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Screening of autochthonous lactic acid bacteria strains from artisanal soft cheese: probiotic characteristics and prebiotic metabolism

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ABSTRACT

Today's growing interest in functional foods is due to their positive impacts on human health. The aim of this study was to evaluate the probiotic properties and prebiotic metabolism of autochthonous lactic acid bacteria from artisanal Serpa cheese. Twenty strains presented better tolerance to stress conditions found in the gastrointestinal tract, highlighting *Lactobacillus brevis* C1Lb21 for its adequate auto-aggregating ability and significantly higher hydrophobicity. However, eight strains were discarded for their antibiotic resistance and biogenic amine production. Finally, prebiotic fermentation study showed that lactulose supported better growth of lactobacilli and induced the production of short-chain fatty acids (SCFA). During lactulose fermentation, *Lb. pentosus* G4Lb7 produced statistically more SCFA, and *Lb. plantarum* G1Lb5 lactic acid. Thus, *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 are promising probiotic candidates, and in combination with lactulose could be used for developing a symbiotic cheese.

1. Introduction

Nowadays, there is a growing interest in nutrition to improve health. Food is not only intended to provide the necessary nutrients but also to beneficially impact on consumers' health (Linares et al., 2017). This matter has increased the awareness and demand for functional food, mainly probiotics, due to the extensive information about their beneficial effects. Lactic acid bacteria (LAB) are the most common microorganisms applied as probiotics. They are a desirable member of the gastrointestinal tract (GIT) microbiota and have the GRAS "Generally Recognised As Safe" status (Linares et al., 2017). Several LAB species have shown probiotic characteristics and it is well-known that they are strain-dependent (Vasiljevic & Shah, 2008). Fermented dairy products have been by far the most investigated as a source of probiotics.

Serpa cheese is a soft-cheese produced in the south of Portugal. In our previous research, the microbial community of this artisanal cheese have been described (Gonçalves Dos Santos, Benito, Córdoba, Alvarenga, & Ruiz-Moyano, 2017; Gonçalves Dos Santos et al., 2018) and LAB were undoubtedly the main microbial group, with viable counts of around 10^8 cfu g^{-1} at the end of the ripening process. LAB are involved in the ripening process of the cheese and contribute to the final organoleptic properties (O'Sullivan & Cotter, 2017). Additionally, the LAB strains present in Serpa cheese may possess probiotic potential.

The primary criteria for selecting strains as suitable probiotics are (i) survival to GIT conditions, (ii) ability to adhere to the intestinal mucosa and colonise the colon, at least temporarily, (iii) capacity to exert potential health benefits on the host, and (iv) verified safety. Another important activity of probiotics is the fermentation of non-digestible carbohydrates into short-chain fatty acids (SCFA), mainly acetate, propionate and butyrate. There is a plethora of evidence regarding the positive benefits of these compounds on human energy metabolism, health and protection against colorectal cancer, inflammatory bowel disorders, obesity and other diseases (LeBlanc et al., 2017; Ríos-Covián et al., 2016). Therefore, studies to select probiotics with an enhanced ability to produce SCFA are relevant.

In addition to probiotic characteristics, it also desirable that these microorganisms are adapted to the fermentation process and storage. Probiotic products should be consumed regularly with an approximate amount of 100 g/day to deliver about 10^9 viable cells into the intestine

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Table 1
Selected LAB isolates from Serpa cheese to study probiotic characteristics.

Selected LAB from Serpa cheese (Gonçalves et al., 2017)		
Species	Nº isolates	Code of acid-tolerant strains
Lactobacillus casei/paracasei	34	V1LB8, A2Lb1
Lactobacillus plantarum	16	C1Lc12, G4Lb1, G2Lb12, B1Lb12, G1Lb5, G2Lb9
Lactobacillus brevis	12	V1Lb10, B1Lb3, C1Lb21, B2Lb5
Lactobacillus curvatus	4	–
Lactobacillus crustorum	4	A3Lb18, V1Lb9
Lactobacillus coryniformi	2	–
Lactobacillus pentosus	2	G4Lb7
Leuconostoc mesenteroides	12	–
Lactococcus lactis	2	–
Enterococcus faecium	14	V2Lb3, A1Lb9, G1Et4
Enterococcus hirae	12	G3Et3
Enterococcus durans	2	G1Et3

in order to positively influence consumer health (Tripathi & Giri, 2014). Cheese is an excellent food matrix to transport probiotic, due to its specific physical and chemical characteristics, such as pH between 5 and 6, high fat content and nutrient availability, and low oxygen content. Since these conditions favour their survival through gastrointestinal transit, the application of probiotics has been optimised in different types of cheeses (Albenzio et al., 2013; Minervini et al., 2012). Therefore, this study aimed to evaluate probiotic properties and prebiotic metabolism of autochthonous LAB isolated from artisanal Serpa cheese, for potential application during its manufacturing process.

