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ORIGINAL ARTICLE

Safety, probiotic and technological properties of *Lactobacilli* isolated from unpasteurised ovine and caprine cheeses

Dobroslava Bujnakova[1] · **Eva Strakova**[1]

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Abstract Eleven *Lactobacillus plantarum* from Slovak ovine and caprine lump and stored cheeses, and from four commercial probiotic and yogurt cultures *(Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*) identified using a Maldi-TOF MS analysis were screened in vitro for selected aspects correlated with safety (antibiotic susceptibility patterns, biochemical and haemolytic activity, presence of genes responsible for biogenic amines production), functional traits (including acid, bile tolerance and antimicrobial activity), ecological roles (ability to produce biofilms), and technological applications (acidification and milk coagulation capacity) for assurance of their quality and diversity. The antibiotic susceptibility showed two *L. plantarum* strains, 19l5 and 18l4, with the presence of the non-wild-type ECOFFs (epidemiological cut-off) for clindamycin and/or gentamicin. All these strains expressed a high acid tolerance at pH 2.5 after a 4 h exposure (bacteria viability varied between 60% and 91%), and bile resistance at 0.3% oxgall ranged from 60% to 99% with no haemolytic activity. Three wild *L. plantarum* strains, 17l1, 16l4, 18l2, had no harmful metabolic activities, and formed strong biofilms that were measured by a crystal violet assay. Simultaneously, the acid cell-free culture supernatant (ACFCS) from *L. plantarum* 18l2 had a marked inhibitory effect on the viability of the pathogens as evaluated by flow-cytometry, and also exhibited fast acidification and milk coagulation. As a result, we conclude that *L. plantarum* 18l2 can be included as part of the created lactobacilli collection that is useful as a starter, or starter adjunct, in the dairy industry, due to its desirable safety and probiotic characteristics, together with rapid acidification capacity compared with other investigated strains from commercially accessible products.

Keywords *Lactobacillus plantarum* · Maldi biotyper · MSP dendrogram · β-Glucuronidase · *Escherichia coli* · *Staphylococcus aureus*

Introduction

After observing that the availability of new engrossing probiotic or starter cultures with the facility to create a manifold of fermented dairy products is currently limited, an effort was made to find potential new strains. Wild lactobacilli strains isolated from unpasteurised dairy products in their natural environment were selected as one of the more competitive options due to their excellent adaptability compared to probiotics isolated from other sources. Moreover, these strains may possess new and unique sensorial properties, and good technological characteristics in comparison with available commercial starter cultures.

Species of the genus *Lactobacillus* are "generally recognised as safe" (GRAS status) due to their long history of safe use as starter cultures in the food industry; their presence in the normal intestinal and urogenital microbiota of humans and animals; and their commercial use as probiotic bacteria (Jamaly et al. 2011). Nonetheless, according to recommendations for the assessment of probiotics, presumptive probiotic or starter cultures should be screened extensively for their functional properties, including acid and bile tolerance, antimicrobial capacity, safety attributes (antibiotic susceptibility patterns, haemolytic and biochemical activity, presence of genes responsible for biogenic amines production) (FAO/WHO 2002; FAO/WHO 2006; Belicová et al. 2013) and

✉ Dobroslava Bujnakova
dbujnak@saske.sk

[1] Institute of Animal Physiology, Slovak Academy of Sciences, Soltesovej 4/6, 040 01 Kosice, Slovak Republic

ecological roles (ability to produce biofilms) (Ortu et al. 2007). Actually, Salvetti et al. (2016) propose combining whole genome sequencing analysis with conventional phenotypic assays with special attention to virulence factors, antibiotic resistance genes, and genes encoding enzymes responsible for undesirable metabolites, and suggest that this approach could become a structured modus operandi, especially for novel strains with only limited or no history of safe use, allowing the correct taxonomic identification of bacterial strains and the accurate evaluation of risk-related gene traits.

For potential applications in the dairy industry, monitoring of acidification capability is required as an important technological property of probiotic starters. In order to reduce incubation time, and related production costs, fast acidification is preferable. Given that this characteristic is not satisfactory in most probiotic strains, efforts to find specific isolates that are adequate for the above-mentioned aspects are receiving special attention. Another possibility is the co-cultivation of a probiotic with the support of starter cultures. However, the antagonistic relationship between the starter and probiotic bacteria can cause significant reductions in the viability of this approach. Therefore, a future perspective would benefit mostly by isolating new probiotic strains that are able to exhibit both health benefits and the required technological characteristics (Mohammadi et al. 2012).

Previous studies have dealt with the common problematic characterisation of wild lactobacilli isolated from traditional Slovak unpasteurised milk products (Berta et al. 2009; Smetanková et al. 2014); however, these studies did not include a comparison with the currently available strains used as probiotics or starter adjuncts.

To achieve our intention of comparing commercial cultures and wild *L. plantarum* isolates from Slovak ovine and caprine lump and stored cheeses, the former were characterised in order to fulfil the basic requirements, including wild-type epidemiological cut-off (ECOFF) antibiotic resistance patterns, absence of haemolytic and harmful biochemical activities, lack of undesirable metabolites such as biogenic amines (histamine, tyramine, putrescine), acid and bile tolerance, antagonistic activity against pathogens, biofilm formation, and acidification capacity, resulting in the most accurate evaluation of their diversity and quality assurance.

Materials and methods

Bacterial isolates and growth conditions

From 32 wild lactobacilli isolates obtained from the Institute of Biotechnology and Food Science, Faculty of Chemical and Food Technology (Slovak University of Technology, Bratislava, Slovakia) and the Dairy Research Institute (Žilina, Slovakia), as well as those recovered from ovine lump cheeses ($n = 21$), ovine stored cheeses ($n = 7$), and caprine lump cheeses ($n = 4$) from various Slovakian regions, 11 strains identified as *L. plantarum* were further screened. The commercial strains included in the experiments were: *L. plantarum* 299v from Probicus (Generica); *Lactobacillus reuteri* from Lacto Seven (Vitabalans Oy, Finland); *L. reuteri* from Reuflor (Italchimici, Belgium); and *Lactobacillus acidophilus* from Danone yogurt (Czech Republic). All of the lactobacilli isolates were routinely grown in MRS medium (Oxoid, Basingstoke, UK) under anaerobic conditions at 37 °C for 48 h. The pathogens used in the experiments were: *Staphylococcus aureus* (*blaZ* positive strain isolated from unpasteurised ovine milk); and an *invasive Escherichia coli DH5a/pCIB10B* (*ibeA* positive strain), which was kindly provided by J.R. Johnson and B. Johnson from the VA Medical Center (Minneapolis, MN). *S. aureus* was grown in Mannitol agar (Oxoid), whilst McConkey agar was used for *E. coli* (Oxoid). Both were incubated at 37 °C for 24 h.

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry bacterial identification

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (Maldi-TOF MS) was performed with a Microflex LT instrument (Bruker Daltonik, Leipzig, Germany) as described by Bessede et al. (2011). To identify the microorganisms, the raw spectra obtained for each isolate were imported into BioTyper software, Version 3.0 (Bruker Daltonik) and compared with the reference spectra in the database.

