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Growth studies of dominant lactic acid bacteria in orange juice and selection of strains to ferment citric fruit juices with probiotic potential

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Abstract
The study aimed to evaluate the ability of dominant lactic acid bacteria (LAB) in orange juice to growth on N-depleted MRS medium supplemented or not with cysteine (mMRS), then to select the most nutritionally promising strains for growth assays in the food matrix and evaluation of beneficial attributes for fruit juice fermentation. *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* were dominant species among the total of 103 LAB isolates as confirmed by multiplex PCR and/or 16 s rDNA sequence analysis. Based on growing lower than 20% and higher than 70% in mMRS (1.0 g/l meat extract, without peptone and yeast extract) with and without cysteine requirement, one *L. brevis* (JNB23) and two *L. plantarum* (JNB21 and JNB25) were selected. These bacteria and the *L. plantarum* strains N4 and N8 (previously isolated from oranges peel) when inoculated in orange juice grew up to 1.0 log cfu/ml for 24 h incubation at 30 °C and mainly produced lactic acid, with strains JNB25 and JNB23 reaching the highest and lowest cell densities in agreement with their nutritional exigency. In addition, all *L. plantarum* strains exhibited antagonistic activity against the majority of tested bacterial pathogens (in opposition to *L. brevis*), ability to grow or survive to pH 3.0 for 3 h, to grow with 0.5% sodium taurocholate, and a decrease after simulated gastrointestinal digestion assay which did not exceed 1.0 or 2.0 log units, depending on the strain. Thus, autochthonous *L. plantarum* strains with ability for overcoming nutritional limitations and beneficial attributes are promising candidates for further investigations as novel probiotic and/or preservative starters to ferment citric fruit juices.

Keywords *Lactobacillus* sp. · Nutritional requirements · Orange juice · Metabolism · Probiotic potential

Introduction

The consumer trend towards fresh or minimally processed, health-promoting, and rich flavor ready-to-eat or -to-drink foods is increasing remarkably [1]. In this regard, lactic acid bacteria (LAB) have been used to preserve and improve the foods quality from centuries [2]. They have been strongly recommended due to their advantages regarding probiotic characteristics and enhancement of fermented food functionality [3]. Some lactobacilli able to survive in the human and animal gastrointestinal tracts have been recognized as probiotics (live microorganisms that confer a beneficial effect on the host when administered in proper amounts) [4].

Orange juice is rich in human health-promoting nutrients and is well accepted by consumers of all age groups [5, 6]. In this regard, the development of new fermented fruit beverages for their shelf life extension and as vehicles for probiotic microorganisms is a growing trend [7–9]. Sixteen strains comprising different *Lactobacillus* species isolated from the pulp of *Mangifera indica* L., or from industrial fruit pulp processing by-products of *Malphigia glabra* L., *M. indica* L., *Annona muricata* L., and *Fragaria ananassa* L. presented potential probiotic features with beneficial effects on health [10]. However,

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research on citric fruit by-products, particularly orange juice, as raw material for isolation of LAB with probiotic aptitude and ability to ferment the natural juice is still scarce [5].

The main criteria currently followed for in vitro selection of LAB intended to be used as probiotics include pH and bile tolerance and the ability to withstand the conditions of the gastrointestinal tract [11]. In addition, LAB must have the ability to survive and grow quickly to a desirable concentration in the fermentation medium. However, this property could be limited by several factors in the natural environmental during processing and storage such as low pH, high dissolved oxygen concentration, intrinsic antimicrobial compounds, and insufficient free amino acid and peptide amounts [12]. Nevertheless, Perez and Saguir [5] documented that *L. plantarum* N4 isolated from orange peel grew rapidly and survived at high cell density (~ 10^9 cfu/ml) in orange juice stored at 30 and 4 °C for 4 weeks respectively, without causing spoilage. The ability of this strain to assimilate mainly complex nitrogen molecules (dipeptides) rather than simple ones in an N-depleted medium could contribute to its development in the natural medium [13]. This fact paves the way for further investigation to explore its preservative and probiotic potentials and of new isolates for juice fruit fermentation. Lactic acid fermentation is a sustainable process that may enhance nutritional properties, sensorial, and health-related aspects of food, extending its shelf life [10].

LAB have complex nutritional requirements, especially concerning to nitrogen sources for growth [14]. The auxotrophy of the *Lactiplantibacillus plantarum* strains N4 and N8 for cysteine, glutamic acid, isoleucine, leucine, threonine, and valine was reported [15]. Among them, cysteine is a relevant amino acid in cellular physiology as it is involved in several functions such as protein synthesis, generation of metabolic energy or redox potential, and resistance to oxidative stress [16]. Its catabolism may liberate hydrogen sulfide (H_2S) which may contribute to human health, or prolong the postharvest storage of fruits and vegetables [17–19]. However, H_2S also possesses deleterious effects on some foods such as fermented vinegar and wine, and in large amounts may cause adverse effects on human health [20, 21].

Therefore, this study was initiated to characterize the ability of dominant LAB species in orange juice to grow on N-depleted MRS medium supplemented or not with cysteine, and then to select the most nutritionally promising strains for growth assays in the food matrix and evaluation of the antimicrobial activities and probiotic potentials. Our findings will enable to gain further insights into nutritional and functional attributes of LAB species to ferment new citric fruit juices.

Materials and methods

Bacterial strains

L. plantarum strains N4 and N8 (Genbank access numbers: AY082883 and AY082884, respectively), isolated from orange peel, were stored at − 20 °C in MRS broth (Britania S.A, Argentina) supplemented with glycerol (30%, v/v). These strains were selected because of (1) ability to growth and survival at high cell density in orange juice stored at 30 and 4 °C for 4 weeks respectively (N4); (2) to assimilate mainly dipeptides as nitrogen sources in an N-depleted medium (N4); (3) ability to growth efficiently in a defined chemically medium where only consumed lysine, arginine, cysteine, threonine, and very low aromatic amino acid values (both strains); and (4) ability to metabolize arginine via the arginine dihydrolase (ADI) pathway (both strains), which would favor its growth in the natural medium without causing spoilage [5, 13, 15, 22].

Isolation and identification of LAB from orange juice

LAB were isolated from juice obtained from oranges at commercial maturity with no visible damage or spoilage purchased from local market of San Miguel de Tucumán (Tucumán, Argentina). Oranges were cut in halves and squeezed to obtain juice using a small-scale commercial juice extractor (Citrus Press 1000, Type HR2783/A, Philips) under asepsis conditions. Juice samples were stored at 30 °C for 7 days for enrichment and proper dilutions were plated onto plate count agar (PCA, Oxoid, Basingstoke, UK) and MRS agar pH 6.5 supplemented with 1.7 µg/ml of pimaricin (MRS-P) (Oxoid Ltd, Basingstoke, England) to suppress yeast growth. All plates were incubated at 30 °C for 72 h under microaerophilic condition. Then, the colonies presumed to be LAB on the MRS-P ($n = 100$) and PCA ($n = 20$) plates were isolated and further characterized. Pure cultures were preserved in MRS broth supplemented with 30% (v/v) glycerol at − 20 °C. Working cultures were prepared from the frozen cultures by two consecutive transfers in MRS broth at 30 °C and re-streaked onto MRS agar.