2. Material and methods

2.1. Bacterial isolates

A total of 116 LAB belonging to the *Lactobacillus, Lactococcus*, *Leuconostoc* and *Enterococcus* genera, isolated from Serpa cheese (Gonçalves et al., 2017), were selected to study their probiotic characteristics (Table 1). LAB were routinely grown in de Man–Rogosa–Sharpe (MRS; Scharlab, Barcelona, Spain) broth at 37 °C under 10% CO_2 for 24 h. All strains were sub-cultured twice before the experiments, harvested at 21,500 × *g* for 5 min at room temperature, washed twice with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA) at pH 7.2, and the cell concentration adjusted to around 10^8 cfu mL^{-1}. For all strains, three biological replicates of the cultures and duplicate assays were conducted.

2.2. Probiotic assays in vitro

2.2.1. Tolerance to simulated GIT

To evaluate the acid tolerance of the LAB *in vitro*, they were exposed to a pH between 2.5 and 3.0 for 2 h. Each isolate was incubated for 2 h in PBS supplemented with 3.5 g L^{-1} of pepsin, and the pH adjusted to 2.5, 2.75, 3.0 or 5.0 (positive control) and measured by counting following the method described by Ruiz-Moyano, Martín, Benito, Nevado, and Córdoba (2008).

The strains resistant to low pH were used to determine the survival during complete gastrointestinal transit, by the method of Bao et al. (2010). Briefly, 30 μL of concentrated strains were inoculated in 270 μL of simulated gastric fluid (3.5 g L^{-1} porcine pepsin (≥400Units/mg protein) and 2 g L^{-1} NaCl) at pH 2.75 for 2 h. Then 30 μL were transfer to 270 μL of in simulated intestinal fluid (1 g L^{-1} trypsin, 5 g L^{-1} bile salt, 2 g L^{-1} pancreatin [all from Sigma St. Louis, MO, USA], 11 g L^{-1} $NaHCO_3$ and 2 g L^{-1} NaCl) at pH 8 during 6 h. The 96 micro-well plates were incubated at 37 °C under 10% CO_2, and the viable bacteria were counted on MRS media after 0, 2, 4 and 6 h. Data were expressed in Log cfu mL^{-1} reduction with respect to the initial inoculum (Time 0 h: 8 Log cfu mL^{-1}). Strains with viable counts below 2 log cfu mL^{-1} compared with the initial inoculums were considered not resistant.

2.2.2. Aggregation activity

The specific cell–cell interactions were determined using the auto-aggregation assay described by Xu, Jeong, Lee, and Ahn (2009). The auto-aggregation was calculated using the following equation:

$$\text{Auto-aggregation (\%)} = (1 - A_t/A_0) \times 100 \quad (1)$$

where A_t represents absorbance at a determined interval (1 or 2 h); and A_0 represents the absorbance at the beginning of the assay (0 h).

2.2.3. Cell surface hydrophobicity

The cell surface hydrophobicity was determined as described Lee and Puong (2002). The decreased absorbance in the aqueous phase was taken as a measure of cell surface hydrophobicity, calculated using the following equation:

$$\text{Hydrophobicity (\%)} = [(A0 - A1)/A0] \times 100 \quad (2)$$

where *A0* and *A1* are the absorbance values before and after extraction with *n*-hexadecane, respectively.

2.3. Safety assays

2.3.1. Antibiotic susceptibility

The selected acid-tolerant strains were tested for antibiotic susceptibility by the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2012). Twelve clinically important antibiotics (Oxoid, Basingstoke, England) were used: ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), trimethoprim (5 μg), erythromycin (15 μg), tetracycline (30 μg), clindamycin (2 μg), chloramphenicol (30 μg), penicillin G (10 U), polymyxin B (300 U), nalidixic acid (30 μg) and vancomycin (30 μg). The diameters of the inhibition zones were measured and the results interpreted according to the criteria proposed by Charteris, Kelly, Morelli, and Collins (1998).

2.3.2. Biogenic amine (BA) production

The capacity of selected strains to produce BA was determined by the improved agar medium, as described by Bover-Cid and Holzapfel (1999). Moreover, the amount and type of BA produced in the improved medium (Bover-Cid & Holzapfel, 1999), without agar and containing 0.25% of each precursor amino acid after incubation for 4 days at 37 °C and 10% CO_2 was measured by high-performance liquid chromatography (HPLC)–electrospray ionisation (ESI)-mass spectrometry, according to the method described by Fernández et al. (2016). BA in samples was distinguished by their mass spectrum and retention time.

