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Probiotic Potential of Lactic Acid Bacteria from Traditional Fermented Dairy and Meat Products: Assessment by *In Vitro* Tests and Molecular Characterization

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Abstract

The aim of the present study was to evaluate the probiotic potential of lactic acid bacteria (LAB) isolated from Greek traditional fermented products. A series of *in vitro* tests that included survival in simulated gastrointestinal conditions (resistance to low pH, bile salts resistance and bile salts hydrolysis) and safety assessment (resistance to antibiotics, haemolytic and antimicrobial activity) were performed to select potential probiotic candidates, while *Lactobacillus rhamnosus* GG and *Lactobacillus casei* Shirota were used as reference strains. Initially, a total of 255 isolates of LAB have been recovered and screened for their survival in simulated gastrointestinal tract conditions and 133 isolates that exhibited moderate or good behavior in these tests were subsequently differentiated and characterized at species level with molecular tools. Pulsed Field Gel Electrophoresis was applied for strain differentiation, while species differentiation was based on restriction analysis of the amplified 16S rRNA gene. Specific multiplex PCR assay targeting the *rec*A genes was applied to resolve the species level of the isolates, belonged to *Lb. plantarum* group. From the 133 isolates, 47 different strains were recovered and were assigned to *Lactobacillus sakei* (14), *Lactobacillus curvatus* (4), *Leuconostoc mesenteroides* (4), *Lactococcus lactis* (4), *Lactobacillus casei* group (1), *Lactobacillus brevis* (1), *Lb. plantarum* (10), *Lb. pentosus* (7) and *Lb. paraplantarum* (2). The identified strains with good behavior to the gastrointestinal tract tests were selected and further evaluated for their safety aspect. In conclusion, 19 out of the 47 identified strains were assessed as well-behaved, under simulated gastrointestinal conditions and also considered as safe, possessing thus desirable *in vitro* probiotic properties similar or better to that of the reference strains. These strains may be considered as good candidates for further investigation at *in vivo* and *in situ* studies to assess their potential health benefits and their performance as novel probiotic starters or adjunct cultures.

Keywords: Probiotic; Lactic acid bacteria; Meat; Dairy; Molecular characterization; PFGE; Multiplex PCR

Introduction

The term probiotic is a quite new word meaning "for life" and it is recently used to name bacteria related with positive effects for humans [1] and animals [2]. The first observation of the positive role of some selected bacteria is ascribed to Elie Metchnikoff, the Russian born Nobel Prize holder who was working at the Pasteur Institute at the beginning of the last century. A generally accepted definition of probiotics recognized by the FAO/WHO, proposes that probiotics are "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" [3]. Members of the genera *Bifidobacterium, Lactobacillus, Streptococcus* and *Enterococcus* are the most frequently used probiotics, although members of the genera *Streptococcus* [4] and *Enterococcus* contain some opportunistic pathogens [5,6].

Several beneficial functions have been suggested for probiotic bacteria e.g., vitamin production [7], cholesterol lowering [8], alleviation of lactose intolerance [9], cancer prevention [10], stimulation of the immune system [11], enhancement of bowel motility [12], relief from constipation [13], prevention and reduction of rotavirus and antibiotic associated diarrhea [14]. Some of these benefits have been proved and established, while other have shown a promising potential in animal models, with human clinical studies required to confirm these claims [15]. It's of great importance to mention that the biological effects revealed from probiotic bacteria are strain specific and there is no universal strain that would provide all the suggested benefits, not even strains of the same species [15].

Foods containing probiotic bacteria fall within the category of functional foods, which are defined as foods claimed to have a positive effect on health. Such products are gaining more widespread popularity and approval throughout the developed world, while increased commercial interest has contributed significantly to the development and expansion of this sector of the market [16]. Despite their increasing economic significance, probiotic functional foods are not specifically regulated by European legislation and currently only Japan, the UK, the USA and the Scandinavian countries have accomplished substantial evolution [17].

Traditional fermented foods represent a rich source of microorganisms. Among fermented foods, dairy products are considered to be the major source of probiotic bacteria isolation with numerous studies confirming this theory [17-19]. Although these products have been exploited in depth as both source and carrier of probiotic lactic acid bacteria, research has been conducted with other

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fermented products as well, such as fruits and vegetables [20], table olives [21,22], fermented cereals [23,24] and fermented meat [25,26].

The aim of the current study was to isolate strains from Greek traditional dairy and meat products and to perform a series of *in vitro* tests to assess their probiotic properties. The isolates that exhibited moderate or good properties at *in vitro* tests, were then differentiated and characterized with molecular tools (PFGE, multiplex PCR), as a part of the selection of new probiotic candidates. The results acquired from this study will be employed in further research focusing on the assessment of the technological properties of the isolated strains for the selection of potential adjunct cultures with improved characteristics in fermented meat and dairy products and food industry in general.