The isolates initially characterized as LAB based on positive Gram staining and negative catalase reaction (3% v/v H_2O_2) were also tested for motility using a phase contrast microscope (Olympus CX41, Japan), spore formation, gas and ammonia production from glucose and arginine respectively, ability to grow under microaerophilic conditions (in BBL GasPak jars, Becton Dickinson, Argentina) at different temperatures (15, 30, 37, and 45 °C), NaCl concentrations (2.5, 5.0, 7.5%, w/v), and pH values (4.0,

7.0, 9.0). Production of dextran from sucrose (5%) was determined on agar medium as described by Savino et al. [23]. The API 50 CHL fermentation test (BioMérieux, Marcy-l'Etoile, France) was performed according to the manufacturer instructions.

LAB species were genetically identified using PCR method and 16S rDNA sequencing. Pure cultures were grown in MRS broth, genomic DNA was extracted, and 16S rDNA was amplified using the prokaryotic 16S ribosomal DNA universal primers 27F/1492R (5′-AGAGTTTGATCCTGGCTCAG-3′/5′-GGTTACCTTGTTACGACTT-3′) [24]. Amplicons were confirmed by agarose gel electrophoresis and then purified using a Prep-A-Gene kit (Bio-Rad, USA) according to manufacturer's instructions. They were sequenced using an Applied Biosystems 3730 XL sequencer by Macrogen Inc. (908 World Meridian Venture Center, #60–24, Gasan-dong, Geumchun-gu, Seoul 153–781, Korea). The sequences were compared with those available in the GenBank database and similitude percentages were calculated after alignment. Strains showing homology of at least 97% were considered belonging to the same species. *L. plantarum*, *Lactiplantibacillus pentosus*, and *Lactiplantibacillus paraplantarum* strains were further distinguished by partial amplification and product comparison of the recA gene according to the method of Torriani et al. [25] as modified by Savino et al. [23]. The amplification products were visualized by ethidium bromide (5 µg/ml) staining after gel electrophoresis. A Biometra TRIOT-Thermoblock was used for PCR reactions.

Media, growth conditions, and culture procedures

Isolates were screened for ability to grow in a minimum N-deficient medium (mMRS), which was prepared by omitting peptone and yeast extract from MRS medium and adding 1 g/l rather than 10 g/l meat extract [13]. At the same time, the effect of cysteine at (g/l) 0.2, 0.5, or 1.0 (mMRSC0.2; mMRSC0.5; mMRSC1.0, respectively) on bacterial growth was determined. All media were adjusted to pH 6.0 with 1 N NaOH before sterilization at 121 °C for 15 min. Cysteine was sterilized by filtration through a nylon membrane (0.22 µm pore size, Millipore, Bedford, MA) and added to the sterilized media. On the other hand, orange juice (OJ) was prepared from oranges with no visible damage or spoilage as above mentioned, except that in this case oranges were washed before juice extraction. After that OJ was clarified (8000 × *g*, 20 min, 4 °C), pasteurized at 80 °C for 5 min, and quickly cooled to 35 °C. Juice was aseptically transferred into sterile glass bottles, and stored at 4 °C for further study. Growth experiments were also carried out using autoclaved juice (121 °C, 5 min) with similar results (data not shown).

For inoculum preparation, cells grown in MRS broth pH 6.0 were harvested at the end of exponential growth phase (10–12 h) by centrifugation, washed twice to avoid carryover of essential nutrients and resuspended in sterile distilled water to an optical density (OD_{560nm}): 1.0. Cell suspensions were used to inoculate at a rate of 2% (v/v) mMRS and orange juice media with ca. 10^7 cfu/ml. Media were incubated statically at 30 °C for 72 (mMRS) or 24 h (OJ). Uninoculated OJ was used as control.

Bacterial growth was monitored by periodic spectrophotometric measurements at 560 nm using a UV–visible spectrophotometer (Biochrom Ltd., Cambridge Science Park, England) during bacterial growth and by the colony-forming unit counts (cfu/ml) by plating 0.1 ml aliquots of serial decimal dilutions in duplicate on MRS agar. The plates were incubated for 48 h at 30 °C. After incubation, typical colonies were counted and results were transformed to logarithmic scale. Nutritional requirements of the tested microorganisms were estimated according to bacterial growth extent (ΔA) in mMRS relative to control medium (MRS). (ΔA) was expressed as the difference in cell concentration (log cfu/ml) between end of exponential growth phase and inoculum (14–16 h).

Chemical analysis

Samples of culture supernatants from bacterial cultures were collected at beginning and 24 h of incubation, and used to determine the residual sugars (glucose, fructose) and organic acids (citric and L-malic) and the D-L-lactic acid, acetic acid, and ethanol production. Quantitative analysis was carried out by HPLC using an ISCO liquid chromatograph (ISCO, Lincoln, NE) as described by Sajur et al. [26]. D- or L-lactic acid isomers were measured by using enzymatic methods (Boehringer Kit, Mannheim, Germany). On the other hand, hydrogen sulfide production was qualitatively determined using a sterile strip of absorbent paper embedded in saturated solution (5% v/v) of lead acetate which was placed in each tube inoculated with test and control media [27]. After 24 h of incubation at 30 °C, a positive result was evidenced by the appearance of a black-brown stain on the paper.

Hemolytic activity

To assess hemolytic activity of the selected LAB strains, each active pure culture was streaked on agar plates containing 5% (v/v) sheep blood, and then incubated at 30 °C for 48 h. After incubation, the presence of green-hued zones, clear zones, and no clear zones around the colonies was examined in order to determine α-hemolysis, β-hemolysis, and γ-hemolysis, respectively. Only strains with γ-hemolysis were considered safe. *Escherichia. coli* ATCC 25,922 and

Staphylococcus aureus ATCC 25,923 were used as positive controls for α- and β-hemolysis, respectively.

Antimicrobial activity screening

LAB were screened for antibacterial activity against six indicator stains, which were reported as foodborne pathogenic and spoilage microorganisms [28–30], including *E. coli* (ATCC 35218), *S. aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enterica* serovar Typhimurium (ATCC 14028), and *E. coli* O157:H12 by the spot agar assay according to González et al. [31]. *E. coli* O157:H12 isolated from ground beef [32] was deposited in Certified Bacteriology Laboratory of National Tucumán University (LABACER, FBQF, UNT) and used as a non-pathogenic surrogate for *E. coli* O157:H7. A 2 µl aliquot collected from each active LAB culture in MRS broth with approximately 7.0 log cfu/ml was spotted on the surface of MRS agar, which was dried at room temperature for 30 min and incubated at 30 °C for 24 h. After colony development, plate was overlaid with 10 ml of tripticase soy broth with 0.8% agar (w/v), seeded with 1% (v/v) of an active overnight culture of the indicator strain (aprox 7.0 log cfu/ml), and incubated at 37 °C for 48 h. Non-inoculated MRS agar was used as a negative control.