2.4. Growth on prebiotic

The LAB selected on the basis of their probiotic properties were tested for growth in the presence of three commercial prebiotics; lactulose (Sigma), short-chain fructooligosaccharide (FOS, Orafti® P95 with a degree of polymerisation (DP) 2–8, Beneo-Orafti, Belgium) and long-chain inulin (Orafti® GR with DP 2–60 and average DP ≥ 10, Beneo-Orafti). Two μL of each LAB strain suspension was inoculated in 200 μL of semi-solid MRS medium containing 0.125 g L^{-1} agar, devoid of glucose, and supplemented with 2 g L^{-1} of each sterile-filtered prebiotic, as the sole carbohydrate source. The positive control for growth consisted of semi-solid MRS supplemented with 2 g L^{-1} glucose or lactose, whereas the negative control was a carbohydrate-free semi-solid MRS. The automated turbidimeter Bioscreen C Microbiology reader (Labsystem, Helsinki, Finland) set up at 37 °C was used to monitor the growth for 96 h by reading the optical density (OD) at

600 nm at regular intervals without shaking. The ability of each strain to grow in the presence of different prebiotics was evaluated by comparing the OD at 600 nm of each carbohydrate with the value obtained on glucose.

2.5. SCFA production

To determine the capacity to produce SCFA, selected LAB strains were grown on modified MRS (mMRS) broth at 37 °C under 10% CO_2 until stationary phase. The mMRS was formulated as commercial MRS devoid of glucose and sodium acetate and supplemented with 2 g L^{-1} of the carbohydrate source (glucose, lactose, lactulose, FOS or inulin). The supernatants of the cultures were obtained by centrifugation of the media at 8000 × *g* for 5 min before filtering through 0.22-μm filters (Thermo Fisher Scientific).

The concentration of lactic acid (D- and L-isomers) in the supernatant was quantified using the enzymatic kit K-DLATE (Megazyme, Bray, Ireland) according to the manufacturer's instructions.

To measure the amount of SCFA, 800 μL of supernatant was mixed with 100 μL of internal standard solution (2-ethyl butyric acid [Sigma] at 5 mM prepared in 12% formic acid) and 100 μL of meta-phosphoric acid solution (16 g L^{-1}). SCFA were extracted with 500 μL of diethyl ether by vortexing for 1 min and centrifugation at 17,000 × *g*, 4 °C for 5 min. One microlitre of the upper phase was injected into a gas chromatograph (model 4890 Series II; Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/split-less injector and a flame ionisation detector. SCFA were separated on a Carbowax™ fused silica capillary column (30 m × 0.25 mm id; 0.25 μm film thickness; (Ohio Valley, Marietta, OH, USA). The initial oven temperature was held at 80 °C for 2 min, and then increased to 200 °C at 20 °C min^{-1} and retained for 12 min. The injector and detector were set at 250 °C. The carrier gas was nitrogen at 1.8 mL min^{-1}. The individual SCFA were identified by comparison of their retention times with those of reference standard mixtures (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, hexanoic acid from Sigma Chemical Co., St Louis, MO, USA). SCFA concentrations were calculated by using the peak area ratio of the analyte to the internal standard (2-ethyl butyric acid), as detailed by Brighenti (1997).

2.6. Statistical analysis

Auto-aggregating and hydrophobicity data were analysed by one-way analysis of variance (ANOVA), and SCFA production by two-way ANOVA, respectively, using SPSS for Windows 21.0 (SPSS, Inc., Chicago, IL, USA). The means were separated by Tukey's honestly significant difference (HSD) test ($p \leq 0.05$).

3. Results and discussion

3.1. Tolerance to the GIT conditions

All LAB isolates tolerated pH 3.0. When the isolates were exposed to pH 2.5, none survived at the levels required. At an intermediate pH (2.75), 20 of the 116 strains exhibited satisfactory levels of viability after exposure for 2 h (Table 2). Previous authors have reported that the viability of LAB is dramatically affected at low pH, especially below pH 2.5 (Zoumpopoulou et al., 2018). The acid tolerance of LAB is strain specific and mediated via several mechanisms, in particular, F0F1-ATPase is an important element in the response and tolerance to low pH in *Lactobacillus* spp. (Corcoran, Stanton, Fitzgerald, & Ross, 2005). In this study, a pH value of 2.75 was found to be highly discriminating and although the pH of the stomach during a meal is normally above this value, this value was set in order to guarantee the viability of the potential probiotic strains.