Materials and Methods

Isolation of LAB and pre-selection of most promising probiotic strains

Traditional Greek dairy products such as feta cheese, manouri cheese and xerotyri cheese, and traditional meat products such as sausages, fermented sausages from Lefkada region, cured beefs and soutzouki (a dry spicy product) were obtained from local markets in Greece.

Samples of 25 g were weighted aseptically, added to 225 ml quarter strength Ringer's solution (LABM, Lancashire, UK) and homogenized in a stomacher (Stomacher 400 circulator, SEWARD LIMITED, Norfolk, UK) for 60 sec at room temperature. Decimal dilutions were prepared and 1 ml of the sample was mixed on De Man-Rogosa and Sharpe agar (OXOID, Hampshire, UK). MRS Agar was used for selection and quantification of LAB population and was incubated at 30°C for 48-72 h. 20% of the colonies were randomly selected and purified from each sample from the appropriate dilution of the growth medium. Pure cultures were stored at -80°C in MRS broth supplemented with 20% (v/v) glycerol (APPLICHEM, Darmstadt, Germany). Before experimental use, each isolate was sub-cultured twice on the appropriate medium and colonies were checked for purity before use. A total of 255 isolates were recovered from feta cheese (9 isolates), manouri cheese (26 isolates) and xerotyri cheese (30 isolates), as well as from sausages (17 isolates), fermented sausages from Lefkada region (89 isolates), cured beefs (67 isolates) and soutzouki (17 isolates). These isolates as well as, 2 reference strains i.e., *Lactobacillus rhamnosus* GG (ATCC 53103) and *Lactobacillus casei* Shirota (ACA-DC 6002), kindly provided by Prof. E. Tsakalidou, Laboratory of Dairy Research, Agricultural University of Athens, were screened for their probiotic potential with a series of *in vitro* tests (screened for their survival in simulated gastrointestinal (GI) tract conditions). 133 out of 255 isolates that exhibited moderate or good behavior in simulated gastrointestinal conditions were subsequently differentiated and characterized at species level with molecular tools.

Pulsed field gel electrophoresis

Pulsed Field Gel Electrophoresis was performed in order to determine LAB differentiation at strain level. In brief, genomic DNA extraction was performed from all isolates as previously reported [27]. The restriction enzyme *Sma*I (10U) (NEW ENGLAND BIOLABS, Ipswich, MA, USA) was used according to manufacturer recommendations for 16 h. Following digestion, restriction fragments were separated in 1% PFGE grade agarose gel in 0.5mM Tris-Borate buffer on a CHEF-DRIII (BIO-RAD, Hercules, CA, USA) equipment with the following running parameters: 6 V/cm, 1 s initial switching time, 10 s final switching time and 16 h total run at 14°C. Gels were then stained with ethidium bromide (0.5 mg/L) in water for 1 h and distained for 2 h before being photographed with GelDoc system. Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA clusteringwith Bionumerics software, version 6.1 (APPLIED MATHS, Sint-Martens-Latem, Belgium).

Identification and characterization of strains

Following PFGE differentiation, the different isolates were subjected to sequence analysis of V1-V3 region of 16S rRNA gene [27]. DNA was extracted according to Doulgeraki et al. [28] and PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to manufacturer instructions. For the differentiation of *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum*, specific multiplex PCR assay targeting the *rec*A gene was employed, while the sizes of the amplicons were 318 bp for *Lb. plantarum*, 218 bp for *Lb. pentosus*, and 107 bp for *Lb. paraplantarum* [29]. The GenBank closest relative accession numbers for the 16S rRNA gene sequences are given in Table 1 for each strain.

Probiotic tests *in vitro*

Survival under simulated human gastrointestinal (GI) tract: The methods that were used to examine resistance of strains to low pH, resistance to bile salts and bile salts hydrolysis are described below and were performed according to Argyri et al. [22] with slight modifications.

Resistance to low pH: In order to examine resistance of strains to low pH, bacterial cells from overnight cultures (18 h), were harvested by centrifugation (10000 g, 5 min, 4°C), washed twice with PBS buffer (pH 7.2) before being re-suspended in PBS solution, with a pH adjusted to 2.5. Resistance to low pH was assessed in triplicates in terms of viable colony counts and enumerated on MRS agar (OXOID, Hampshire, UK) after incubation at 37°C under stirring conditions, for 0, 0.5, 1, 2 and 3 h, reflecting the corresponding time which food spends in the stomach. The isolates that exhibited final counts ≥ 10^3 cfu/ml or ≥ 10^6 cfu/ml at low pH for 3 hours, were considered to have moderate or good resistance, respectively, to this test and were selected for strain differentiation, characterization and safety assessment tests. For the final selection of the identified strains, the criterion of counts ≥ 10^6 cfu/ml at low pH for 3 hours was set.