Genotype bacterial identification

Amplification of DNA was carried out using genus-specific primers LbLMA 1-rev and R16–1. The PCR mixture and cycle parameters were set according to Dubernet et al. (2002), and PCR amplification was carried out in a C-1000 Thermal Cycler (Bio-Rad, Hercules, CA). For species identification, *Lactobacilli* were first separated by multiplex PCR (with primers Ldel-7, LU-1′, LU-3′, LU-5, Lac-2) into four groups based on the nucleotide sequences of the 16S–23S rRNA intergenic spacer region and adjacent 23S rRNA gene; the finally selected *L. plantarum* 18l2 was then identified with species-specific primers (Lpla-3 and Lpla-2; ~248 bp amplicon) as described Song et al. (2000) for confirmation of Maldi-TOF MS results.

Phenotype and genotype determination of antibiotic resistance

The minimal inhibitory concentrations (MICs) of *L. plantarum*, *L. reuteri* and *L. acidophilus* strains towards 8 antibiotics were determined by the microdilution method, using the microtiter VetMIC Lact-1 panel for the susceptibility

testing of bacteria (Statens Veterinarmedicinska Anstalt, Uppsala, Sweden) according to the ISO 10932/IDF 223 standard (2010). The MICs values (μg/mL) were interpreted in compliance with the recent FEEDAP (Panel of Additives and Products or Substances Used in Animal Feed) document of the EFSA (European Food Safety Authority) updating the criteria used in the assessment of antibiotic bacterial resistances of human or veterinary importance (EFSA 2012), as well as with the ECOFF values defined by the ACE-ART Project results. MIC values surpassing microbiological breakpoints were additionally verified using MIC strip tests (Liofilchem, Roseto degli Abruzzi, Italy).

PCR reactions to confirm the presence of antimicrobial resistance determinants was carried out by using primers for gentamicin resistance [aac(6′)-aph(2′)-Ia] according to Vakulenko et al. (2003) and *linA* primers (Lina et al. 1999) for lincosamide resistance.

Haemolytic activity

Haemolytic activity was analysed as described by Maragkoudakis et al. (2006). Fresh lactobacilli cultures were streaked onto Columbia agar plates containing 5% (w/v) sheep blood (DISMED, Kysta, Slovakia), and were incubated for 48 h at 30 °C. The blood agar plates were read according to the following criteria: β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).

Acid resistance and bile tolerance

Acid resistance and bile tolerance were analysed according to the method of Anderson et al. (2010) with minor modifications. Cultures of lactobacilli were propagated in MRS broth overnight at 37 °C under anaerobic conditions and then inoculated with a concentration 10^6 cfu/mL into MRS broth with the pH adjusted to 2.5 using hydrochloric acid (HCl) with MRS containing 0.3% oxgall (Difco), and with the normal MRS broth as a control. Survival rates were assessed after 4 h incubation by plating 100 μL appropriately diluted culture onto MRS agar. Quantification was performed after 48 h incubation in the same conditions as previously described. Each determination was conducted in duplicate.

Metabolic activity

Metabolic activities were monitored using the apiZYM system (BioMérieux, Craponne, France) according to Arora et al. (1990), whereas histidine (*hdc*), tyrosine (*tyrdc*), ornithine (*odc)* decarboxylases and agmatine deiminase (*agdi*) were evaluated by a multiplex PCR for the detection of the four genes responsible for the production of biogenic amines (histamine, tyramine and putrescine), as described by Coton et al. (2010).

Biofilm formation assay

The biofilm formation assay used a method modified from that of Toledo-Arana et al. (2001), as previously described by Bujňáková and Kmeť (2012). Biofilm formation capacity was evaluated by the measurement of absorbance changes at λ=570 nm in a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT). The results are shown as the average values of A_{570} (absorbance at λ = 570 nm) from eight replicated measurements ± standard deviation (SD). The results were interpreted according to the following scheme: $A_{570} < 0.1$ – the biofilm production was considered as low; in the case of A_{570} between 0.1–0.2 – the biofilm production was considered as moderate; and in a case of $A_{570} > 0.2$ – the biofilm production was considered as strong.

Preparation of cell-free culture supernatant from lactobacilli cultures

The lactobacilli were adjusted to McFarland Standard 1 suspensions that corresponded to $1.5–3 \times 10^8$ cfu/mL in PBS (phosphate-buffered saline); 1 mL of these suspensions was then inoculated into 9 mL MRS broth and incubated at 37 °C. After 24 h of incubation, cultures were centrifuged at 4000 *g* for 10 min. The supernatants were collected and then filter-sterilised using a 0.22 mm membrane syringe filter (Millipore, Carrigtwohill, Ireland) and 100 μL was inoculated into MRS agar as a control where no live cells were present. This acid cell-free cultured supernatant (ACFCS) was used for the experiment without further treatment because the neutralisation to pH 7.0 using 10 mM NaOH entirely destroyed the activity and the treatment with proteolytic enzymes (2 h at 37 °C in the presence of 1 mg/mL of trypsin or proteinase K) had no effect on the antimicrobial activity that was preliminary screened by an agar well diffusion assay (data not shown).

Detection of lactobacilli antibacterial activity using flow-cytometric dead/live staining analysis

After a preliminary antimicrobial activity evaluation screened by an agar well diffusion assay (data not shown), the ACFCS from the 18l2 isolate was selected based on the best results for a flow-cytometric dead/live staining analysis. For comparison purposes, four commercial probiotic and yogurt cultures including the species *L. plantarum*, *L. reuteri* and *L. acidophilus* were selected, and were evaluated using the same quantitative protocol. *E. coli* and *S. aureus* (about 10^6 cfu/mL) were treated with 10% or 2.5% of ACFCS and incubated at 37 °C for 24 h. Untreated and heat-treated bacteria were used as a positive and negative control. For flow cytometry, the samples were diluted in filtered saline with the addition of an appropriate mixture of microspheres, propidium iodide (PI) and SYTO 9. The measurements were performed using the

FACSCalibur™ (BD, Biosciences) instrument equipped with an air-cooled argon ion laser providing 15 mW at 488 nm combined with a 635 nm red-diode laser, and were then analysed with the BD CellQuest™ Pro Software (BD, Biosciences). All parameters were collected as logarithmic signals. The forward scatter (FSC) photodiode signal was set to E01, while the voltage on the photomultiplier (PMT) tube was set on side scatter (SSC) to 353 mV, FL1 to 460 mV, FL2 to 520 mV, FL3 to 500 mV and FL4 to 800 mV. Unstained and single-stained cells were used for the differentiation of the bacterial cells from debris and a background signal in the corresponding density plots. On this basis, the primary threshold was set to FSC (332 mV) and the secondary to SSC (101 mV). The spectral overlap between the emitted fluorescence was eliminated by adjusting for compensations (FL2–72% FL1; FL3–42% FL2). For each sample, 100,000 events were acquired at low flow rate (12 μL/s). The rate of events in the flow was generally below 3000 events/s. For absolute bacterial counting, 6 μm diameter microspheres at a concentration of 1.0×10^8 beads/mL in deionised water with 2 mM sodium azide (Molecular Probes, Eugene, OR) were used. To distinguish between living and dead populations, cells were stained with two nucleic acid stains: red fluorescence PI and green fluorescence SYTO 9. The absolute counts were determined as a ratio of whole bacteria and/or living or dead bacteria numbers to the numbers of the microspheres and their dilution factor according to the formula:

$$\frac{[(\#\ of\ events\ in\ the\ bacterial\ region) \times (dilution\ factors)]}{[(\#\ of\ events\ in\ the\ bead\ region) \times 10^{-6}]} = bacteria/\mathrm{mL}$$