The acid-tolerant strains were subjected to *in vitro* digestion. Most of the selected strains showed good survival to complete GIT transit (Table 2). However, two of the twenty strains, *Lb. casei/paracasei* strains V1Lb8 and A2Lb1, decreased their viability dramatically when they were exposed to simulated gastric fluid at pH 2.75 for 90 min, followed by 120 min in simulated intestinal fluid. These results are consistent with those previously recovered from other probiotic *in vitro* testing where most of the strains were more resistant to bile salts than low pH (Han, Kong, Chen, Sun, & Zhang, 2017; Ruiz-Moyano et al., 2008; Zoumpopoulou et al., 2018).

3.2. Capacity to colonise the intestine

The percentage of auto-aggregation after incubation for 1 and 2 h are presented in Fig. 1A. In general, the values varied between 1.43 and 9.14% at 1 h, and 2.70–17.14% at 2 h, respectively. All strains increased the auto-aggregation percentages with the incubation time, in congruence with other researchers (García-Cayuela et al., 2014; Han et al., 2017). Among strains, significant differences were observed ($p \leq 0.05$). At 2 h, seven of the twenty strains, *Lb. brevis* C1Lb21 and B2Lb5, *Lb. crustorum* A3Lb18, *E. faecium* V2Lb3, A1Lb9 and G1Et4, and *E. durans* G1Et3, exhibited better auto-aggregation, with values over 12%, corresponding to a noteworthy capacity based upon previous studies in LAB (García-Cayuela et al., 2014; Han et al., 2017).

There was high variability in the hydrophobicity capacity, which ranged from 5.42 to 76.50% (Fig. 1B). *Lb. brevis* C1Lb21 exhibited the highest hydrophobicity (76.50%). The results obtained for both cell surface properties did not always show the same trend in each strain, as already noted by García-Cayuela et al. (2014). Adhesion is a very complex mechanism influenced by multiple factors. Auto-aggregation capacity is afforded by complex physical and chemical interactions, as well as being influenced by environmental conditions (Collado, Meriluoto, & Salminen, 2008; García-Cayuela et al., 2014; Goh & Klaenhammer, 2010).

3.3. Safety aspects

3.3.1. Antibiotic susceptibility

Nowadays, antibiotic resistance is considered a serious concern in medicine. According to the European Food Safety Authority (EFSA), intrinsic resistance in bacteria present a minimal risk for horizontal spread whereas acquired resistance is considered a high risk (EFSA, 2012). The antibiotic susceptibility of selected LAB strains against 12 common antibiotics is shown in Table S1. All lactobacilli strains were found susceptible or moderately susceptible to penicillin G, chloramphenicol, erythromycin, tetracycline, ampicillin, gentamycin and clindamycin. In addition, it was found resistance of 11 lactobacilli strains to kanamycin, all *Lb. brevis* and *Lb. casei/paracasei* strains to polymyxin B, and three strains, *Lb. brevis* B1Lb2, *Lb. casei/paracasei* V1lb8 and A2Lb1, to trimethoprim. In general, although there may be differences between species and strains, most *Lactobacillus* spp. are considered to be naturally resistant to vancomycin, nalidixic acid, kanamycin, polymyxin B and trimethoprim. In this context, the observed patterns of antibiotic susceptibility agree with the results obtained in the literature for these lactobacilli species, and they can be used in fermented food (Abriouel et al., 2015). The resistance profiles of enterococci from food or clinical isolates vary widely, containing many acquired traits. Enterococci are naturally resistant to cephalosporins, low level aminoglycosides (kanamycin and gentamycin), polymixins, lincomycin, clindamycin and often quinolones (nalidixic acid) (Sharma, Tomar, Goswami, Sangwan, & Singh, 2014). The major concern was the detection of acquired resistance to trimethoprim in three enterococci strains, G3Et3, A1Lb9 and V2Lb3, and especially to relevant clinical antibiotics, such as tetracycline (in two strains, A1Lb9 and G3Et3) and vancomycin (in G1Et4). Consequently, these enterococci strains were not selected.

Table 2
Tolerance of bacterial strains to low pH, and complete gastrointestinal transit. Data are expressed in Log cfu mL^{-1} reduction with respect to the initial inoculum (Time 0 h: 8 Log cfu mL^{-1}).