Resistance to bile salts: Bacterial cells from overnight cultures (18 h), were harvested by centrifugation (10000 g, 5 min, 4°C), washed twice with PBS buffer, (pH 7.2), before being re-suspended in PBS solution (pH 8.0), containing 0.5% (w/v) bile salts (OXOID, Hampshire, UK). Resistance to bile salts was assessed in triplicates in terms of viable colony counts and enumerated after incubation at 37°C under stirring conditions, for 0, 1, 2 and 4 h reflecting the corresponding time that food spends in the small intestine. The isolates that exhibited final counts ≥ 10^3 cfu/ml or ≥ 10^6 cfu/ml in bile salts for 4 hours, were considered to have moderate or good resistance, respectively, to this test and were selected for strain differentiation, characterization and safetyassessment tests. For the final selection of the identified strains, the criterion of counts ≥ 10^6 cfu/ml in bile salts for 4 hours was set.

Bile salts hydrolysis: Fresh bacterial cultures were streaked on MRS agar in triplicates containing 0.5% taurodeoxycholic acid-TDCA (SIGMA, Missouri, USA). The hydrolysis effect was evaluated by different colony morphology (partial hydrolysis) in comparison to the control MRS plates, after 48 h of anaerobic incubation at 37°C.

Safety assessment of the selected strains: The strains that had good behavior to the aforementioned GI tract tests were selected and further evaluated for their potential haemolytic activity, antimicrobial activity and resistance to antibiotics according to Argyri et al. [22].

Strain	Closest Relative	GenBank accession number of the closest relative	Identity
Lactococcus lactis T4	*Lc. lactis* subsp. *lactis*	LC153549	100%
Lactococcus lactis T12	*Lc. lactis*	KU248781	100%
Lactococcus lactis T17	*Lc. lactis* subsp. *lactis*	LC153549	100%
Lactococcus lactis L167	*Lc. lactis* subsp. *cremoris*	LC129537	100%
Leuconostoc mesenteroides T15	*Lc. mesenteroides*	KT722833	99%
Leuconostoc mesenteroides T25	*Lc. mesenteroides*	KT722833	99%
Leuconostoc mesenteroides L246	*Lc. mesenteroides*	KT722833	100%
Leuconostoc mesenteroides L258	*Lc. mesenteroides*	KT722833	100%
Lactobacillus plantarum L32	*Lb. plantarum*	KX082943	100%
Lactobacillus plantarum T48	*Lb. plantarum*	KX074205	99%
Lactobacillus plantarum T71	*Lb. paraplantarum*	LC090476	100%
Lactobacillus plantarum T75	*Lb. plantarum*	KR078354	100%
Lactobacillus plantarum L79	*Lb. paraplantarum*	LC090476	100%
Lactobacillus plantarum L81	*Lb. plantarum*	KR025393	100%
Lactobacillus plantarum L119	*Lb. plantarum*	KX074205	100%
Lactobacillus plantarum L125	*Lb. plantarum* subsp. *plantarum*	KP763941	100%
Lactobacillus plantarum L132	*Lb. plantarum* subsp. *plantarum*	KP763941	100%
Lactobacillus plantarum T571	*Lb. paraplantarum*	LC090476	100%
Lactobacillus pentosus L33	*Lb. plantarum*	KP763939	100%
Lactobacillus pentosus L41	*Lb. plantarum*	KR025402	100%
Lactobacillus pentosus L45	*Lb. plantarum*	KX082943	99%
Lactobacillus pentosus L49	*Lb. plantarum*	KP764187	100%
Lactobacillus pentosus L83	*Lb. plantarum*	KX074205	99%
Lactobacillus pentosus L138	*Lb. plantarum* subsp. *plantarum*	KP763946	100%
Lactobacillus pentosus L219	*Lb. plantarum*	KP887104	100%
Lactobacillus paraplantarum L207	*Lb. plantarum*	KX082940	99%
Lactobacillus paraplantarum L247	*Lb. plantarum*	KT722828	99%
Lactobacillus sakei L9	*Lb. sakei*	EU755262	99%
Lactobacillus sakei L31	*Lb. sakei*	KT351714	99%
Lactobacillus sakei L35	*Lb. sakei*	LC129551	100%
Lactobacillus sakei L129	*Lb. sakei*	EU755262	99%
Lactobacillus sakei L155	*Lb. sakei*	LC129551	99%
Lactobacillus sakei L156	*Lb. sakei*	KT351714	99%
Lactobacillus sakei L157	*Lb. sakei*	LC129551	100%
Lactobacillus sakei L160	*Lb. sakei*	LC129551	99%
Lactobacillus sakei L164	*Lb. sakei*	LC129551	99%
Lactobacillus sakei L165	*Lb. sakei*	LC129551	100%
Lactobacillus sakei L168	*Lb. sakei*	LC129551	99%
Lactobacillus sakei L171	*Lb. sakei*	LC129551	99%
Lactobacillus sakei L197	*Lb. sakei*	LC129551	100%
Lactobacillus sakei L205	*Lb. sakei*	KT351714	99%
Lactobacillus curvatus L209	*Lb. curvatus*	LC129556	100%
Lactobacillus curvatus L245	*Lb. curvatus*	LC129556	100%
Lactobacillus curvatus L248	*Lb. curvatus*	LC129556	100%
Lactobacillus curvatus L363	*Lb. curvatus*	LC129556	100%
Lactobacillus brevis T47	*Lb. brevis*	KT285603	100%
Lactobacillus casei group T26	*Lb. casei*	KU315405	99%