Acidification capacity

Overnight cultures of lactobacilli strains were inoculated into 10 mL sterile MRS broth (pH 6.9) at a concentration of 10^6 cfu/mL, and cultivated anaerobically at 30 °C. After initial measurement, the pH value was monitored with a pH meter (Jenco Electronics, San Diego, CA) after 6 h, 12 h, 24 h, 36 h and 48 h. Every measurement was conducted in triplicate, and the results shown as the average pH value ± standard deviation (SD). Another assay was performed under the same conditions with sterile cow's milk.

Results and discussion

Maldi-TOF MS bacterial identification

All tested strains reached a BioTyper log (score) >2.3, indicating their highly probable identification at species level. From the dendrogram generated by MALDI Biotyper (Fig. 1), it can be seen that all selected lactobacilli isolates are placed <400 on the y-axis value. A distance level of <500 on the y-axis determines a similarity level of individual strains, and confirms the correct species identification (Sauer et al. 2008). The software also divided the strains into three clusters according to the identified species, together with the corresponding reference strains included in the database. Despite the fact that DNA techniques are considered more accurate for bacterial identification than protein fingerprinting, the available literature indicates that misidentification by Maldi-TOF MS is most probably associated with an insufficient number of reference strains available in the Maldi-TOF MS spectral database, and optimization of extraction protocols for difficult-to-treat samples is undoubtedly important for increased accuracy of identification by Maldi-TOF MS (Bizzini et al. 2011).

Maldi-TOF MS identification has been used routinely in our laboratory from 2010 and the methodology of sample preparation, especially for *Lactobacilli*, is empirically well designed. Moreover, our database is continually updated and currently contains 225 *Lactobacilli* belonging to 14 species.

Genotype bacterial identification

All tested strains gave positive results with genus-specific primers (LbLMA 1-rev and R16–1). Separation by multiplex PCR into four groups based on the nucleotide sequences of the 16S–23S rRNA intergenic spacer region and adjacent 23S rRNA gene showed that 11 strains identified by Maldi-TOF MS as *L. plantarum* belonged to group IV, as described by Song et al. (2000). The finally selected *L. plantarum* 18l2 was subjected to PCR with species-specific primers (Lpla-3 and Lpla-2) gave positive ~248 bp amplicons, which confirmed Maldi-TOF MS results.

Phenotype and genotype determination of antibiotic resistance

According to the EFSA and the FEEDAP panel, all microorganisms used for feed and fermented food production must have a specified susceptibility to reference antibiotics (EFSA 2012). Due to the potential transfer of antibiotic resistance genes, those strains harbouring acquired resistance patterns should not enter the food chain; or, more precisely, they must be excluded from it.

The majority of the 11 wild and commercial isolates included in the present evaluation were susceptible to the selected antibiotics. Two isolates (19l5, 18l4) showed non-wild-type ECOFFs, with MIC values for gentamicin that were higher than 64 μg/mL, and isolate 19l5 was resistant to clindamycin with an MIC value higher than 8 μg/mL (Table 1). Despite the fact that these values are comparable with the transmissible determinants, it is important to also verify them at the molecular level. The PCR results did not confirm a positive

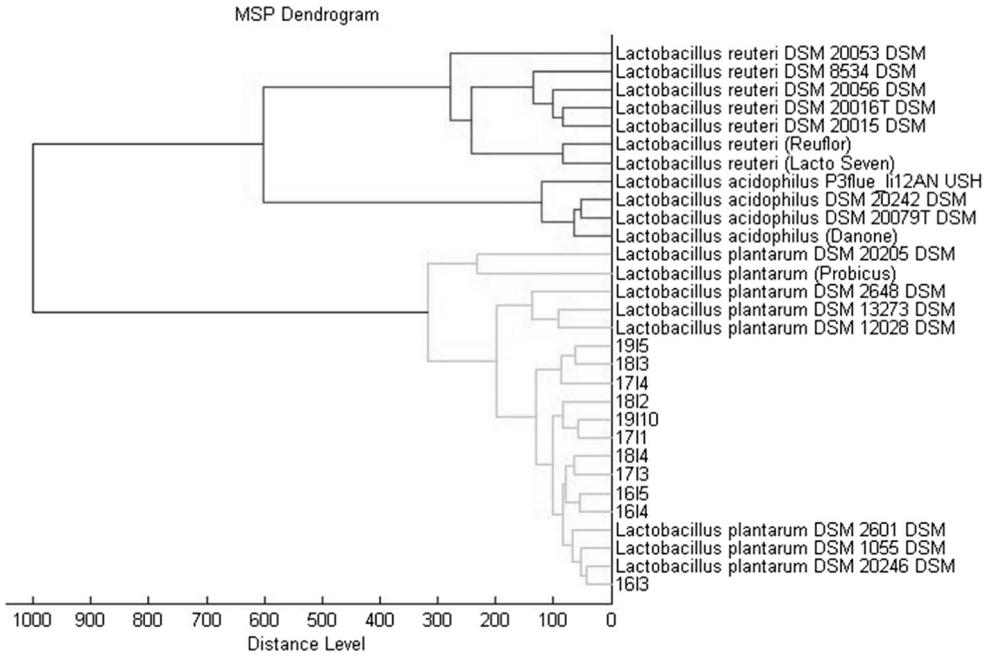


Fig. 1 Main spectrum (MSP) dendrogram of matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectral profiles generated by the MALDI Biotyper. Strains clustering with distance level < 500 could be classified up to species level

correlation between phenotype and genotype resistance, which was possibly caused by the presence of other determinants. For example, a comparable clindamycin resistant phenotype without a genotypic confirmation was observed by Charteris et al. (1998) and Cauwerts et al. (2006). The gene encoding the aminoglycoside-modifying enzyme is very rarely present in lactobacilli, and until now has been described only twice (Tenorio et al. 2001; Bujňáková et al. 2014); both cases were in lactobacilli isolated from the intestinal tract of chickens. Also, a previously published study concerning the antimicrobial susceptibility of microflora from Slovak ovine cheese (Kmeť and Drugdová 2012) did not confirm any antibiotic resistance in wild lactobacilli.