LAB strains		pH tolerance (2.75)			Tolerance to gastointestinal transit			
					Gastric juice (pH2.75) 1.5 h	Intestinal juice		
		1 h	1.5 h	2 h		2 h	4 h	6 h
Lb. plantarum	C1Lc12	0	0	0	0	1	1	1
	G4Lb1	1	1	2	0	0	0	0
	G2Lb12	0	1	1	0	0	0	0
	B1Lb2	0	1	1	1	1	1	2
	G1Lb5	1	1	1	0	0	0	0
	G2Lb9	1	1	2	1	1	1	1
Lb. brevis	V1Lb10	0	1	1	1	2	2	3
	B1Lb3	1	1	1	0	0	0	0
	C1Lb21	0	0	0	0	1	1	1
	B2Lb5	0	0	1	0	1	1	1
Lb. casei/paracasei	V1Lb8	1	2	2	2	3	5	8
	A2Lb1	1	1	1	1	3	4	7
Lb. crustorum	A3Lb18	0	1	1	0	0	1	1
	V1Lb9	0	1	1	0	0	0	0
Lb. pentosus	G4Lb7	1	1	2	1	1	1	1
E. faecium	V2Lb3	0	1	1	0	0	0	0
	A1Lb9	1	1	1	1	1	1	1
	G1Et4	0	0	0	0	0	0	1
E. durans	G1Et3	0	0	0	0	0	0	0
E. hirae	G3Et3	0	0	0	0	0	0	0

3.3.2. BA production

The ability of the LAB strains to produce BA *in vitro* is presented in Table S2. Of the 20 strains, three *Lactobacillus* spp. (*Lb. brevis* B1Lb3 and B2Lb5, and *Lb. crustorum* V1Lb9) and the five *Enterococcus* spp. strains were positive on the decarboxylase medium. The HPLC-MS analysis confirmed that all positive strains produced levels ranged from 523.61 to 4167.67 mg L^{-1} of tyramine and tryptamine between 1.58 and 14.46 mg L^{-1}. Production of BA by potential probiotics strains is not a desirable property due to its toxic effect on consumer health (Gardini, Özogul, Suzzi, Tabanelli, & Özogul, 2016). So, the eight positive BA amine strains were not suitable as probiotic candidate.

3.4. Prebiotic growth and SCFA production

The capacity of the 12 pre-selected LAB to grow *in vitro* on three commercial prebiotics and positive substrate controls (glucose and lactose) is showed in Fig. S1.

In general, all LAB strains reached a maximum OD 600 nm values in lactulose comparable to the positive controls, although *Lb. brevis* C1Lb21 and V1Lb10 presented a slower growth rate and a longer lag phase. In the FOS or inulin, the growth was lower and more variable between strains than in lactulose. Interestingly, *Lb. casei/paracasei* V1Lb8 showed relevant growth on FOS and inulin while intermediate growth was observed in the remaining strains, except for *Lb. brevis* strains *Lb. plantarum* B2Lb1 and *Lb. crustorum* A3Lb18, which showed low ability to use these prebiotics (Fig. S1). Contrary to the expected, LAB growth was, generally, greater with inulin than FOS, despite the higher DP of inulin. We suppose that this result might be influenced by the relatively higher purity of FOS (Orafti® P95). The capacity to ferment lactulose is widespread between lactobacilli species, and β-galactosidase activity has been related to lactulose hydrolysis and metabolisation (Mao et al., 2014; Sharma & Kanwar, 2018). However, FOS utilisation is generally more specific. Our results suggest that lactulose is a suitable prebiotic to support lactobacilli growth. In addition, this prebiotic has demonstrated to be selective to avoid pathogenic bacterial growth (Sharma & Kanwar, 2018), improve the response of probiotics to acid and bile stresses (Adebola, Corcoran, & Morgan, 2014) and exert a positive effect in the treatment of intestinal disorders (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004; Vilela, Torres, Ferrari, Lima, & Cunha, 2008).

Table 3 shows lactic acid and SCFA production by carbon source obtained from the mean values of the 12 selected strains. Overall, lactic acid was the principal metabolite detected ranged from 15.086 mM in FOS to 120.697 mM in lactose. The acetic acid ranged from 6.500 mM in FOS to15.471 in lactulose. Small amounts of butyric, isobutyric, propionic and isovaleric acids were detected. The limited capacity of most of the LAB strains to ferment FOS and inulin was evidenced by the low lactic acid and SCFA production. Interestingly, lactulose statistically increased the production of SCFA and induced a noteworthy level of lactic acid, plus a lower ratio of D-lactic acid, which has been associated with potential health problems. SCFA display distinct positive physiological effects on the host. Butyric acid has been the most widely studied for its anti-carcinogenic and anti-inflammatory effects, although acetic acid and propionic acid also exhibit health-promoting actions (Russell, Hoyles, Flint, & Dumas, 2013).