Table 1: Species identification, Results obtained after sequencing of the variable V1-V3 region of the 16S rRNA genes as well as the closest relative and its accession number from the GenBank.

Antimicrobial activity against pathogens: All strains were tested in triplicates for antimicrobial activity against 3 *Listeria monocytogenes* strains (FMCC-B-129, FMCC-B-131, FMCC-B-133), 1 *Salmonella enterica* subsp. *enterica* serovar Enteritidis strain (FMCC B-56 PT4), 1 *Staphylococcus epidermidis* strain (FMCC B-202 C5M6), kindly provided by the laboratory of Food Microbiology and Biotechnology (Food Microbiology Culture Collection of the Agricultural University of Athens) 1 *Escherichia coli* strain (ATCC 25922) and 1 *Staphylococcus aureus* strain (ATCC-25923). Fresh overnight bacterial MRS culture supernatants of the tested LAB strains were harvested by centrifugation (10000 g, 15 min, 4°C), adjusted to pH 6.5 and then sterilized by filtration (0.22 μm). The cell free culture supernatants (CFCs) of the tested LAB strains were screened for antimicrobial activity using the well diffusion assay. Initial inoculum of 10^6 cfu/ml of the target strain

was incorporated into soft agar (1% w/v) plates of the appropriate for the target strain medium. CFCs (50 µl) were transferred in holes (5 mm diameter) drilled into the agar. The plates were incubated at 37°C and were examined for growth-free zones (diameter) around the well. The antibiotic kanamycin (30 µg/ml) was used as positive control, while MRS broth adjusted to pH 6.5 was the negative control.

Haemolytic activity: Fresh bacterial cultures were streaked on Columbia agar plates (OXOID, Hampshire, UK) in triplicates containing 5% (w/v) of horse blood and incubated for 48 h at 30°C. Blood agar plates were examined for signs of α-haemolysis (green-hued zones around colonies), β-haemolysis (clear zones around colonies) or γ-haemolysis (no zones around colonies).

Antibiotic resistance: For testing antibiotic resistance of the strains selected by the previous phenotypic tests, microdilution broth was used. Bacterial strains were inoculated (1% v/v) in MRS broth supplemented with antibiotics (vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, tetracycline, chloramphenicol) at various concentrations (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024 µg/ml) and examined in triplicate for growth in a microplate reader (OD 610 nm) following an incubation period of 24 h at 30°C.

Results and Discussion

Isolation of LAB, strain differentiation and characterization

The total of 255 isolates that were recovered, were initially screened for their survival in simulated gastrointestinal conditions and the 133 isolates that exhibited moderate or good behavior, were subsequently differentiated and characterized at species level.

The application of PFGE analysis to the 133 isolates resulted in 47 different fingerprints (Figure 1). The cluster analysis of PFGE *Sma*I digestion fragments of the LAB isolates showed two major clusters as it seen on Figure 1. From the two clusters, the upper cluster was found to contain 4 strains belonging to *Ln. mesenteroides,* which were recovered from both dairy and meat samples. On the other hand, no specific information could be provided from the clustering in the second branch, which included isolates of different genus and species recovered from different sources of dairy and meat products. The sequence analysis of the different 47 strains, revealed the presence of *Lactobacillus sakei* (14), *Lactobacillus curvatus* (4), *Leuconostoc mesenteroides* (4), *Lactococcus lactis* (4), *Lactobacillus casei* group (1), *Lactobacillus brevis* (1) and *Lactobacillus plantarum* group (19). For the differentiation of isolates assigned to *Lb. plantarum* group, multiplex PCR assay targeting to the *rec*A gene was employed and resulted in 10 *Lb. plantarum*, 7 *Lb. pentosus* and 2 *Lb. paraplantarum* strains. The prevalence of different identified species detected in the different samples is summarized in Table 2.

Species	Source		
	Dairy samples	Meat samples	Total
Lactobacillus sakei		14	14
Lactococcus lactis	3	1	4
Lactobacillus curvatus		4	4
Leuconostoc mesenteroides	2	2	4
Lactobacillus casei group	1		1
Lactobacillus brevis	1		1
Lactobacillus plantarum	4	6	10
Lactobacillus pentosus		7	7
Lactobacillus paraplantarum		2	2
Total	11	36	47

Table 2: Source of the selected strains. LAB strains isolated from different dairy and meat products and selected according to their probiotic potential.