Haemolytic activity

None of the tested strains showed α- and/or β-haemolytic activity when grown in Columbian sheep blood agar. The tested strains showed γ-haemolysis, or, more precisely, showed no haemolytic activity. Given that haemolysin is one

Table 1 Minimum inhibitory concentration (MIC) (μg/mL) of *Lactobacilli* isolates to eight antibiotics determined by the VETMIC Lact 1 system

Strain	Antibiotics							
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm
L. plantarum 17l1	0.5	16	8	1	16	0.12	0.03	4
L. plantarum 17l4	0.5	8	4	0.5	8	0.12	0.03	2
L. plantarum 18l3	1	16	8	2	0.5	0.06	0.03	2
L. plantarum 16l5		2	8	4	8	0.12	0.5	4
L. plantarum 17l3	0.5	4	2	0.5	8	0.12	0.5	2
L. plantarum 18l2	1	16	8	2	0.5	0.06	0.03	2
L. plantarum 16l3	1	16	8	4	1	0.06	0.03	2
L. plantarum 16l4	0.5	32	16	1	8	0.12	0.03	2
L. plantarum 19l5	64	16	8	4	1	0.12	8	2
L. plantarum 19l10	0.5	64	4	0.5	8	0.06	0.03	2
L. plantarum 18l4	64	16	4	2	16	0.12	0.03	4
L. plantarum* 299v *(Probicus)	**0.5**	**8**	**2**	**1**	**8**	**0.25**	**0.12**	**2**
L. reuteri (Lactoseven)	**0.5**	**2**	**1**	**0.5**	**8**	**0.03**	**0.03**	**4**
L. reuteri (Reuflor)	**0.5**	**2**	**0.5**	**0.5**	**8**	**0.03**	**0.03**	**4**
L. acidophilus (Danone)	**1**	**16**	**2**	**2**	**4**	**0.5**	**0.12**	**2**

Gm-gentamicin; Km-kanamycin; Sm-streptomycin; Nm-neomycin; Tc-tetracycline; Em-erythromycin; Cl-clindamycin; Cm-chloramphenicol

In grey, MIC values surpassing the microbiological breakpoints defined by the EFSA (2012)

Bold type in the table represents results of commercially available probiotic and yogurt strains

of the potential virulence factors, whose method of action is the disruption of the cell membranes of red and white blood cells due to the formation of pores in their phospholipid bilayers, this feature should consequently also be taken into account when ascertaining the absence of potential toxicity.

Acid resistance and bile tolerance

Nine presumptive probiotic strains of *L. plantarum* from the ovine and caprine lump and stored cheeses were screened for their survival at a low pH (pH 2.5) and in the presence of bile salts (0.3% oxgall). All strains expressed a high acid tolerance at a pH 2.5 after a 4 h exposure (the bacteria viability varied between 74% and 91%). The viable cell counts decreased by about 0.65–1.62 log cfu/mL for all strains after 4 h incubation at pH 2.5, and the residual counts were > 10^4 cfu/mL. The bile resistance at 0.3% oxgall varied from 89% to 99%. The viable cell counts decreased by about 0.08–0.6 log cfu/mL for all strains after 4 h of incubation at 0.3% oxgall and the residual counts were more than 10^5 cfu/mL. The bacterial viability of the commercial probiotics and yogurt culture varied between 60% and 87% after 4 h incubation at pH 2.5; and 60–85% in the presence of 0.3% oxgall (Table 2). The performance of each of our isolates was comparable, or preferable, to those of the commercial probiotics. Primarily, *L. plantarum* 299v and *L. reuteri* from Reuflor expressed a lower competence for survival under the acid and oxgall conditions used in our experiments.

An acid- and bile-resistant phenotype is considered as one of the essential functional attributes of probiotic strains, which includes the synthesis of a variety of proteins and multiple mechanisms of the acid tolerance response, and therefore allows the bacteria to reach the gastrointestinal tract (GIT) in sufficient quantities (van de Guchte et al. 2002). Since this feature is strain-specific, every potential probiotic strain must be exposed to conditions that simulate those in the upper GIT. Their survival is quantified after the exposure for a time period corresponding with the transit through an upper GIT. A high tolerance to the low pH and bile salts that simulate the conditions of the human GIT is therefore considered as important selection criteria (Klingberg et al. 2005). Specifically, strains that are extremely sensitive to acid and bile are unusable for probiotic or technological applications; and a necessary feature of such strains is at least moderate resistance (Morelli 2007). However, very little or no information is available about how in vitro findings correlate with the in vivo behaviour of consumed bacteria in the GIT (Maragkoudakis et al. 2006).

Metabolic activity

The examined strains displayed various enzyme profiles, as summarised in Table 3. Some of the results (for isolates 17l1, 18l2) were presented in a previously published study (Kološta et al. 2014), where they were evaluated from a technological

Table 2 Effect of acid and bile on viability of *Lactobacilli* isolates. *SD* Standard deviation

Strain	Viable counts of bacteria (log cfu/mL ± SD)			
	pH 2.5		0.3% oxgall	
	t_0 [a]	t_4 [b]	t_0 [a]	t_4 [b]
L. plantarum 17l1	6.77 ± 0.14	6.04 ± 0.10 (89%)	6.33 ± 0.21	6.14 ± 0.17 (96%)
L. plantarum 17l4	6.55 ± 0.22	5.87 ± 0.12 (89%)	6.05 ± 0.12	5.97 ± 0.22 (99%)
L. plantarum 18l3	6.78 ± 0.05	5.45 ± 0.07 (80%)	6.56 ± 0.07	6.45 ± 0.25 (98%)
L. plantarum 16l5	5.99 ± 0.14	4.47 ± 0.11 (74%)	5.75 ± 0.18	5.17 ± 0.10 (89%)
L. plantarum 17l3	6.48 ± 0.22	4.97 ± 0.20 (77%)	6.54 ± 0.02	5.97 ± 0.20 (91%)
L. plantarum 18l2	7.58 ± 0.07	5.98 ± 0.12 (79%)	6.48 ± 0.09	5.78 ± 0.12 (89%)
L. plantarum 16l3	7.41 ± 0.17	5.77 ± 0.12 (78%)	6.41 ± 0.13	5.99 ± 0.18 (93%)
L. plantarum 19l10	6.14 ± 0.25	4.52 ± 0.04 (74%)	6.54 ± 0.15	6.32 ± 0.24 (96%)
L. plantarum 16l4	7.32 ± 0.03	6.67 ± 0.05 (91%)	7.22 ± 0.05	6.97 ± 0.12 (97%)
L. plantarum 299v (Probicus)[c]	5.45 ± 0.24	3.30 ± 0.20 (61%)	5,87 ± 0.19	3.53 ± 0.21 (60%)
L. reuteri (Lactoseven)[c]	6.61 ± 0.13	5.75 ± 0.25 (87%)	6.45 ± 0.26	5.26 ± 0.23 (81%)
L. reuteri (Reuflor)[c]	6.94 ± 0.33	4.15 ± 0.15 (60%)	6.98 ± 0.09	5.43 ± 0.37 (78%)
L. acidophilus (Danone)[c]	6.74 ± 0.24	5.90 ± 0.15 (87%)	6.99 ± 0.32	5.95 ± 0.09 (85%)

[a] t0 - Viable counts (log cfu/mL ± SD) of individual strain at 0 h

[b] t4 - Viable counts (log cfu/mL ± SD) of individual strain at 4 h (% viability of *Lactobacilli* strains)