Regarding the strain capacity, Fig. 2 shows the mean values and statistical differences between the organic acids produced by the pre-selected LAB strains in lactulose and carbohydrate source controls. Significant differences ($p \leq 0.05$) were found between strains in the amount of SCFA produced in lactulose. For all tested strains, acetic acid was the second major metabolite, with approximately 10-fold less concentration than lactic acid, except for *Lb. pentosus* G4Lb7 that produced a similar amount of both metabolites. For the remaining compounds detected, the concentrations were approximately similar to each other, with values around 0.10 mM. Besides acetic acid, *Lb. pentosus* G4Lb7 also produced significantly higher ($p \leq 0.05$) amounts of butyric, isobutyric, propionic and isovaleric acids while similar patterns were found among the other strains. Among them, *Lb. casei/paracasei* A2Lb1 stood out due to its high production of propionic acid comparable to *Lb. pentosus* G4Lb7, and slightly higher than average amount of the other SCFA, although this strain was not resistant to GIT transit (Table 2).

With respect to lactic acid production in lactose and glucose, *Lb. plantarum* G2Lb9 produced the highest amounts ($p \leq 0.05$), with values around 175 mM, followed by a second group of strains composed of *Lb. plantarum* G1Lb5, C1Lc12 and G4Lb7, *Lb. casei/paracasei* V1Lb8 and *Lb. pentosus* G4Lb7, with values ranging from 130 to 155 mM. Lactic acid production from lactose fermentation by cheese microbiota has