The antimicrobial properties of the *Lactobacillaceae* strains were tested on cell-free supernatants (CFSs) by the well diffusion method [33]. Strains were incubated in MRS broth-pH 6.0 for 12 h at 30 °C, centrifuged (8000 × *g*, 10 min, 4 °C), and the supernatants sterilized by filtration (0.22 µm pore-size filters). An aliquot was separated to be used as non-treated control, while the remaining CFSs were neutralized (pH 6.50 ± 0.10) with NaOH (1 N) for organic acid production determination and divided in equal portions for assays with catalase (5.0 mg/ml) and α-chymotrypsin (20 mg/ml) (Sigma-Aldrich) at 37 °C for 1 h. Fifty-microliter aliquots of bacterial supernatants were dispensed into wells (5 mm diameters and depth) in MRS agar plates overlaid with 10 ml of MRS soft agar (0.8% w/v) inoculated with the indicator strain. The plates were kept at 4 °C for 1 h for supernatant diffusion and then incubated at 37 °C for 48 h. The antibiotic gentamicin (30 µg/ml) and MRS broth (pH 6.5) were used as positive and negative controls, respectively. The antagonistic activity was recorded as the growth inhibition zone diameter (mm) around each spot or well.

Acid and bile tolerance and simulated gastrointestinal digestion

The modified methods of Clark et al. [34] and Gilliland et al. [35] were used to evaluate the acid and bile tolerance respectively. Bacterial cells grown in MRS broth were harvested at the end of exponential growth phase (10–12 h) by centrifugation, washed with sterile distilled water, and resuspended in MRS broth adjusted to different pH values 2.0, 3.0, 4.0, and 6.5 (control) using 1 N HCl or NaOH. On the other hand, the ability to grow in bile presence was evaluated using sodium taurocholate salt (Sigma-Aldrich, USA), which after dissolving in distilled water, was sterilized through 0.22 µm pore-size filters and added to sterile MRS broth at 0.15, 0.30, and 0.50% (v/v). Experimental media were inoculated at 2% (v/v) and incubated under microaerophilic condition at 37 °C for 6 h to mimic the intestinal conditions.

The resistance to gastric and intestinal digestions was sequentially assessed according to the protocol described by Babot et al. [36], for which solutions simulating gastric and intestinal juices were prepared. Bacterial suspensions were incubated in each condition at 37 °C for 2 h. After exposure, each cell suspension (100 µl) was stained with propidium iodide (PI, 1 mg/ml) and 4′,6´-diamidino-2-phenylindole (DAPI, 0.1 mg/ml) solutions to enumerate dead/damaged and total cells, respectively, at 100 × magnification with a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with appropriated filters. Pepsin and pancreatin were purchased from Merck and MP Biomedicals (Solon, USA), respectively. PI, DAPI, and other chemicals were from Sigma-Aldrich.

Statistical analysis

All experiments were carried out in triplicate, and each sample was analyzed in duplicate. The results were expressed as mean ± standard deviation (SD). Shapiro–Wilk normality test was used to fit the normal distribution data. Significant differences between averages ± SD were determined using ANOVA followed by Tukey's test. Values were considered significantly different when $P < 0.05$. Data from antimicrobial activity; pH variation, pH and bile salt tolerance, simulated gastrointestinal digestion, and parameters in orange juice were subjected to permutation analysis using PermutMatrix [37], in order to explore the main variations among autochthonous strains through hierarchical cluster analysis. Dissimilarity was measured based on Euclidean distance.

Results

Isolation and identification of dominant species from orange juice

After transferring, fresh juice contained initial bacterial levels determined on MRS-P and PCA plates of 2.91 ± 0.11 and 4.07 ± 0.21 log cfu/ml respectively. Colonies on the PCA plates were small, gray or white, catalase negative suggesting they were LAB, although yeast colonies were also found. Cell counts reached a maximum mean value of

8.62 ± 0.35 log cfu/ml (8.57 ± 0.31 and 8.67 ± 0.39 in PCA y MRS-P media respectively) at 4 days. Then, it decreased to 7.02 ± 0.26 log cfu/ml in 7 days at 30 °C. One hundred twelve (100 from MRS-P and 12 from PCA) out of 120 colonies (93.3%) obtained at day 4 were classified as LAB and constituted the dominant element. 95.5% of them were rods occurring singly, in pairs, and short chains, while the remaining isolates showed the typical *Leuconostoc*-like ovoid cell shape. LAB isolates were divided in group I—homofermentative lactobacilli, negative for arginine ($n = 40$); group II—heterofermentative lactobacilli, positive for arginine ($n = 67$); group III—heterofermentative coccus, negative for arginine ($n = 5$). A total of twenty representative strains (7/40, 12/67, and 1/5 of groups I, II, and III, respectively) were phenotypically and genotypically (multiplex PCR assay and the 16S rRNA sequence analysis) identified as belonging to *L. plantarum* species, *Levilactobacillus brevis* species, and *Leuconostoc* genus (Table 1). Sequences determined in this study for *L. plantarum* JNB25, *L. brevis* JNB7, *L. brevis* JNB16, *L. brevis* JNB1, *L. brevis* JNB3, and *L. brevis* JNB23 were deposited in the Genbank databases (JQ741971, JX218939, JX218940, JX218941, JX218942, and JQ741972 respectively). The *L. plantarum* and *L. brevis* strains were nutritionally characterized in a poor MRS medium (mMRS).

Growth assays of LAB in minimum medium

The tested strains showed growth rates (μ_{max}) and reached growth extents (ΔA) varying from 0.51 to 0.90 h^{-1}, and 0.90 to 2.7 log cfu/ml in MRS medium (control) respectively. In general, the growth parameters significantly decreased when cultivated in mMRS. In this condition, the tested strains were classified into three nutritional groups according to growth extent (ΔA) relative to control (Table 2): (I) [(ΔA) < 29%], the microorganisms were considered with high nutritional exigencies (HE), and it was constituted by the twelve *L. brevis* strains with JNB23 reaching the highest biomass production (18%^{bY} relative control^{aV}); (II) [29% < (ΔA) ˂ 70%], the microorganisms were considered with moderate nutritional exigencies (ME), and it was formed by four *L. plantarum* strains with JNB19 growing to the highest cell density (62%^{bY} relative control^{aZ}); and (III) [(ΔA) ≥ 70%], the microorganisms were considered with low nutritional exigencies (LE), and it was constituted by three *L. plantarum* strains with JNB25 growing in similar way as the control^{aZ}.