The aforementioned species are related with the microbiota of spontaneous fermentation of dairy and meat products in previous studies. More specifically, *Lc. lactis*, *Ln. mesenteroides* and *Lb. plantarum* are identified as the most frequently isolated species in fermented dairy products [30,31]. Furthermore, *Lb. casei* group strains as well as *Lb. brevis* are recovered from dairy samples in previous studies [30,31]. It has to be noted that *Leuconostoc* strains naturally play an important role in the development of flavor in fermented products, although they display a weak competitive ability during milk fermentation, because of their complex nutritional necessities [30]. Several researchers have investigated the biodiversity of fermented meat products and most of the studies reveal that *Lb. sakei* and *Lb. curvatus* are the predominant microflora of such products [32-35]. Additionally, *Ln. mesenteroides* [32,36,37] and *Lc. lactis* [38] are detected in fermented meat samples. Other species isolated include *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* [34-40].

In vitro tests related to probiotic potential

Probiotics must remain viable during their passage in the gastrointestinal tract in population levels of 10^6-10^7 cfu/g in order to deliver the health benefits [22]. The acid environment of the stomach and the inhibitory effects of bile salts secreted in the duodenum are the major obstacles against probiotic survival. The *in vitro* evaluation of the survival of the potential probiotic strains in simulated GI tract conditions may only be necessary in predicting the actual *in vivo* survival of a strain when consumed in a non-protected way [19].

Survival under simulated human gastrointestinal tract conditions: The isolates that exhibited final counts ≥ 10^3 cfu/ml at low pH for 3 hours and ≥ 10^3 cfu/ml in bile salts for 4 hours were considered to have moderate or good resistance to these tests and were selected for strain

Strain	MICs[a] (µg/ml)						
	V	G	K	S	E	T	C
Lc. lactis T4	32[R]	32	32	32	<1	2	1
Lb. plantarum L32	512	32[R]	128[R]	256	<1	16	1
Lb. plantarum T48	512	8	32	16	<1	16	1
Lb. plantarum T71	≥ 1024	32[R]	64	256	<1	128[R]	1
Lb. plantarum T73	≥ 1024	32[R]	32	256	<1	16	1
Lb. plantarum L79	512	4	32	32	<1	8	1
Lb. plantarum L119	≥ 1024	4	16	256	<1	128[R]	1
Lb. plantarum L125	≥ 1024	8	32	64	<1	32	2
Lb. plantarum L132	512	32[R]	128[R]	256	<1	32	1
Lb. plantarum T571	≥ 1024	2	32	64	<1	32	1
Lb. pentosus L33	≥ 1024	16	32	64	<1	8	1
Lb. pentosus L41	≥ 1024	32[R]	64	256	<1	64	1
Lb. pentosus L45	512	4	32	64	<1	8	1
Lb. pentosus L49	≥ 1024	4	32	64	<1	8	1
Lb. pentosus L83	≥ 1024	4	32	32	<1	8	1
Lb. paraplantarum L207	512	8	64	64	<1	8	0.5
Lb. sakei L35	512	8	32	64	<1	4	0.5
Lb. sakei L165	256	16	32	64	<1	2	1
Lb. brevis T47	64	8	32	16	<1	16[R]	1
Lb. casei Shirota	≥ 1024	16	4	128[R]	2[R]	16[R]	8[R]
Lb. rhamnosus GG	≥ 1024	16	256[R]	32	<1	2	4

[R]Resistant according to the EFSA's breakpoints [54]; V: vancomycin, G: gentamycin, K: kanamycin, S: streptomycin, E: erythromycin, T: tetracycline, C: chloramphenicol. [a]MIC: minimum inhibitory concentration

Table 3: Antibiotic resistance of the 19 selected strains. MIC values for the selected strains according to the breakpoints set by EFSA [54].