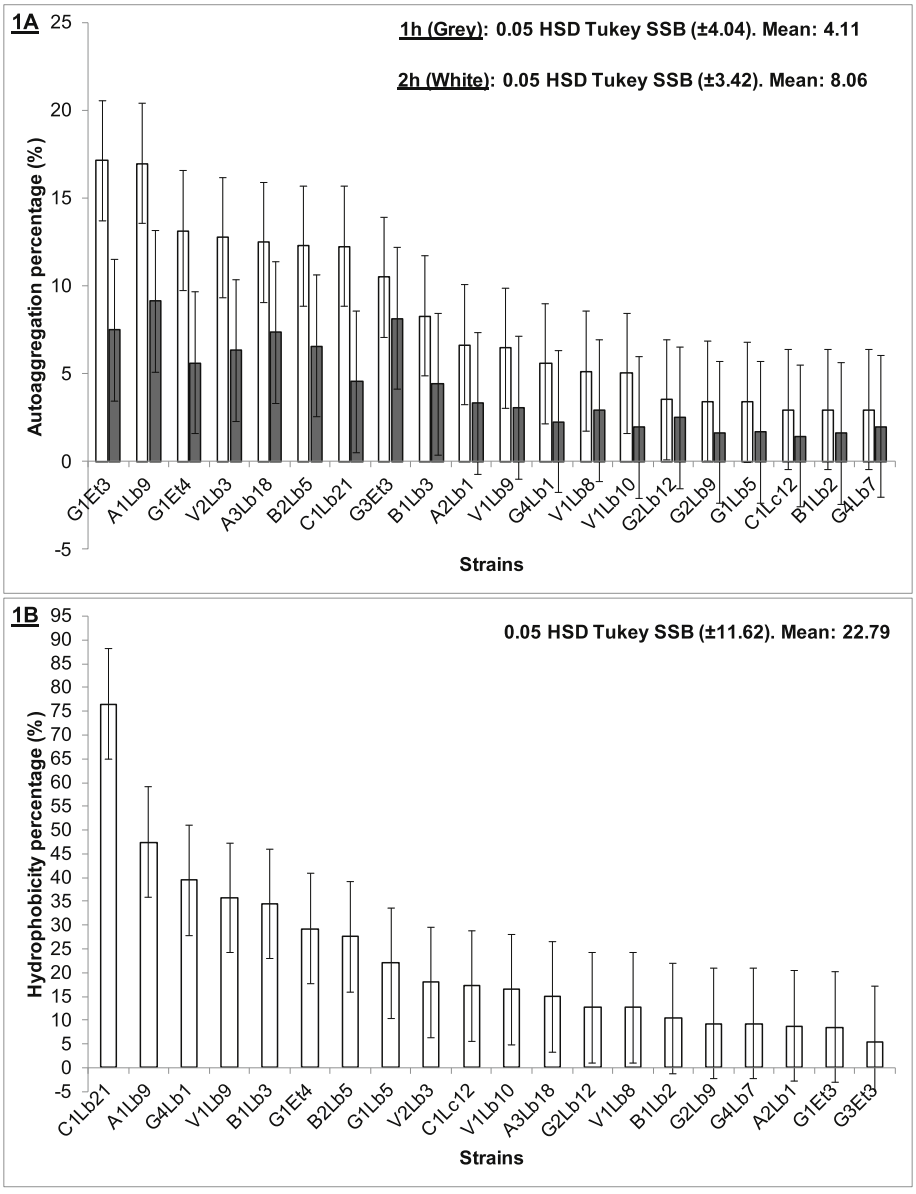

Fig. 1. (**A**) Auto-aggregation percentages of LAB strains after 1 h (grey bars) and 2 h (white bars) and (**B**) hydrophobicity capacity against hexadecane. SSB: statistical significance bar using Tukey's HSD test ($p \leq 0.05$).

Table 3
Amount of lactic acid and short-chain fatty acids (SCFA) by carbohydrate source. Each data is the average values obtained from the 12 selected strains.

Carbon source		Organic acids (mM)							
		Lactic acid	L-lactic acid	D-lactic	acetic	Propionic	butyric	Isobutyric	Isovaleric
Lactose	**Mean**	**120.697**^{a*}	**63.596**^{b}	**57.100**^{a}	**7.098**^{b}	**0.057**^{b}	**0.0788**^{b}	**0.051**^{b}	**0.037**^{b}
	Std. Deviation	29.517	21.410	34.518	3.990	0.010	0.007	0.014	0.008
Glucose	**Mean**	**116.335**^{b}	**72.204**^{a}	**44.130**^{b}	**6.503**^{b}	**0.053**^{b}	**0.079**^{b}	**0.047**^{b}	**0.038**^{b}
	Std. Deviation	32.863	26.865	27.845	3.647	0.011	0.012	0.007	0.006
Lactulose	**Mean**	**92.016**^{c}	**62.866**^{b}	**29.149**^{c}	**15.471**^{a}	**0.130**^{a}	**0.113**^{a}	**0.080**^{a}	**0.055**^{a}
	Std. Deviation	31.265	28.650	20.246	4.699	0.078	0.045	0.051	0.021
Inulin	**Mean**	**21.590**^{d}	**15.192**^{c}	**6.398**^{d}	**6.708**^{b}	**0.049**^{c}	**0.060**^{c}	**0.038**^{c}	**0.030**^{c}
	Std. Deviation	30.778	28.255	6.770	5.322	0.048	0.044	0.038	0.022
FOS	**Mean**	**15.086**^{e}	**12.999**^{c}	**2.086**^{e}	**6.500**^{b}	**0.041**^{c}	**0.058**^{c}	**0.036**^{c}	**0.027**^{c}
	Std. Deviation	32.633	32.187	1.843	5.551	0.030	0.042	0.037	0.020
$P \leq 0.05$		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tukey HSD IC		± 4.174	± 3.158	± 2.630	± 1.299	± 0.009	± 0.006	± 0.007	± 0.005

* ^{a,b,c,d,e} For a given organic acid, values (column) with different superscript letters are significantly different ($p \leq 0.05$).