[c] Results of commercially available probiotic and yogurt strains

Table 3 Enzyme activities of *Lactobacilli* isolates assayed by the apiZYM system

Strains	Enzyme assayed [a,b]												
	Naphtol-AS-BI-phosphohydrolase	Acid phosphatase	Esterase (C4)	Esterase-lipase (C8)	Leu-arylamidase	Val-arylamidase	Cys-arylamidase	α-Galactosidase	β-Galactosidase	β-Glucuronidase [b]	α-Glucosidase	β-Glucosidase [b]	α-Fucosidase
L. plantarum 17l4	20	30	20	20	30	30	5	0	≥40	5	20	≥40	5
L. plantarum 16l5	20	20	20	30	30	≥40	5	0	30	5	10	30	10
L. plantarum 17l3	5	10	20	20	20	≥40	5	0	5	10	5	30	20
L. plantarum 18l3	20	20	10	20	≥40	≥40	10	30	30	0	5	10	5
L. plantarum 16l3	5	5	5	5	≥40	≥40	5	≥40	≥40	10	20	30	5
L. plantarum 19l10	10	20	0	20	≥40	≥40	10	≥40	≥40	5	10	30	10
L. plantarum 16l4	10	10	10	10	≥40	≥40	5	20	≥40	0	0	0	20
L. plantarum* 299v *(Probicus)	**10**	**5**	**5**	**5**	**20**	**20**	**10**	**0**	**20**	**0**	**≥40**	**0**	**0**
L. reuteri (Lacto Seven)	**0**	**20**	**20**	**5**	**20**	**10**	**0**	**20**	**≥40**	**0**	**30**	**0**	**0**
L. reuteri (Reuflor)	**0**	**20**	**5**	**5**	**20**	**10**	**0**	**20**	**≥40**	**0**	**30**	**0**	**0**
L. acidophilus (Danone)	**20**	**20**	**5**	**0**	**30**	**30**	**10**	**0**	**≥40**	**0**	**≥40**	**0**	**0**

[a] Enzyme activity measured as the approximate nanomoles of substrate hydrolysed during 4-h incubation

[b] harmful enzyme activities

In grey *L. plantarum* strains without harmful enzyme activities

Bold type in the table represents results of commercially available probiotic and yogurt strains

point of view concerning mostly the enzymatic activity connected with flavour development and cheese ripening; this study focusses on recommended safety criteria.

From a safety point of view, three types of *L. plantarum* (17l1, 18l2 and 16l4) showed very low or no undesirable enzymatic activity. Another six isolates, which exhibited high β-glucuronidase and β-glucosidase activities associated with detrimental effects in the colon by the release of aglycones and de-conjugating glucuronic acid-conjugated carcinogens (Delgado et al. 2007, 2008), were discarded from further screening.

By contrast, β-galactosidase activity is a desirable feature in probiotic strains. Lactose intolerance (β-galactosidase deficiency) is linked to an inability to break down lactose in the upper regions of the small intestine, which is then utilised by indigenous microbiota (Vrese et al. 2001). The above-mentioned strains had very high β-galactosidase activities (≥40 nmol substrate hydrolysed) that are associated with a positive impact on the alleviation of lactose intolerance (Hussain et al. 2008), and these might therefore be used as a dietary adjunct to aid in moderate lactose intolerance in the gut. One strain (16l4) also showed moderate α-fucosidase activity, which can help in long-term intestinal colonisation (Monteagudo-Mera et al. 2011). In relation to dairy production, arylamidase activity is responsible for flavour development due to the cleavage of single amino acid residues from the oligopeptides (Neelakantan et al. 1999). Moreover, esterase and lipase activity is important in the production of enzyme-modified cheese or for accelerated cheese ripening (Katz et al. 2002).

The enzyme activities of the commercial probiotics were mainly comparable with the tested isolates, except for their α-fucosidase activity, which showed zero values. The undesirable enzyme activities, such as β-glucosidase, β-glucuronidase, α-chymotrypsin and N-acetyl-β-glucosaminidase, were not detected and the advisable β-galactosidase activity reached a high level (≥40 nmol substrate hydrolysed).

Furthermore, the lactobacilli were tested by multiplex PCR for detection of the four genes responsible for the production of three biogenic amines (histamine, tyramine and putrescine), and the results showed no presence of these genes. Since biogenic amines are present in all living organisms, and play a role in synaptic transmissions and blood pressure control, and, in addition, they are precursors to hormones, it is known that their presence in higher doses may cause toxicity. They are produced in a small amount in the GIT, but the main source of these amines is via the food chain (Russo et al. 2012) through bacterial metabolism with possible specific amino acid decarboxylase activities, and, thus, there is a potential to synthesise biogenic amines that could be accumulated in dairy (fermented) products. The use of starter cultures with a lack of decarboxylase activity contributes significantly to the reduction of biogenic amines in food. Hence, this attribute may become the one of the safety aspects in a rigorous preliminary screening using well-characterised starter cultures, in order to exclude those strains with the undesirable potential to produce biogenic amines and minimise the potential health risks to consumers (EFSA 2011).

Biofilm formation

The three lactobacilli isolates (*L. plantarum* 16l4: A_{570} = 0.274 ± 0.022; *L. plantarum* 17l1: A_{570} = 0.295 ± 0.015 and *L. plantarum* 18l2: A_{570} = 0.264 ± 0.025) had the ability to produce strong biofilms on abiotic surfaces ($A_{570} > 0.2$). On the other hand, the commercial strains showed a moderate biofilm forming capacity (*L. plantarum* from Probicus: A_{570} = 0.118 ± 0.024; *L. reuteri* from Lacto Seven: A_{570} = 0.178 ± 0.027; *L. reuteri* from Reuflor: A_{570} = 0.175 ± 0.019; *L. acidophilus* from Danone yogurt: A_{570} =0.149 ± 0.017). This capability of probiotic strains is associated with an immune modulation of the host organism, both at the local and the organism level. Bacterial strains living in a biofilm community are more resistant to external influences due to the production of an extracellular matrix. Biofilm-forming probiotics and the strength of the resident microbiota form an integral part of the mucosal barrier, and thus enhance the colonisation resistance against pathogens (Jones and Versalovic 2009). Borges et al. (2013) suggested this trait as an option for inhibiting pathogenic biofilm formations. The choice of new probiotic strains should therefore include the screening of this feature, and should consider it as essential. Although the biofilm formation on abiotic surfaces cannot definitely replace evaluation of adhesion ability to biotic surfaces, some researchers have shown a statistically significant positive correlation between adhesive power to biotic surfaces, and the ability to form biofilm on abiotic surfaces (Martín et al. 2008; Pompilio et al. 2008, 2010).

Besides, in their research article Arena et al. (2017) highlight the importance of biofilm formation in starter cultures. They note that, although the biofilms formed on food and food processing plants usually spoil the products (Flemming and Wingender 2010), nevertheless, in some manufacture, biofilms are advantageous for food technology (Licitra et al. 2007; Didienne et al. 2012). Lactic acid bacteria biofilms can inhibit spoilage and potentially pathogenic microorganisms (Mariani et al. 2007), improve the properties of the final product, extend its shelf life, and thus contribute to food safety.

