFU/mL, the ability to withstand gastric acidity (pH 2.5 for 1 h) was strain-dependent. Only *L. paracasei* SJC117 and *L. brevis* SJC120 maintained counts above 5 log CFU/mL, whereas the remaining isolates dropped to between 2 and 4.6 log CFU/mL. This is noteworthy because survival through the stomach is often the most restrictive step for orally delivered probiotics; a reduction of 3–6 log units at pH 2.5 is commonly observed even in robust strains, and the ability to retain >5 log CFU/mL indicates strong acid tolerance [53,54]. The threshold of 10⁶ CFU/mL in the small bowel is widely accepted to ensure that a sufficient number of viable cells reach the intestinal epithelium to exert health benefits [53]. The fact that only SJC117 and SJC120 isolates remained close to or above this threshold after the gastric challenge suggests they are the most promising candidates for further probiotic development, although all isolates remain viable under intestinal stress, which is encouraging.

The surface properties, however, revealed a different pattern. All isolates displayed relatively low cell surface hydrophobicity (<20% adhesion to both chloroform and ethyl acetate), indicating a predominantly hydrophilic cell surface. While high hydrophobicity is often used as a rapid indicator of potential adhesion to intestinal mucosa and epithelial cells, low values do not preclude effective mucosal interaction, as adhesion is a multifactorial process mediated by specific surface proteins, lipoteichoic acids, and exopolysaccharides that can compensate for a hydrophilic surface [55]. Despite the low hydrophobicity, the isolates

exhibited favourable autoaggregation and coaggregation traits, which are desirable for competitive exclusion of pathogens. Notably, *L. paracasei* SJC117 showed no autoaggregation yet displayed moderate to high coaggregation ($\approx 32\text{--}33\%$) with all four tested pathogens (*L. monocytogenes*, *E. coli*, *S. aureus* and *S. Typhimurium*). The absence of autoaggregation in strains SJC117 and SJC119, coupled with their robust coaggregation with pathogenic bacteria, is consistent with previous reports for other lactobacilli [56]. This observation suggests a cell-surface architecture that favours heterotypic (bacteria–pathogen) over homotypic (bacteria–bacteria) interactions. Such a phenotype may be mediated by surface-exposed adhesins or EPS that exhibit greater affinity for pathogen surface moieties than for self-surface determinants. Comparable patterns of coaggregation without autoaggregation, despite low cell-surface hydrophobicity, have been documented [56], supporting the hypothesis of a surface organisation that preferentially promotes interspecies interactions. Future studies focusing on the molecular characterisation of cell wall-associated proteins and EPS structure will be essential to unravel the mechanisms governing these adhesive properties. Meanwhile, isolates such as *L. paracasei* SJC115 and SJC116 demonstrated both autoaggregation and good coaggregation, potentially reflecting a greater capacity for biofilm formation. Overall, despite low hydrophobicity, the adhesive and anti-pathogenic traits support the probiotic functionality of these strains, with SJC117 and SJC120 emerging as the most robust candidates based on combined acid tolerance, intestinal survival, and pathogen coaggregation. Further in vivo studies are warranted to confirm these properties.

The simultaneous production of multiple bioactive metabolites represents a key strength of the strains analysed in this study. The ability to produce EPS was strain- and sugar-dependent; two isolates (SJC115 and SJC117) produced EPS with all four sugars tested, while others were more selective. This trait is technologically advantageous for improving food texture and water retention while also conferring prebiotic and immunomodulatory effects on the host. A limitation of our EPS assessment is that it was only qualitative (slime colony and ropy phenotype); quantitative determination of EPS yield (e.g., by phenol sulfuric acid method) will be performed in follow-up studies. Recent reviews highlight that LAB-derived EPS can act as prebiotics, modulate the immune system, and provide antioxidant and hypocholesterolemic benefits, making them highly desirable in functional food formulations [57]. In addition, ACE-inhibitory activity was high ($>90\%$) for SJC115, SJC116, SJC117, and SJC120, values that compare favourably with those reported for other dairy-derived lactobacilli and indicate a strong potential for blood pressure regulation via the renin–angiotensin system. For instance, milk fermented by *Lactocaseibacillus rhamnosus* GG and *Lactobacillus delbrueckii* ssp. *bulgaricus* has been shown to achieve up to 79% ACE inhibition through the release of bioactive peptides during fermentation, underscoring the antihypertensive potential of proteolytic LAB in dairy matrices [58].