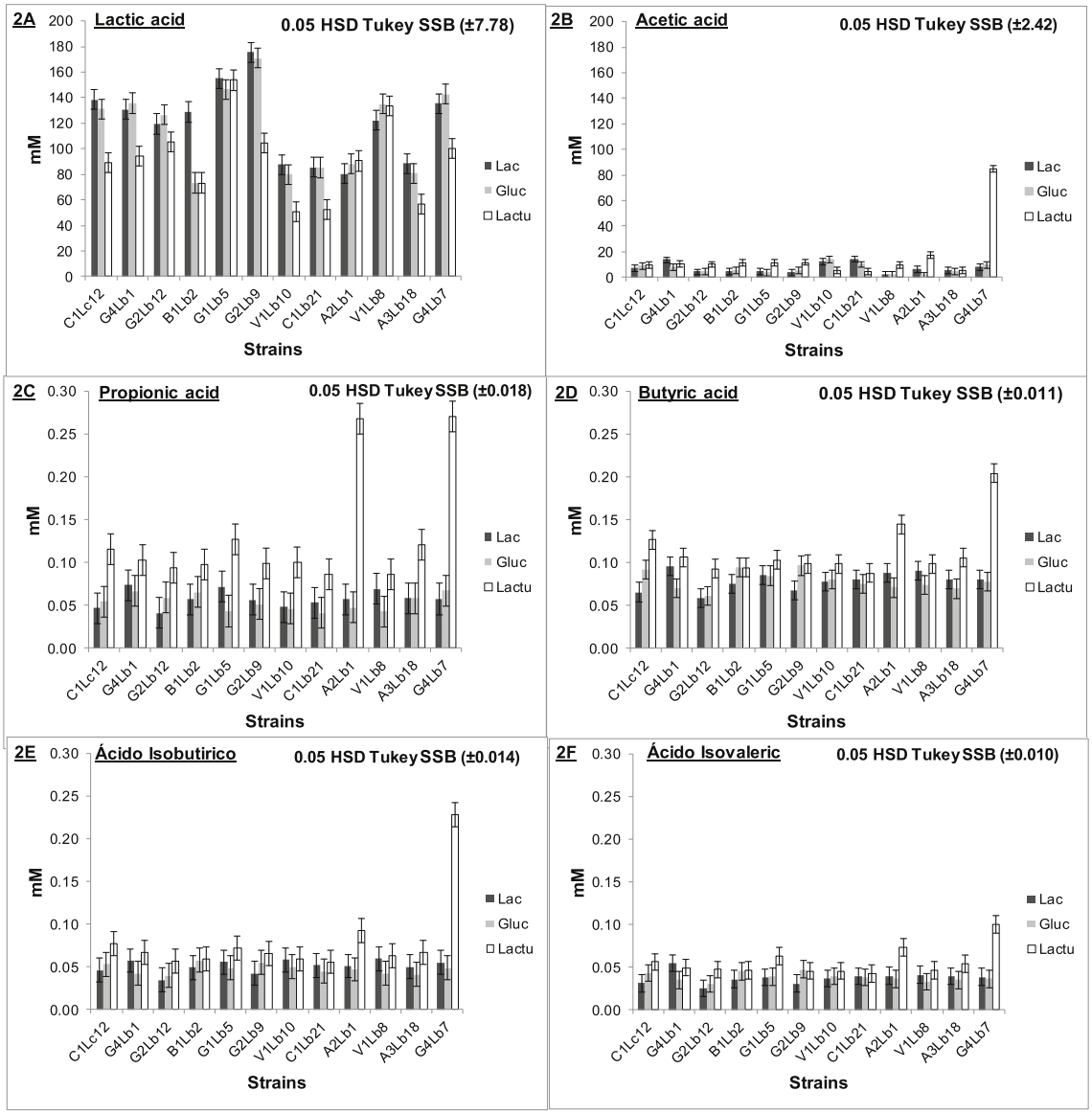

Fig. 2. Quantification of lactic acid and short-chain fatty acids by selected LAB in glucose (Gluc), lactose (Lac) and lactulose (Lactu). (**A**) Lactic acid, (**B**) acetic acid, (**C**) propionic acid, (**D**) butyric acid, (**E**) isobutyric acid and (**F**) isovaleric acid. SSB: statistical significance bar using Tukey's HSD test ($p \leq 0.05$).

technological applications during cheese ripening, to drop the pH and provide an acidic environment, which, in turn, ensures food safety and favours the coagulation process (McSweeney, 2004). In lactulose, *Lb. plantarum* G1Lb5 produced the greatest amounts of lactic acid ($p \leq 0.05$), reaching levels of 153 mM, without statistical difference from glucose and lactose. The other strains, except for the two *Lb. casei/paracasei,* exhibited an evident decrease in the production of this metabolite. Lactulose is not consumed in the upper part of the intestine and can stimulate the growth of probiotics in the colon and lactic acid production. Although, in general, LAB produced low amounts of the most interesting SCFA for human health, it must be considered that a potential probiotic will be a member of the intestinal microbiota where it may establish possible cross-feeding interactions with lactate-consuming butyrate-producing colon bacteria (Moens, Verce, & De Vuyst, 2017). Thus, to select a potential probiotic, aside from SCFA production capacity in assay conditions, it is also relevant to consider their ability to produce lactic acid from non-digestible oligosaccharides, as this may contribute to increasing the daily SCFA amount in the colon environment, by the activity of the intestinal microbiota.

In conclusion, considering their tolerance to gastrointestinal transit, capacity to colonise the intestine, safety properties and prebiotics metabolism, *Lb. brevis* C1Lb21, *Lb. plantarum* G1Lb5 and *Lb. pentosus* G4Lb7 are promising candidates for their use in a new functional cheese. The development of a symbiotic commercial cheese with lactulose prebiotic to support LAB growth and induce SCFA production could positively impact on human health.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.108388.

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