Lactobacilli antibacterial activity detection by using flow-cytometric dead/live staining analysis

The representative commercially available lactobacilli and the wild isolates (17l1, 18l2 and 16l4) were initially screened by agar well diffusion assay (data not shown) for the biological nature of the antibacterial compounds that were produced. The use of untreated ACFCS showed it to be the most competent agent against representative Gram-negative bacteria, such as *E. coli*; and for Gram-positive bacteria, such as *S. aureus.* Therefore, the following experiments were carried out using ACFCS in a flow-cytometric dead/live staining analysis, with the application of ACFCSs to those strains with the highest antibacterial activity from the preliminary screening.

The ability to inhibit the growth of pathogenic bacteria varied broadly among the lactobacilli, and, together with adhesion, these functional characteristics are used to select potentially probiotic bacteria. Lactobacilli are able to protect the host organisms against eventual colonisations of pathogenic bacteria (Mego et al. 2005) by using different mechanisms (Fazeli et al. 2004, 2006; Walencka et al. 2008). One possible mechanism is the production of various antimicrobial metabolites, which can be divided into two major groups: low molecular mass compounds, such as organic acids with a broad inhibitory activity; and antimicrobial peptides (called "bacteriocins"), with a generally narrow spectrum of activity (Schoster et al. 2013). Some *Lactobacillus* strains are also able to aggregate with the pathogenic bacteria (Kmeť and Lucchini 1999; Bujňáková and Kmeť 2002; Bujňáková et al. 2004).

The different treatments of the 18l2 culture supernatant allowed us to determine the types of the antibacterial metabolites. The antagonistic activity of *L. plantarum* 18l2 against pathogens could be explained by the production of organic acids, such as lactic acid (whose inhibition was pH-dependent), because the inhibitory effect was entirely destroyed by adjusting the supernatant pH to 7.0, and treatment with catalase, trypsin and proteinase K had no effect on antibacterial activity. Similar results were obtained in a study by Hacin et al. (2008), who tested lactobacilli that produced lactic and acetic acids with an inhibition ability against common porcine pathogens. Hütt et al. (2006) once again found a correlation between decreased pH and the amount of lactic acid produced, along with the intensity of antimicrobial activity of the probiotic strains. De-Keersmaecker et al. (2006) likewise reported that the antimicrobial activity of *Lactobacillus rhamnosus* against *Salmonella typhimurium* was due to a lactic acid accumulation. Arena et al. (2016) noted that antimicrobial activity may even be due to organic acids similar to lactic acid, for example phenyl lactic acid. Furthermore, organic acids could increase the activity of other antibacterial metabolites, which might require acidification and/or acid-mediated cell membrane disruption to exert a visible antagonistic effect. Many other works have documented the fact that the production of organic acids is considered as the main mechanism mediating the lactobacilli antimicrobial activity (Makras et al. 2006; Belicová et al. 2013). The release of organic acids produced by lactobacilli cause an acidification of the cytoplasm and dissipation of the pH gradient, and along with the inhibition of nutrient transport, subsequently also result in bacterial cell death, as suggested by Blom and Mørtvedt (1991).

Two food-borne pathogenic bacteria, *E. coli* and *S. aureus,* which are frequently responsible for food poisoning, were treated with *L. plantarum* 18l2 ACFCS at two different concentrations (2.5 and 10%). Viability was assessed by the use of a Live/Dead BacLight Bacterial Viability and Counting Kit

that contained fluorescent dyes that selectively penetrate the cells depending on the integrity of their cytoplasmic membrane. The nucleic acid stain Syto9 has the ability to permeate all cells; whilst PI is a membrane-impermeable stain that enters just those cells with damaged cell walls. In this way, it is possible to observe the viable populations (Syto9 positive), populations of injured cells (Syto9 and PI positive) and the population of dead cells (PI positive) (Berney et al. 2007). The SYTO 9 and PI stains are excited by the 488 nm spectral line of an argon-ion laser, and the fluorescence exhibited can be detected in the green and red channels, while the background remains non-fluorescent. The cell type, physiological condition and Gram character influence the amount of red-fluorescent staining exhibited by injured and dead bacteria. The viability of the examined pathogens was measured after 24 h of exposure to ACFCSs. Our results showed that the 10% concentration of ACFCS from the chosen isolate 18l2 had a marked antimicrobial effect, in comparison with the 2.5% ACFCS, which had a considerably lower response on the bacterial viability (data not shown). The number of live *S. aureus* in the strain after application of *L. plantarum* 18l2 ACFCS decreased from the original 97.09% (absolute numbers 1.18×10^9 cfu/mL)(Fig. 2a–Q2) to 0.00% (Fig. 2c–Q2). The number of injured *S. aureus* increased from 2.58% in the untreated *S. aureus* (absolute numbers 2.87×10^7 cfu/mL) (Fig. 2a–Q4) to 58.16% in the treated *S. aureus* (absolute numbers 4.19×10^8 cfu/mL) (Fig. 2c–Q4), and the number of dead bacteria increased from 0.01% (Fig. 2a–Q3) to 40.41% in the treated samples (absolute numbers 2.91×10^8 cfu/mL) (Fig. 2c–Q3). The application of the 10% ACFCS from the starter culture of *L. acidophilus* (Danone) showed a similarly marked anti-microbial effect on Gram-positive *S. aureus*, and also caused an increment in the number of injured bacteria from 2.58% in the untreated *S. aureus* to 92.21% (absolute numbers 1.08×10^9 cfu/mL) (Fig. 2d–Q4). The other commercial probiotics induced a decrement of live *S. aureus* from the original 97.09% to numbers in the range of approximately 45–77% (absolute numbers in range 3.26×10^8–7.02×10^8 cfu/mL) (Fig. 2e, f, g–Q2) and an increment of injured bacteria at values ranging from 21% to 52% (absolute numbers were in the range of 1.91×10^8–4.12×10^8 cfu/mL) (Fig. 2e, f, g–Q4).