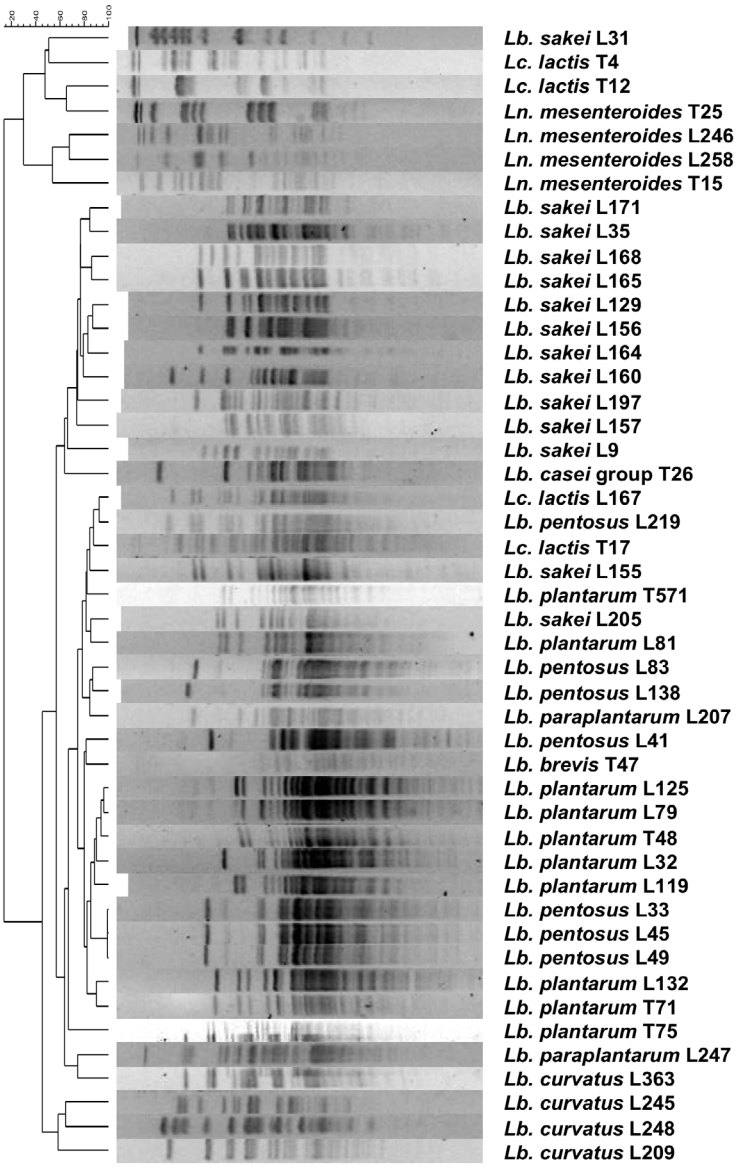

Figure 1: Cluster analysis of PFGE results. Smal digestion fragments of the lactic acid bacteria recovered from different dairy and meat samples calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

differentiation, characterization and safety assessment tests. Since bile salts resistance test resulted in <3 log reduction for the total of isolates, the main criterion for the selection of the isolates was the resistance to low pH. As a result, 133 isolates out of 255 met both criteria and were further characterized with molecular tools, resulting to 47 identified strains that were selected and further studied.

Resistance to low pH: 133 isolates out of 255, exhibited final counts ≥ 10^3 cfu/ml at low pH for 3 hours. Regarding the 47 identified strains, the viable counts of most *Lb. plantarum* and *Lb. pentosus* strains showed higher resistance to low pH than *Ln. mesenteroides* and most of *Lc. lactis* strains which their final counts indicated the lowest resistance (10^3 cfu/ml). Furthermore, variability in the final viable counts of *Lb. sakei* strains after exposure to low pH for 3 hours was observed. Totally, 19 strains showed good resistance (>6 log cfu/ml) to low pH (*Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83) (Figure 2). These results are in agreement with other studies, where *Lactobacillus* strains are able to maintain their viability when exposed to low pH values (2.5-4.0) [19,22], while other researchers have reported strains of *Lb. plantarum* with lower ability to survive at low pH [24] *In vitro* assays propose to select acid resistant strains including exposure to pH-adjusted PBS [19,25], incubation in gastric juice [41,42], or the use of GIT simulator [43]. The survival of potential probiotic strains to stomach juice is determined by their intrinsic resistance to the hostile environment, but also on the ingestion vector and its contents. As a result, foods with a high level of fat and the presence of certain proteins in the food may provide additional protection to the bacteria from gastric acid and therefore increase survival to gastric transit [44]. In the current study, pH value of 2.5 was used, in order to select potential probiotic strains. Such low pH value is very selective and although it is not the most common pH