The production of conjugated linoleic acid (CLA) was particularly elevated in SJC117 and SJC120 ($>3.5\text{ }\mu\text{g/mL}$), which is notable because CLA has been associated with anti-carcinogenic, anti-adipogenic, antidiabetic, and anti-inflammatory activities. Microbial biosynthesis of CLA by LAB is increasingly regarded as a practical and safe approach for enriching foods with this health-promoting compound, with strain type, pH, temperature, and incubation time being key determinants of yield and isomer profile [59]. Furthermore, glutamate decarboxylase (GAD) activity, responsible for the biosynthesis of γ -aminobutyric acid (GABA), was strongly positive only in SJC117 and SJC120, moderately positive in SJC115, SJC116, and SJC119, and absent in SJC114. GABA is a non-proteinogenic amino acid with well-documented hypotensive and anxiolytic effects, and its microbial production in fermented foods is highly sought after. As reviewed comprehensively, LAB in

fermented dairy foods can synthesise GABA through GAD activity, with beneficial effects on cardiovascular health, anxiety reduction, diabetes control, and sleep improvement [60].

The multifunctional profile of *L. paracasei* SJC117—combining putative BLIS, broad-spectrum antifungal activity, and high levels of ACE-inhibitory peptides, CLA, and GABA—is uncommon among cheese-derived LAB. While several studies have explored the biotechnological potential of *L. paracasei* from diverse sources [61–65], few report such a comprehensive combination of traits in a single isolate.

Carneiro et al. [66] identified bacteriocinogenic *L. paracasei* and *Pediococcus* spp. from Bulgarian feta and Brazilian prato cheeses with strong anti-*Listeria* activity and good bacteriocin stability, but these strains lacked concurrent antifungal activity. Similarly, Falfán-Cortés et al. [61] and Dinev et al. [62] reported *L. paracasei* strains with antibacterial activity against foodborne pathogens, yet neither study documented BLIS production or activity against *Aspergillus* spp. By contrast, our SJC117 and SJC120 inhibited all tested pathogens, retained activity after pH neutralisation (suggestive of BLIS), and exhibited strong antifungal activity against both *Penicillium* and *Aspergillus* species.

Regarding bioactive metabolites, Shukla et al. [65] reported 78% ACE inhibition by *L. paracasei* M11, while our strains achieved >85%. Ayag et al. [67] found that high GABA production in Turkish cheeses was restricted to *Lactiplantibacillus plantarum*, making SJC117 and SJC120 rare GABA-producing *L. paracasei* dairy isolates. Moiseenko et al. [68] demonstrated that *L. paracasei* improved CLA and ACE-inhibitory activity in yoghurt, but no single strain combined these traits with antifungal and BLIS production.

The unique advantage of SJC117 lies in its integrated multifunctionality: putative BLIS production, broad antifungal activity, high ACE inhibition, moderate CLA production, and strong GAD activity. This convergence of antimicrobial, technological, and health-promoting properties in a single cheese-derived *L. paracasei* isolate is, to our knowledge, rarely documented in the literature [69–71]. These distinguishing features likely reflect adaptation to the specific ecological niche of São Jorge PDO cheese and position SJC117 as an exceptionally versatile candidate for integrated biopreservation and functional food strategies. Furthermore, the simultaneous production of high EPS, potent ACE inhibition, elevated CLA, and strong GABA-generating capacity in SJC117 and SJC120 aligns with recent trends in LAB research, where strategies to enhance the production of multiple bioactive metabolites—including short-chain fatty acids, bacteriocins, vitamins, and EPS—are being pursued to maximise probiotic and technological benefits [51,72].