Cysteine addition did not significantly increase the *L. plantarum* strains growth in mMRS, and reduced between 23 and 38% the final biomass of the JNB2, JNB10, and JNB13 strains in mMRSC1.0 relative to control. By contrast, cysteine stimulated the *L. brevis* isolates growth between 50% and up to 400% depending on strain and its initial concentration (for instance 412.5% for JNB24 in mMRSC1.0). On the other hand, all *L. plantarum* strains produced variable H_2S levels in mMRSC1.0 and/or mMRSC0.5, while some *L. brevis* weakly produced it (Table 2).

Based on these results, the two best *L. plantarum* (JNB21 and JNB25) and *L. brevis* JNB23 with the highest biomass yields and weak- or no hydrogen sulfide production from at- or down- 0.5 g/l cysteine were selected for further research. The *L. plantarum* strains N4 and N8, previously isolated from orange peel, were also included.

Table 1 Identification of lactic acid bacteria isolated from fermented orange juice by phenotypic and genotypic techniques

Group	Identification techniques					
	Phenotypic*	NII	16s gene[a]	NII	PCR[b]	NII
I ($N = 40$; $n = 7$)	*L. plantarum*	7	*L. plantarum*	1	*L. plantarum*	7
II ($N = 67$; $n = 11$)	*L. brevis*	11	*L. brevis*	5	-	0
III ($N = 5$; $n = 1$)	*Leuconostoc*	2	*Leuc. mesenteroides*	1	-	0

N, isolates total number; *n*, representative isolates total number for identification; *NII*, number of identified isolates

*Results for group I: DL-lactic acid isomers; growth at 15 and 45 °C, at pH values of 4.0 and 9.0, and at 2.5% NaCl; and patterns in the API 50 CHL showing 99.7–99.9% with *L. plantarum* species

Results for group II: D or DL-lactic acid isomers; growth at pH values of 4.0 and 9.0, at 2.5% of NaCl, and at 15 or 37 °C; and patterns in the API 50 CHL showing 99.9% similarity with *L. brevis* species

Results for group III: cocci arranged in chains, gas and D-lactic acid isomer, growth at 15 °C but not at 45 °C, slime layer production, and growth in NaCl presence

[a]The samples of NII were only analyzed as representatives for each group

[b]Multiplex PCR assay only resulted in a 318 bp single amplicon for *L. plantarum* species

(-) genera/species that was not identified by the technique

Table 2 Growth and sulfur hydrogen production of *Lactobacillaceae* species in deficient medium (mMRS)[a] and supplemented with cysteine at 0.2, 0.5, and 1.0 g/l (mMRSC0.2/0.5/1.0 respectively) at 30 °C

Strain	MRS	mMRS		mMRSC0.2		mMRSC0.5		mMRSC1.0		Class
LP	ΔA^{b}	ΔA^{b}	H_2S^{c}	ΔA^{b}	H_2S^{c}	ΔA^{b}	H_2S^{c}	ΔA^{b}	H_2S^{c}	
JNB2	2.12 ± 0.01^{aZ}	0.62 ± 0.00^{bZ}	-	0.66 ± 0.00^{bZ}	-	0.61 ± 0.00^{bcZ}	+	0.45 ± 0.00^{dZ}	+ +	ME
JNB10	2.15 ± 0.01^{aZ}	1.56 ± 0.00^{bX}	-	1.64 ± 0.00^{bX}	+ +	1.64 ± 0.00^{bX}	+ +	1.19 ± 0.01^{cX}	+ +	LE
JNB13	2.27 ± 0.00^{aZ}	1.01 ± 0.00^{bY}	-	1.05 ± 0.01^{bY}	-	0.92 ± 0.00^{bY}	+ +	0.63 ± 0.00^{cY}	+ +	ME
JNB19	2.24 ± 0.00^{aZ}	1.40 ± 0.02^{bV}	-	1.44 ± 0.01^{bVX}	-	1.33 ± 0.01^{bV}	+ +	1.23 ± 0.02^{bX}	+ +	ME
JNB20	2.01 ± 0.02^{aZ}	1.23 ± 0.01^{bYV}	-	1.23 ± 0.03^{bYV}	-	1.21 ± 0.02^{bV}	+ +	1.11 ± 0.01^{bX}	+ + +	ME
JNB21	2.23 ± 0.02^{aZ}	1.58 ± 0.01^{bX}	-	1.64 ± 0.01^{bX}	-	1.65 ± 0.01^{bX}	+	1.45 ± 0.00^{bX}	+ +	LE
JNB25	2.72 ± 0.01^{aZ}	2.25 ± 0.02^{bT}	-	2.26 ± 0.02^{bT}	-	2.23 ± 0.02^{bU}	+	2.08 ± 0.01^{bV}	+ +	LE
LB										
JNB1	1.53 ± 0.01^{aZ}	0.16 ± 0.00^{bZ}	-	0.24 ± 0.00^{cZ}	-	0.27 ± 0.00^{cZ}	-	0.29 ± 0.00^{cZ}	-	HE
JNB3	1.59 ± 0.00^{aZ}	0.15 ± 0.00^{bZ}	-	0.28 ± 0.00^{cZ}	-	0.35 ± 0.00^{cdZ}	-	0.41 ± 0.00^{dZ}	-	HE
JNB5	1.28 ± 0.01^{aY}	0.16 ± 0.00^{bZ}	-	0.28 ± 0.00^{cZ}	-	0.69 ± 0.00^{dY}	+	0.82 ± 0.00^{dY}	+	HE
JNB7	1.60 ± 0.02^{aZ}	0.31 ± 0.00^{bY}	-	0.46 ± 0.00^{cY}	-	0.52 ± 0.00^{cX}	-	0.51 ± 0.00^{cXV}	-	HE
JNB8	1.35 ± 0.00^{aY}	0.17 ± 0.00^{bZ}	-	0.32 ± 0.00^{cZ}	-	0.66 ± 0.00^{dY}	+	0.78 ± 0.00^{dY}	+	HE
JNB15	1.38 ± 0.00^{aY}	0.18 ± 0.00^{bZ}	-	0.30 ± 0.00^{cZ}	-	0.73 ± 0.00^{dY}	+	0.89 ± 0.00^{dY}	+	HE
JNB16	1.56 ± 0.00^{aZ}	0.19 ± 0.00^{bZ}	-	0.33 ± 0.00^{cZ}	-	0.42 ± 0.00^{dZ}	-	0.53 ± 0.01^{dV}	-	HE
JNB17	1.17 ± 0.01^{aX}	0.13 ± 0.00^{bZ}	-	0.27 ± 0.00^{cZ}	-	0.37 ± 0.00^{cdZ}	-	0.47 ± 0.00^{dV}	+	HE
JNB18	1.06 ± 0.00^{aX}	0.13 ± 0.00^{bZ}	-	0.26 ± 0.00^{cZ}	-	0.35 ± 0.00^{cZ}	-	0.47 ± 0.00^{dV}	+	HE
JNB23	2.09 ± 0.02^{aV}	0.38 ± 0.00^{bY}	-	0.93 ± 0.00^{cX}	-	1.09 ± 0.00^{cdV}	+	1.25 ± 0.01^{dU}	+	HE
JNB24	0.90 ± 0.01^{aX}	0.08 ± 0.00^{bV}	-	0.21 ± 0.00^{cZ}	-	0.31 ± 0.00^{cdZ}	-	0.41 ± 0.00^{dZ}	+	HE