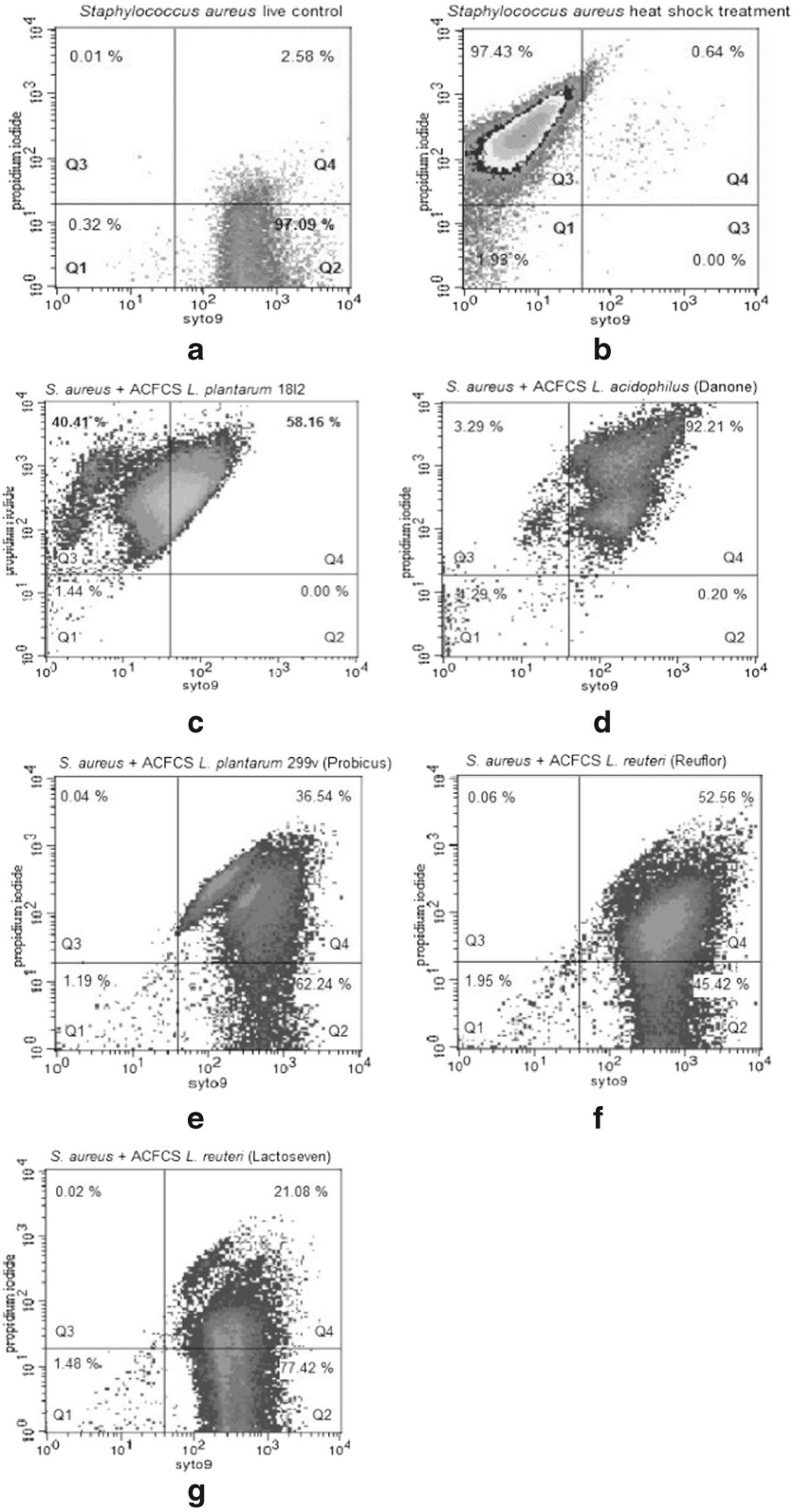
Similarly, *E. coli* viability diminished after application of *L. plantarum* 18l2 from 83.93% (absolute numbers 1.00×10^9 cfu/mL) (Fig. 2h–Q2) to 13.80% (absolute numbers 1.49×10^8 cfu/mL) (Fig. 2j–Q2). The number of injured *E. coli* increased from 12.42% in the untreated sample (absolute numbers 1.29×10^8 cfu/mL) (Fig. 2h–Q4) to 76.02% in the treated *E. coli* (absolute numbers 7.47×10^8 cfu/mL) (Fig. 2j–Q4). The second major inhibitory activity was shown by the *L. acidophilus* from Danone, which caused a decrement of live *E. coli* to 12.94% (absolute numbers 1.32×10^8 cfu/mL)(Fig. 2k–Q2); and *L. reuteri* from Lacto Seven with a loss of the *E. coli* viability to 19.04% (absolute numbers 2.48×10^8 cfu/mL)(Fig. 2n–Q2). Meanwhile, the *L. plantarum* 299v from Probicus and the *L. reuteri* from Reuflor decreased the number of live *E. coli* to 31.60% and 33.32%, respectively (absolute numbers 3.11×10^8 cfu/mL and 4.12×10^8 cfu/mL) (Fig. 2l and m–Q2).

The results confirmed a decrease of *E. coli* viability by almost six times, and an absolute diminution of live *S. aureus* after the application of the 10% ACFCS from the 18l2 isolate. A comparable result was obtained only after the application of a 10% concentration of ACFCS from *L. acidophilus*, commonly in the case of Gram negative *E. coli* and Gram positive *S. aureus*. The aforementioned results indicate that the *L. plantarum* 18l2 ACFCS is a potent antimicrobial agent against *E. coli* and *S. aureus* bacteria. Therefore, it fulfils one of the functional criteria, and could became one of the strategies for suppressing or preventing food contamination and infections.

Acidification capacity

The *L. plantarum* 18l2 isolate reduced the pH of the sterile cow's milk from pH 6.59 to pH 6.0 after 6 h, and to pH 5.25 after 24 h, with a milk coagulation ability after 12 h growth and the advisable lactic acid production (0.22 g 100 g^{-1}) as described in a previous study by Kološta et al. (2014). In the present study, the acidification ability of the above-mentioned strain was compared with the commercially available culture *L. acidophilus* from Danone, which caused a reduction in the pH value from 6.6 ± 0.10 to 6.0 ± 0.05 after 24 h of incubation. The other isolates from the market had a comparable or lower acidification capacity to *L. acidophilus* under the same conditions (*L. plantarum* 299v from Probicus: pH = 5.5 ± 0.21; *L. reuteri* from Reuflor: pH = 5.4 ± 0.14; *L. reuteri* from Lacto Seven: pH = 6.0 ± 0.04). A similar situation was found in the MRS medium, where the best results for our isolate 18l2 were recorded with a reduction in pH value from 6.9 ± 0.24 to 3.5 ± 0.12 after 24 h of incubation. The acidification capacity of the market cultures was less pronounced (*L. plantarum* 299v from Probicus: pH = 5.27 ± 0.21; *L. reuteri* from Reuflor: pH = 4.2 ± 0.14; *L. reuteri* from Lacto Seven: pH = 4.3 ± 0.04; *L. acidophilus* from Danone: pH = 4.6 ± 0.10).

The lactobacilli acidification ability with a strong pH reduction of milk allows researchers to ascertain that the strain is a candidate for a good starter culture in the fermentation process (Kostinek et al. 2007; Oguntoyinbo 2007). Generally, most probiotic strains exhibit poor acidifying and coagulation capacity in milk; however, our isolate 18l2 was a good acidifier along with a reduction of the pH in the range of 3.5–5.25 after 24 h incubation in MRS or sterile cow's milk, together with rapid milk coagulation.