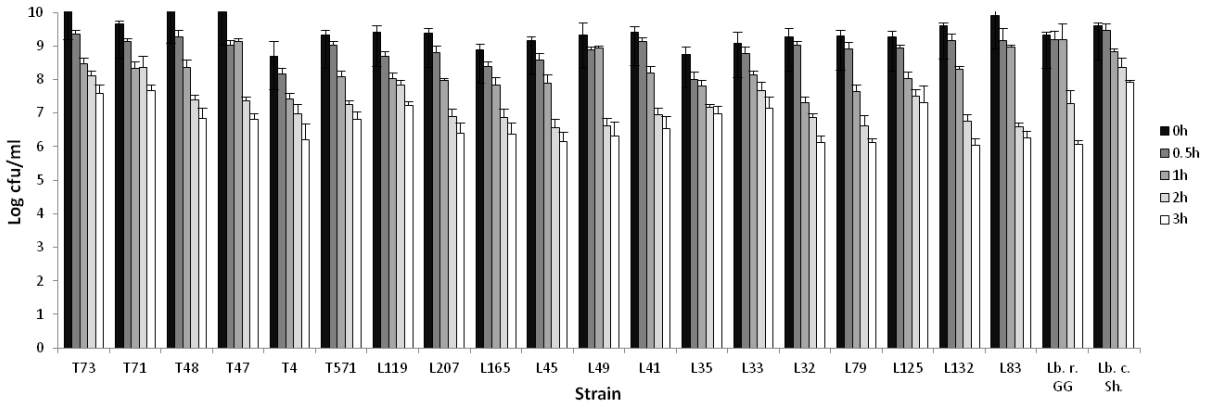

Figure 2: Results for low pH resistance for the selected strains. Resistance to low pH after 0, 0.5, 1, 2 and 3 h of the selected strains *Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83 and the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG (Error bars indicate standard deviation from three replications).

Strains	Test				
	Low pH (SR%)	Bile Salts (SR%)	Bile Salts Hydrolase	Haemolytic activity	Antibiotic Resistance
Lc. lactis T4	71.46	90.02	0	γ	V
Lb. plantarum L32	66.20	94.20	1	γ	G,K
Lb. plantarum T48	67.89	98.81	1	γ	-
Lb. plantarum T71	79.48	97.36	1	γ	G,T
Lb. plantarum T73	74.51	96.17	1	γ	G
Lb. plantarum L79	65.88	93.90	1	γ	-
Lb. plantarum L119	76.91	95.95	0	γ	T
Lb. plantarum L125	78.94	96.49	1	γ	-
Lb. plantarum L132	62.81	78.65	1	γ	G
Lb. plantarum T571	73.18	94.87	0	γ	-
Lb. pentosus L33	78.83	93.96	0	γ	-
Lb. pentosus L41	69.43	93.35	0	γ	G
Lb. pentosus L45	67.18	91.40	0	γ	-
Lb. pentosus L49	67.74	94.22	0	γ	-
Lb. pentosus L83	63.10	91.46	1	γ	-
Lb. paraplantarum L207	68.20	87.11	0	γ	-
Lb. sakei L35	79.86	94.14	1	γ	-
Lb. sakei L165	71.65	87.27	0	γ	-
Lb. brevis T47	69.93	98.63	0	γ	T
Lb. casei Shirota	82.54	98.18	0	γ	S,E,T,C
Lb. rhamnosus GG	65.11	99.26	0	γ	K

V: Vancomycin; G: Gentamycin; K: Kanamycin; S: Streptomycin; E: Erythromycin; T: Tetracycline; C: Chloramphenicol. [a]Survival rate after 3 h in low pH; [b]Survival rate after 4 h in bile salts

Table 4: Results from all tests in vitro for the 19 selected strains. Detailed results from the strains with probiotic potential according to *in vitro* tests in comparison with the reference strains *Lb. casei* Shirota and *Lb. rhamnosus* GG.

value encountered in the stomach, it guaranties the isolation of the very acid-tolerant strains [25].

Resistance to bile salts: The majority of the isolates were found to be highly resistant to bile salts even after 4 hours of exposure. Amongst the 47 identified strains, the viability of 40 strains was retained with minor reduction in viable counts (<1 log cycle), while 7 strains (*Lb. sakei* L168, L165, L155, *Lb. curvatus* L363, *Lb. casei* group T26, *Lb. plantarum* L132 and *Lb. paraplantarum* L207) showed approximately a reduction of <2.5 logs after 4 h of exposure to bile salts.

Tolerance to bile is one of the most essential attributes for probiotic bacteria, as it ascertains their ability to survive in the small intestine, and accordingly their ability to play a functional role as probiotics [45]. Bile response is a complex phenomenon, involved a variety of processes. Active efflux of bile salts/acids, bile salt hydrolysis and changes in the design/composition of cell membrane and cell wall, seem to be the most basic bile-specific mechanisms for resistance in *Lactobacillus* species [45].

Suggested concentration of bile salts for probiotics is between 0.15-0.5%, as it is the range of the physiological concentrations that are met in the GIT [46]. It has to be noted that, the majority of the strains survive well in such bile conditions, suggesting a potential recovery of the initial levels during the passage of the small intestine [19]. Furthermore, studies point out the huge variability in bile resistance that can be encountered within a species or genus [47], revealing that bile tolerance is a strain-dependent feature and tolerances of species cannot be universal [48].