[a]mMRS without yeast extract and peptone and with 1 g/l meat extract

[b]Difference in cell count (log cfu/ml) between growth at the end of exponential phase and inoculum (mean value: 7.52 ± 0.02 log cfu/ml). Values are the means of three independent experiments. Different letters within file$^{(a–d)}$ and column$^{(Z–T)}$ indicated significance differences with a $P < 0.05$

[c]-, not detected; +, weakly detected; + +, moderately detected; + + +, strongly detected

Abbreviations: *LP*, *L. plantarum*; *LB*, *L. brevis*; *LE*, low nutritionally exigent; *ME*, moderate nutritionally exigent; *HE*, high nutritionally exigent

Growth and metabolic activity of selected strains in orange juice

Table 3 shows the behavior of the five selected strains in pasteurized OJ (pH 3.60 ± 0.07) incubated at 30 °C for 24 h. No bacterial growth was observed in uninoculated OJ medium. In inoculated OJ, the cell densities increased from 7.50 log cfu/ml to values ranging from 8.57 to 9.17 log cfu/ml with *L. plantarum* JNB25 and *L. brevis* JNB23 reaching the highest and lowest cell densities at 24 h, respectively. In addition, all tested strains consumed glucose and L-malic and citric acids, although at different extents, and mainly produced lactic acid. Only the strain N4 utilized little initial fructose. The analytical balance of total formed lactate from consumed glucose or glucose + fructose (only in the case of strain N4) was between 2.8 and 3.6 mmol/mmol. However, considering that the additional of L and D- lactic acids was formed from L-malic and citric acids (molar ratio 1:1), a theoretical stoichiometry recovery of hexose(s) metabolism of approximately 2 or 1 mmol of lactic acid produced per mmol of sugar(s) consumed was obtained for homolactic and heterolactic behaviors respectively. The acetic acid yielded accounted for 82–90% citric acid degraded for *L. plantarum* strains, while *L. brevis* JNB23 recovered ~ 58% and produced ethanol accounted for 79% of the consumed glucose under experimental condition. No qualitative hydrogen sulfide production was detected.

Probiotic-related characteristics

Hemolytic and antimicrobial activities

None of the five tested LAB showed α-hemolytic and ß-hemolytic activity when grown on blood agar plates. Thus, these strains could be considered interesting candidates for further research as starter cultures or probiotics for fermenting fruit juice.

Table 4 shows antimicrobial activities of the five LAB strains against indicator bacteria by spot agar test (SAT) and well diffusion method (WDM). In the SAT the *L. plantarum* strains proved, in general, strong antagonistic effects against *E. coli* ATCC 35218, *P. aeruginosa* ATTC 27853,

Table 3 Growth and parameters analyzed in selected strains in orange juice medium after 24 h incubation at 30 °C

Strain[a]	ΔA[b]	Sugar and organic acids (mmol/l)							$Y_{s/lacd}$[d]
		Glucose (92.61 ± 1.6)[c]	Fructose (114.72 ± 1.6)[c]	Citric Ac (43.89 ± 1.3)[c]	Malic Ac (14.08 ± 0.4)[c]	D- lactic ac (ND)	L- lactic ac (ND)	Acetic ac (ND)	
N4	1.40[a]	79.07 ± 3.8[a]	105.13 ± 3.1[a]	22.94 ± 0.9[a]	6.67 ± 0.2[a]	65.15 ± 2.0[a]	15.63 ± 0.0[a]	19.11 ± 1.0[a]	1.81
N8	1.37[ab]	61.73 ± 8.1[b]	110.98 ± 2.0*[ab]	15.82 ± 1.1[b]	2.51 ± 0.3[b]	106.78 ± 0.9[b]	18.97 ± 0.3[b]	23.08 ± 1.1[a]	2.27
JNB21	1.26[b]	31.89 ± 2.0[c]	113.89 ± 5.0*[b]	8.29 ± 0.3[c]	0.13 ± 0.0[c]	155.90 ± 1.0[c]	20.57 ± 1.4[b]	31.73 ± 0.1[b]	1.97
JNB25	1.67[c]	44.83 ± 6.5[c]	115.13 ± 4.1*[b]	8.13 ± 0.5[c]	0.19 ± 0.0[c]	127.66 ± 0.6[d]	19.56 ± 0.1[b]	30.92 ± 0.1[b]	1.93
JNB23	1.07[d]	34.46 ± 2.2[c]	116.17 ± 2.5*[b]	8.10 ± 0.8[c]	0.16 ± 0.0[c]	58.93 ± 0.8[a]	61.46 ± 0.5[c]	20.67 ± 0.5[a]	1.18

[a]All strains belonged to *L. plantarum* species except *L. brevis* JNB23

[b]Difference in cell count (log cfu/ml) between the end of exponential phase and mean initial value: 7.50 ± 0.25 log cfu/ml

[c]Initial concentration (mM) of glucose, fructose, citric acid, and L-malic acid (ac). Lactic and acetic acids were not detected (ND)

[d]Ys/lac represents sugar(s) bioconversion yield to lactic acid (D plus L-isomers): total lactic acid formed minor D and L-lactic acids recovered from citrate and L-malic acid metabolism (1:1 mM) respectively/consumed glucose or glucose + fructose (mM)

Ethanol was only formed in culture incubated with *L. brevis* JNB23 (45.3 ± 1.45 mM). No hydrogen sulfide was qualitatively detected

Values are the means of three independent experiments. Those with asterisk (*) in the same column were significantly different relative to initial time. Different letters within column indicated significance differences with a $P \leq 0.05$

Table 4 Antimicrobial activity of selected strains isolated from oranges peel (N4 and N8) and fermented juice (JNB21, JNB25, and JNB23) against several bacteria