Staphylococcus aureus live control
0.01 %
2.58 %
0.32 %
97.09 %
Q1
Q2
Q3
Q4
propidium iodide
syto9
a
Staphylococcus aureus heat shock treatment
97.43 %
0.64 %
1.93 %
0.00 %
b
S. aureus + ACFCS L. plantarum 18l2
40.41 %
58.16 %
1.44 %
0.00 %
c
S. aureus + ACFCS L. acidophilus (Danone)
3.29 %
92.21 %
7.29 %
0.20 %
d
S. aureus + ACFCS L. plantarum 299v (Probicus)
0.04 %
36.54 %
1.19 %
62.24 %
e
S. aureus + ACFCS L. reuteri (Reuflor)
0.06 %
52.56 %
1.95 %
45.42 %
f
S. aureus + ACFCS L. reuteri (Lactoseven)
0.02 %
21.08 %
1.48 %
77.42 %
g

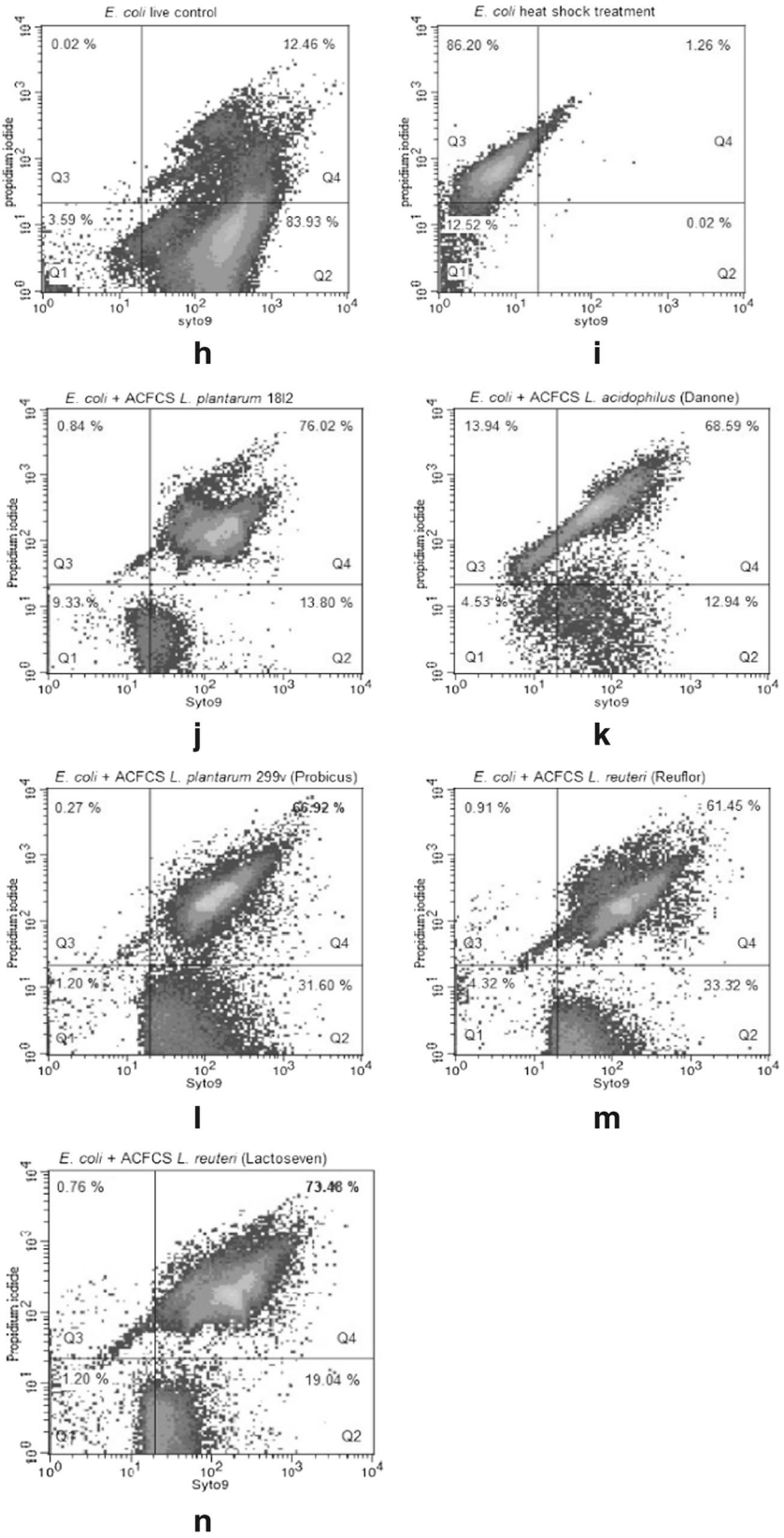


Fig. 2 continued.

◀ **Fig. 2a–n** LIVE/DEAD BacLight Bacterial Viability and Counting Kit in combination with Flow Cytometry for detection of antimicrobial activity of individual lactobacilli ACFCSs. Dual parameters of *Staphylococcus aureus* (**a**–**g**) or *Escherichia coli* (**h**–**n**) density plots are represented by fluorescent Syto 9 vs. PI intensities. *S. aureus* and *E. coli* strains were incubated with and/or without 10% ACFCS of *L. plantarum* 18 l2, *L. plantarum* 299v from Probicus, *L. reuteri* from Reuflor and Lactoseven and *L. acidophilus* from Danone (for more details, see Materials and methods). Quadrants Q1–Q4 represent populations Q1-debris, Q2-live (SYTO 9 positive), Q3-dead (PI positive), Q4-injured (double stained SYTO 9 and PI) bacteria. (A) untreated- live *S. aureus* control; (B) heat treated -dead *S. aureus* control; (H) untreated- live *E. coli* control; (I) heat treated- dead *E. coli* control

Conclusion

Our in vitro results indicate that Slovak ovine and caprine cheeses could be interesting sources for the isolation of bacterial strains, with some of desirable features of market accessible probiotic and yogurt cultures. More specifically, after a comparison with the commercial and wild strains, *L. plantarum* 18l2 showed better in vitro probiotic and safety characteristics, including high acid and bile tolerance; inhibition of known Gram-negative and Gram-positive pathogens; biofilm formation and the lack of acquired antibiotic resistance; harmful metabolic activities, such as α-chymotrypsin, N-acetyl-β-glucosaminidase, β-glucuronidase and β-glucosidase; no presence of the genes responsible for decarboxylation; and no haemolytic activities. *L. plantarum* 18l2 also exhibits features suggesting its survival under gastrointestinal conditions and an attachment to surfaces, and these characteristics indicate its preferable adaptation to different niches, with various stresses, and thereby enable its long-term colonisation. The aforementioned strain likewise demonstrates rapid acidification capacity along with milk coagulation after 12 h growth while simultaneously, as was previously described, this isolate produces a sufficient amount of lactic acid and no acetic acid, all of which is advantageous for its application in the dairy industry. Furthermore, on the basis of the observed metabolic activities, it can be expected to exhibit a suitable flavour production ability. Its inhibition activities against the representative Gram positive and Gram negative bacteria suggests that the above-mentioned strain could participate in the maintenance of microflora and avoid the overgrowth of food-contaminating and pathogenic species in unpasteurised or pasteurised products.

Finally, the above-mentioned strain can be included as part of the created lactobacilli collection, which could be useful as starters or starter adjuncts in the manufacturing of dairy products with validated safety patterns, along with the desired functional traits, and described in a previously published research article (Kološta et al. 2014) with defined important technological properties.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest in this study.

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