A limitation of this study is the reliance on in vitro assays; further validation using food matrices (e.g., cheese challenge tests) and in vivo models would be necessary to confirm the in situ efficacy and health benefits. Additionally, whole-genome sequencing could identify the genetic basis for putative BLIS production and confirm the absence of acquired resistance genes or biogenic amine clusters.

5. Conclusions

The present study characterised six LAB isolates from São Jorge PDO cheese with respect to their technological, safety, antimicrobial, and probiotic potential. All isolates proved technologically suitable for dairy applications, displaying broad carbohydrate fermentation and an absence of lipolytic activity, although proteolytic activity was detected in two isolates (SJC115 and SJC119). The enzymatic profiles observed support their use as starter or adjunct cultures, with the capacity to enhance lactose digestion, flavour development, ripening efficiency, and potentially the release of bioactive compounds.

Safety assessment revealed γ -haemolysis, no DNase or gelatinase activity, and a generally favourable antibiotic susceptibility profile. Nevertheless, whole genome sequencing (WGS) is recommended to exclude transferable resistance genes and biogenic amine clus-

ters. Notably, *L. paracasei* SJC117 and *L. brevis* SJC120 produced putative BLIS, as evidenced by retained antibacterial activity after neutralisation of the supernatant, while all isolates exhibited strong antifungal activity against common cheese spoilage moulds.

Under simulated gastrointestinal conditions, all isolates survived the intestinal phase (bile salts and pancreatin), maintaining viable counts above the probiotic threshold of 10^6 CFU/mL; however, only SJC117 and SJC120 withstood the gastric challenge (pH 2.5, 1 h) with counts remaining above 5 log CFU/mL. Despite low cell-surface hydrophobicity (<20%), the isolates displayed favourable autoaggregation and pathogen-coaggregation properties, indicative of probiotic functionality. While all isolates produced EPS and ACE-inhibitory metabolites; SJC117 and SJC120 stood out as the highest producers of CLA (>3.5 µg/mL) and GABA. Taken together, *L. paracasei* SJC117 combines strong antimicrobial activity (BLIS and antifungal) with superior gastrointestinal tolerance and an exceptional profile of bioactive compounds, positioning it as a particularly promising candidate for biopreservation and functional dairy applications. Nevertheless, further in vivo validation and genomic safety assessment are necessary before its industrial application can be recommended.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ACE	Angiotensin-converting enzyme
<i>A. chevalieri</i>	<i>Aspergillus chevalieri</i>
<i>A. flavus</i>	<i>Aspergillus flavus</i>
<i>B. cereus</i>	<i>Bacillus cereus</i>
BLIS	Bacteriocin-like inhibitory substances
<i>B. spizizenii</i>	<i>Bacillus spizizenii</i>
CFS	Cell-free supernatant
CFU	Colony-forming unit
CLA	Conjugated linoleic acid

<i>E. coli</i>	<i>Escherichia coli</i>
EPS	Exopolysaccharides
GAD	Glutamate decarboxylase
GABA	gamma-aminobutyric acid
LAB	Lactic acid bacteria
<i>L. parabuchneri</i>	<i>Lentilactobacillus parabuchneri</i>
<i>L. paracasei</i>	<i>Lactocaseibacillus paracasei</i>
<i>L. brevis</i>	<i>Levilactobacillus brevis</i>
<i>L. innocua</i>	<i>Listeria innocua</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
MRS	De Man, Rogosa, and Sharpe
MSG	monosodium glutamate
NSLAB	Non-starter lactic acid bacteria
OD	Optical density
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. brevicompactum</i>	<i>Penicillium brevicompactum</i>
<i>P. commune</i>	<i>Penicillium commune</i>
PCA	Plate Count Agar
PDO	Protected Designation of Origin
PLA	Phenyllactic acid
<i>P. nordicum</i>	<i>Penicillium nordicum</i>
QPS	Quality Presumption of Safety
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. enterica</i>	<i>Salmonella enterica</i>

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