Strain		*E. coli*		*S.* Typhimurium ATTC 14028	*P. aeruginosa* ATTC 27853	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212
		ATCC 35218	O157:H12				
		Inhibition in the AST[a]					
LP N4		+ + +	+ +	+	+ + + +	+	+ + +
LP N8		+ + +	+ +	+	+ + +	+	+ + +
LP JNB21		+ + +	+	-	+ +	+ + +	+ + +
LP JNB25		+ + + +	+	-	+ + +	+ + +	+ + +
LB JNB23[b]		-	-	-	-	+	+ +
	pH of CFS[c]	Inhibition zone (mm) in the WDM[c]					
LP N4	4.28[ab]	13.6[a] (5.6)	8.9[a] (5.1)	5.2[a] (3.0)	14.4[a] (7.0)	3.0[a] (0.0)	12.2[a] (5.7)
LP N8	4.33[ad]	13.4[a] (6.2)	9.2[a] (5.6)	5.6[a] (3.2)	13.6[ab] (5.5)	3.2[a] (0.0)	12.6[a] (5.0)
LP JNB21	3.89[bc]	13.7[a] (0.0)	3.0[b] (0.0)	0.0 (0.0)	11.0[c] (0.0)	12.1[b] (0.0)	12.4[a] (0.0)
LP JNB25	3.67[c]	15.3[b] (0.0)	3.0[b] (0.0)	0.0 (0.0)	12.3[b] (0.0)	13.0[b] (0.0)	13.0[a] (0.0)
LB JNB23	4.42[d]	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	3.4[a] (0.0)	5.0[b] (0.0)

[a]Antimicrobial activity determined by agar spot test (AST): **(-)** inhibition zone < 5 mm; **(+)** inhibition zone > 5 mm; **(++)** inhibition zone > 10 mm; **(+++)** inhibition zone > 15 mm; (+ + + +) inhibition zone > 20 mm

[b]This was excluded for further assay with cell free supernatant (CFS)

[c]Well diffusion method (WDM). CFSs were obtained from overnight cultures in MRS broth. CFSs were also tested at neutral pH () or, at neutral pH in the presence of catalase or of α-chymotrypsin. Strains N4 and N8 lost completely activity in catalase treated neutralized-CFS (data not shown). Values are the means of three independent experiments. Different letters within column[(a–c)] indicated significance differences with a $P < 0.05$. Abbreviations: *LP*, *L. plantarum*; *LB*, *L. brevis*

and *E. faecalis* ATCC 29212 (inhibition zone diameters > 15 mm), and inhibitions weak to strong against *E. coli* O157:H12, *S.* Typhimurium ATTC 14028, and *S. aureus* ATCC 25923 (inhibition zones varying between < 5.0 mm and 13.5 ± 0.9 mm) depending on strain and its origin (fruit or juice). Only the N4 and N8 strains exhibited inhibition on

S. Typhimurium ATCC 14028 growth, which was the most resistant pathogen for all studied *L. plantarum*. In the case of *L. brevis* JNB23, it only displayed weak and moderate inhibitions on *S. aureus* and *E. faecalis* growths (inhibition zone diameters < 10.0 mm), respectively. Although with lower growth inhibition zone diameters (between 2.7 ± 0.1 and 15.3 ± 0.7 mm), a similar trend was observed when CFSs of the five LAB strains were screened for antibacterial activity by the WDM. Again, CFSs of the *L. plantarum* strains N4 and N8 were capable of inhibiting weakly *S.* Typhimurium ATCC 14028 growth, while strains JNB21 and JNB25 were the most active against *S. aureus* ATCC 25923. D-lactic acid concentration determined in CFSs varied between 84.1 ± 3.1 and 112.1 ± 6.9 mM for *L. plantarum* strains and 32.0 ± 1.2 mM for *L. brevis* JNB23. No inhibitory activity was observed with neutralized CFSs (NCFSs) treated with catalase or α-chymotrypsin except for strains N4 and N8. In these cases, their NCFS or α-chymotrypsin treated-NCFS exhibited low antibacterial activities against both *E. coli* strains, *S.* Typhimurium ATTC 14028, *P. aeruginosa* ATTC 27853 or *E. faecalis* ATCC 29212, while catalase treated-NCFS completely loosed this activity, suggesting that both *L. plantarum* strains were capable of synthetizing hydrogen peroxide.

Acid and bile tolerance, and simulated gastrointestinal digestion

Table 5 shows growth/survival of the five tested strains in MRS medium adjusted to different pH values and added with different taurocholate salt concentrations (0.15, 0.30, and 0.50%). The *L. plantarum* strains isolated from orange peel persisted with a viability loss of ~ 1.0 log within 6 h at pH 2.0 and were able to grow ~ 0.60 log cycle at pH 3.0. By contrast, the three juice strains showed a complete viability loss after 3 h at pH 2.0 and survived but without growing at pH 3.0. However, at pH 4.0 all the tested strains grew between 0.6 and 1.0 log cfu/ml with JNB25 and N8 reaching the highest cellular densities after 6-h incubation and separating from the other three strains.

Regarding the bile tolerance, all tested strains efficiently grew with all the assayed concentrations within 6-h incubation. However, the *L. plantarum* strains N8 and JNB21 showed growth ~ 15% lower relative to control, while the remaining strains grew in a similar way as the control without bile salt.

Interestingly, when live and dead bacteria of the five tested strains were counted at the end of the simulated gastrointestinal digestion assay (SGD), no viability loss by up to 3.0 log was observed: N4, N8, or JNB25 showed log reductions lower than 1.0; JNB21 between 1.0 and 2.0 units; and *L. brevis* JNB23 between 2.0 and 3.0 units (data not shown).

Permutation analysis based on the main analyzed properties of the seven *Lactobacillaceae* strains showed two main clusters (Fig. 1), cluster A only formed by *L. brevis* JNB23 and another one integrated by the *L. plantarum* strains which were grouped in 2 sub-clusters depending on their origin (JNB21 or JNB25, and N4 or N8 from juice and orange peel respectively). However, strain JNB25 showed similar characteristics of growth in orange juice and survival rate after SGD compared to cluster B strains, and was located at a closer distance of them than strain JNB21.