Bile salts hydrolysis: Concerning bile salt hydrolysis (BSH), 11 strains demonstrated partial bile salt hydrolase activity, recorded as differentiated colony morphology on TDCA-MRS agar when compared to the control MRS agar plates. These strains were *Lb. plantarum* L132, L125, L81, L32, T48, T71, T73, *Lb. pentosus* L83, *Lb. sakei* L35 and L168 and *Lc. lactis* T12. The rest of the tested strains did not exhibit bile salt hydrolase activity, while the growth of 2 strains (*Lb. curvatus* L363, *Ln. mesenteroides* T25) was completely inhibited in the presence of 0.5% (w/v) taurodeoxycholic acid.

There are many studies confirming that BSH activity of probiotics is associated with hypocholesterolemic effect [49,50]. BSH-active probiotic strains exert the aforementioned effect through deconjugation that leads to decreased solubility and lower reabsorption of bile salts and in the excretion of larger quantities of free bile acids in feces. Complementary, deconjugation of bile salts could result in a decrease in serum cholesterol to substitute that misplaced in feces or by decreasing the cholesterol solubility, following absorption of cholesterol through the intestinal lumen [50]. Furthermore, microbial BSH function in the detoxification of bile salts, increase the intestinal survival and persistence of producing strains and possibly the profitable effects related to the strain [51]. On the other hand, there is still essential work to be carried out on BSH activity, concerning its mechanism of action in order to prevent other risks that may be caused by the excessive use of probiotics, including sepsis or colon cancer due to the secondary bile salts that are produced [52].

Safety assessment

Antimicrobial activity against pathogens: None of the supernatants of the selected LAB strains and the 2 reference probiotic strains obtained at adjusted pH of 6.5, inhibited the growth of the pathogenic strains tested (3 *Listeria monocytogenes*, 1 *Salmonella enterica* subsp. *enterica* serovar Enteritidis, 1 *Staphylococcus epidermidis*, 1 *Escherichia coli* and 1 *Staphylococcus aureus*) by the use of well-diffusion assay, leading to the assumption that no bacteriocin-like action exists. These results are in accordance to previous studies [19,22,53].

One of the functional properties involved in the characterization of probiotic bacteria is the capability of producing antimicrobial compounds such as organic acid, short chain fatty acids and bacteriocins [22]. Antimicrobial ability of probiotics is also associated with the enhancement of the intestinal barrier function [46]. Nonetheless, the *in vitro* production of antimicrobial substances alone, cannot provide us with reliable outcomes concerning the probiotic behavior *in vivo* [46].

Haemolytic activity: Absence of haemolytic activity is considered as safety criterion for the selection of a probiotic strain. In our study, none of the selected examined strains exhibited α- or β-haemolytic activity, when grown in Columbia blood agar, whereas all strains were γ-haemolytic (no haemolysis). These results are similar with previous observations where all of the tested strains [54,55] or most of them are γ-haemolytic [19,22].

Antibiotic resistance: The Minimum Inhibitory Concentrations (MICs) detected for the selected strains and the 2 reference probiotic strains, are presented in Tables 3 and 4. Strains are considered resistant when they exhibit MIC values higher than those established by the European Food Safety Authority [56]. Variable susceptibility to antibiotics was observed, according to the breakpoints set by EFSA (2012), even for strains of the same species. All LAB strains showed resistance to vancomycin, similarly to the findings of previous reports [19,22,57], although a specified breakpoint is absent for these genus strains. 5 strains were found to be resistant to gentamycin and 4 to tetracycline, including the reference strains. Lower resistance to erythromycin and chloramphenicol was observed for the majority of the tested strains with the reference strain *Lb. casei* Shirota to be the only resistant for both antibiotics. For kanamycin and streptomycin moderate susceptibility was exhibited with 3 strains to be resistant to kanamycin and 1 to streptomycin, despite the fact that MIC's were not low enough.

The antibiotic resistance of potentially probiotic bacteria is controversial and various opinions have been stated so far. For instance, resistance to specific antibiotics might be desirable for some probiotic strains that are involved in antibiotic-induce diarrhea [58]. On the other hand, LAB as probiotics enter human intestines in large numbers and are able to interact with the intestinal microbiota and therefore, they have the potential to transfer genes to other bacteria, even to pathogenic ones [59]. For safety reasons, the resistance observed to specific antibiotics has to be chromosomally encoded and not inducible or transferable. As accepted by EFSA [60], intrinsic resistance and resistance due to mutation of chromosomal genes exerts low risk of horizontal dissemination and such probiotic strains should be acceptable for food consumption, whereas acquired resistance mediated by added genes may confer a risk for public health [61].

In conclusion, certain strains were found to possess desirable probiotic properties *in vitro*. In more detail, 19 strains (*Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83) were found to have desirable probiotic properties alike or superior of the 2 reference probiotic strains examined, too. The selected strains are good candidates for further investigation with *in vivo* and *in situ* studies, to elucidate their potential health benefits and their performance as novel probiotic starters and adjunct starters in food fermentation processing.

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Conflicts of interest

The authors declare no conflict of interest.

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