Table 5 Growth and/or survival of selected strains after 3 and 6 h incubation in MRS adjusted at low pH values and supplemented with different sodium taurocholate concentrations

		Viable counts (Log cfu/ml)*						
Strain	Time	pH values				Sodium taurocholate (%)		
	(h)	6.5	2.0	3.0	4.0	0.15	0.30	0.50
LPN4	3	7.83 ± 0.22^{aA}	6.29 ± 0.23^{aB}	7.08 ± 0.22^{aC}	7.28 ± 0.19^{aC}	7.95 ± 0.17^{aA}	7.80 ± 0.14^{aA}	7.73 ± 0.18^{aA}
	6	9.15 ± 0.33^{bA}	6.01 ± 0.16^{aB}	7.52 ± 0.15^{bC}	7.81 ± 0.13^{bC}	8.65 ± 0.26^{bA}	8.66 ± 0.23^{bA}	8.58 ± 0.30^{bA}
LPN8	3	7.66 ± 0.31^{aA}	6.40 ± 0.11^{aB}	7.16 ± 0.20^{aA}	7.33 ± 0.33^{aA}	7.50 ± 0.14^{aA}	7.42 ± 0.18^{aA}	7.35 ± 0.10^{aA}
	6	8.83 ± 0.26^{bA}	5.98 ± 0.10^{bB}	7.68 ± 0.10^{bC}	8.01 ± 0.25^{aB}	8.17 ± 0.15^{bB}	8.19 ± 0.24^{bB}	8.07 ± 0.11^{bB}
LPJNB21	3	7.41 ± 0.23^{aA}	ND	7.00 ± 0.20^{aA}	7.12 ± 0.21^{aA}	7.67 ± 0.14^{aA}	7.38 ± 0.11^{aA}	7.37 ± 0.19^{aA}
	6	8.53 ± 0.26^{bA}	ND	6.99 ± 0.22^{aB}	7.59 ± 0.31^{aB}	7.87 ± 0.19^{aB}	7.58 ± 0.15^{aB}	7.55 ± 0.13^{aB}
LPJNB25	3	7.70 ± 0.31^{aA}	ND	6.98 ± 0.17^{aB}	7.13 ± 0.12^{aB}	7.81 ± 0.10^{aA}	7.74 ± 0.15^{aA}	7.51 ± 0.11^{aA}
	6	9.11 ± 0.27^{bA}	ND	6.96 ± 0.09^{aB}	7.98 ± 0.14^{bB}	8.77 ± 0.25^{bA}	8.67 ± 0.23^{bA}	8.59 ± 0.36^{bA}
LBJNB23	3	7.74 ± 0.16^{aA}	ND	7.02 ± 0.19^{aB}	7.24 ± 0.16^{aB}	7.70 ± 0.19^{aA}	7.87 ± 0.12^{aA}	7.75 ± 0.10^{aA}
	6	8.82 ± 0.20^{bA}	ND	6.91 ± 0.12^{aB}	7.60 ± 0.23^{aB}	8.47 ± 0.19^{bA}	8.40 ± 0.30^{bA}	8.41 ± 0.19^{bA}

*Initial count: order of 10^7 ufc/ml. Different letters within column $^{(a,b)}$ and file $^{(A,B)}$ indicate significant differences for each strain in a stress condition (pH or bile salt) with respect to control condition (MRS adjusted to pH 6.5) respectively ($P < 0.05$). *ND*, not detected. Values are the means of three independent experiments ± standard deviation

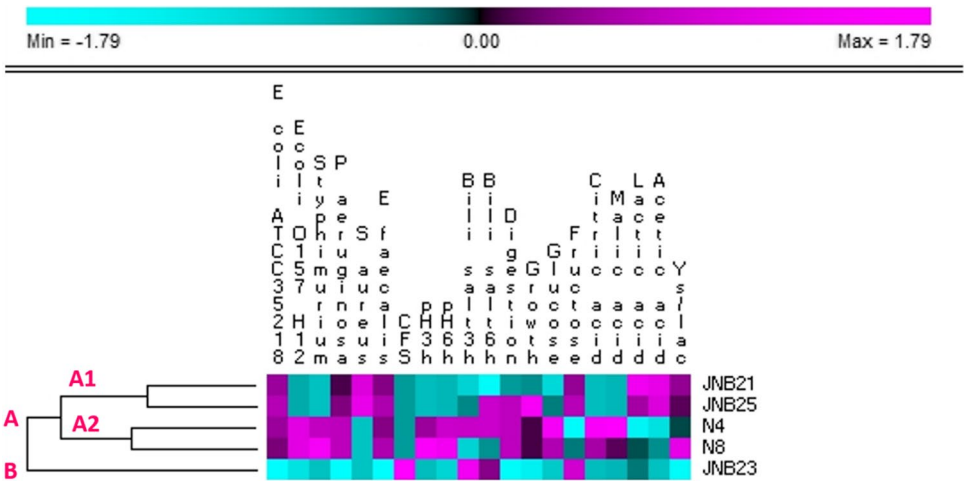

Fig. 1 Antimicrobial activity of CFS on both *E. coli* strains, *S.* Typhimurium, *P. aeruginosa*, *S. aureus*, and *E. faecalis* growth (mm), pH of CFS, pH and bile salt tolerance at 3 and 6 h (log cfu/ml), simulated digestion assay (rate survival, %), growth in orange juice (log cfu/ml), sugars and organic acids in orange juice at 24 h (mM), and bioconversion yield ($Y_{s/lac}$, mM) of selected *Lactobacillaceae* strains (JNB21, JNB23, JNB25, N4, and N8). Euclidean distance and McQuitty's criterion (weighted pair group method with averages) were used for clustering. The color corresponds to normalized mean data levels from low (cyan) to high (magenta). The color scale, in terms of units of standard deviation, is shown at the top

Discussion

In our study, LAB were mainly isolated from orange juice at 4 days of storage at 30 °C even though yeast colonies were also transferred from peel to juice indicating their antimicrobial potential and ability to growth in the acid juice. At this time, the following ranking of LAB species was identified: *L. brevis* > *L. plantarum* > *Leuconostoc* sp., indicating the prevalence of the two first species in the autochthonous LAB microbiota of orange juice. Similar results were reported in other raw fruit and vegetables with *L. plantarum*, *L. brevis*, *Lactobacillus fermentum*, and *Lactobacillus paracasei* (recently reclassified as *Lacticaseibacillus paracasei*) cited among the most frequent lactobacilli [9, 38, 39]. On the other hand, a good proliferative activity of *L. plantarum* in citrus juice in contrast to *Lactococcus lactis* and *Lactobacillus casei* (recently reclassified as *Lacticaseibacillus casei*) was documented [40]. In this study, the *L. brevis* strain dominance in orange juice could be related to its better adaptation to medium conditions and ability to initiate growth more rapidly than other LAB. However, these strains (HE) presented a higher demand of complex sources rich in nitrogen, such as yeast extract and peptone [13], than *L. plantarum* ones when incubated in mMRS medium. This result suggests that orange juice composition is an important factor in enabling the *L. brevis* strains growth requiring growth factors. According to Hébert et al. [41], *L. brevis* has consistently required folic acid for growth, while niacin, pantothenic acid, and pyridoxal auxotrophies appeared to be common to most lactobacilli. By contrast, the *L. plantarum* strains showed differences on nutritional classification (ME or LE), which was in agreement with the variability in the growth factor requirement patterns observed within this species and even strains isolated from same ecological niche [14]. This finding predicts the importance of differentiating them, and hence selecting the most promising nutritionally candidate(s) (such as strains JNB25 and JNB21) to grow in the natural medium with scarce amino acid content. Additionally, all *L. brevis* strains required cysteine to improve the growth in mMRS, possibly linked to its efficient incorporation into cell material for biomass formation. Nevertheless, its stimulatory effect led to biomass yields with statistically significant differences^{U,V,Y,Z}, suggesting variability in their amino acid requirement pattern. However, in all cases there was another growth factor(s) besides sulfur amino acid that prevented maximum growth in cysteine-enriched mMRS media. By contrast, cysteine was considered a non-necessary amino acid for all *L. plantarum* isolates grown under experimental conditions. Moreover, its negative impact on some bacterial growths in mMRSC1.0 could be related to the pathway inhibition involved in the essential amino acid biosynthesis [42]. This finding highlights as cysteine can positively or negatively affect the bacterial growth depending on species and strain.

Our results also suggested that *L. brevis* strains mainly utilized cysteine through assimilative via in concordance with their high nutritional exigency as low hydrogen sulfide levels were detected for some strains in mMRSC0.5/1.0. By contrast, all *L. plantarum* strains produced variable hydrogen sulfide at- or –higher than 0.5 g/l cysteine. Hence, the assessment of this property must be taken into account when *L. plantarum* strain collections are screened for fruit juice

fermentation as it at high concentration is considered off-odor [43]. However, we theorized that the cysteine content would not be enough to spoil orange juice when fermented by the *L. plantarum* strains in study (except JNB10) as previously reported for *L. plantarum* N4 [5]. This was confirmed when the four *L. plantarum* (JNB21, JNB25 from juice and N4 and N8 from orange fruit) and *L. brevis* JNB23 rapidly grew in orange juice incubated at 30° C to values required for probiotic products, without hydrogen sulfur production. However, some statistical differences in their final biomass indicated the usefulness of the growth assay in deficient nutritional conditions as preliminary selection criterion for the most suitable strains. Our results of the Argentina oranges and juice' LAB strains also revealed that (1) glucose was the preferred sugar to grow; (2) high lactic acid levels recovered from glucose confirmed their high lactic acid potential; and (3) acetic acid was exclusively formed from citrate catabolism (molar ratio ~ 1.0), except for *L. brevis* (0.57). In this case glucose and citrate metabolism could be involved in biosynthesis reactions in line with its higher nutritional demand. Saguir and Manca de Nadra [44] reported in *Oenococcus oeni* from wine their beneficial effect for the growth rate, the biomass formed and to fill in the amino acid requirements in deficient nutritional condition. In this study, the rapid growth and the significant organic acid production of the five tested strains in orange juice could explain their dominance in the natural medium when were isolated.

Between studied beneficial properties, our results demonstrated that the four tested *L. plantarum* strains displayed a broth and significant inhibitory activity against the tested foodborne pathogen and spoilage bacteria in opposition to *L. brevis* JNB23 (only had weak/moderate on gram-positive pathogens). *S.* Tiphymurim ATTC 14,028 was the most resistant pathogen for all, apart from other gram-negative bacteria depending on LAB species and strain, except for N4 and N8 (*S. aureus*). In general, their antagonistic effect depended on a significant degree on the presence of live cells as it was reduced in CFSs presence, as also reported by Huang et al. [45] for *L. plantarum* ZDY 2013. The neutralization of the tested LAB supernatants indicated that the antagonistic effects were completely or mainly due to their pH-lowering capacity and organic acid production such as D-lactic acid. Thus, *L. plantarum* strains with activity against the majority of tested indicator bacteria including *S.* Typhimurium, *E. coli* (G−), and/or *S. aureus* (G+) produced the greatest CFS-pH reductions and organic acid production in concordance with that observed in orange juice. However, in the case of the *L. plantarum* strains N4 and N8, the antibacterial activity nature against indicators, including *E. coli* O157:H12 and *S.* Typhimurium ATTC 14,028, would be ascribed to the cumulative effect of acidity plus hydrogen peroxide synthesis. Numerous reports have documented the antagonistic activity of potentially probiotic LAB strains associated to organic acids, but not hydrogen peroxide production [45–47], which was here demonstrated for the first time, in *L. plantarum* strains from oranges. The antimicrobial effect of hydrogen peroxide may result from the sulfhydryl group oxidation causing enzymes denaturing, from the membrane lipid peroxidation and also to be a precursor for the bactericidal free radical's production which can damage DNA [48]. Results obtained highlighted the selected *L. plantarum* strains potential to be implicated in fruit juice biopreservation as they were able to rapidly grow in orange juice with high potential lactic acid and to inhibit pathogen and spoilage microorganism and for expression of a probiotic effect for the host. Another interesting finding was the relationship among the tested LAB strains for nutritional and antibacterial properties, as *L. brevis* JNB23 failed to grow in mMRS without cysteine and had weak antagonistic action in opposition to *L. plantarum* strains.

Regarding the acid tolerance, even though there were strain-specific variations under experimental conditions, all they were able to grow or survive at pH equal or higher than 3.0. This reflects an adaptive response to natural habitat [49], and predicts high survival possibility in in vivo tests. On the other hand, all tested strains grew at high cell densities with taurocholate salt, even up to 0.3%, despite having few chances to be exposed to bile in the natural habitat. This is particularly important as 0.3% is considered to be a critical concentration for the selection of resistant strains to bile [50]. Kimoto-Nira et al. [51] described the resistance of *L. lactis* ssp. *lactis* G50 isolated from vegetable to 0.3% bile but not to higher concentrations. The high sodium taurocholate resistance of the tested strains would be related with the ability to deconjugate it as reported in LAB isolated from human gastrointestinal tract [11].

Coincidently, all tested strains exhibited good survival rates to simulated gastrointestinal digestion (SGD) although with strain-specific variations. Finally, the permutation analysis based on the main studied parameters clearly separated the *L. plantarum* strains from *L. brevis* one, with *L. plantarum* N4, N8, and JNB25 showing the best characteristics of growth and lactic acid bioconversion yield in orange juice, antibacterial activity, and SGD tolerance.

Conclusion

L. brevis and *L. plantarum* phenotypically and genotypically identified were the dominant species in orange juice. However, all *L. brevis* strains required complex sources rich in nitrogen, and cysteine to grow under stressing nutritional condition in opposition to *L. plantarum* ones, especially those classified as LE. A great goal of this study was that the four selected *L. plantarum* strains exhibited ability to grow

rapidly in the natural medium without pH adjustment and showed high fermentative potentials. In addition, all were able to inhibit bacterial pathogens, to survive or grow after 3 h exposure to pH 3.0, to grow in 0.5% sodium taurocholate presence for 6 h, and enduring the unfavorable SGD conditions. Thus, autochthonous *L. plantarum* strains with ability for overcoming nutritional limitations in nitrogen sources are promising candidates for further investigations of novel strains to ferment functional citric fruit juices, without causing spoilage.

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Declarations

Consent to participate The authors gave informed consent to their inclusion in this study.

Conflict of interest The authors declare no competing interests.

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