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

Foteini G Pavli1,2, Anthoula A Argyri1, Olga S Papadopoulou1, George-John E Nychas5, Nikos G Chorianopoulos* and Chrysoula C Tassou1

1Institute of Technology of Agricultural Products, Hellenic Agricultural Organization-DEMETER, Sof. Venizelou 1, Lycoovissi, 14123, Greece
2Laboratory of Food Microbiology and Biotechnology, Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, 11855, Athens, Greece

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 Shirotia 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 recA 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. paraplanterum (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...
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 properties at in vitro tests to assessed their probiotic properties. The isolates that exhibited 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 ≥ 10^7 cfu/ml or ≥ 10^8 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 ≥ 10^7 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, 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 ≥ 10^7 cfu/ml or ≥ 10^8 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 ≥ 10^7 cfu/ml at low pH for 3 hours was set.

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. paraplantrum, specific multiplex PCR assay targeting the recA 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. paraplantrum [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 ≥ 10^7 cfu/ml or ≥ 10^8 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 ≥ 10^7 cfu/ml at low pH for 3 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].
All strains were tested for 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 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 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. CFUs (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 inculcated (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 Smal 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 *recA* 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.

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 10^6-10^9 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 ≥ 10^6 cfu/ml at low pH for 3 hours and ≥ 10^5 cfu/ml in bile salts for 4 hours were considered to have moderate or good resistance to these tests and were selected for strain isolation.

![Table 2](image)

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

| Species                   | Source           | Dairy samples | Meat samples | Total |
|---------------------------|------------------|---------------|--------------|-------|
| *Lactobacillus sakei*     | 14               | 14            |              | 28    |
| *Lactococcus lactis*      | 3                | 1             | 4            | 7     |
| *Lactobacillus curvatus*  | 4                | 4             |              | 8     |
| *Leuconostoc mesenteroides* | 2             | 2             | 4            | 6     |
| *Lactobacillus casei group* | 1              | 1             |              | 2     |
| *Lactobacillus brevis*    | 1                | 1             |              | 2     |
| *Lactobacillus plantarum* | 4                | 6             | 10           | 16    |
| *Lactobacillus pentosus*  | 7                | 7             |              | 14    |
| *Lactobacillus paraplantarum* | 2             | 2             |              | 4     |
| **Total**                 | 11               | 36            | 47           |       |

| Strain                      | MICs (μg/ml) | V | G | K | S | E | T | C |
|-----------------------------|--------------|---|---|---|---|---|---|---|
| *Lc. lactis* T4             | 32<sup>a</sup> | 32| 32| 32| <1| 2| 1 |   |
| *Lb. plantarum* L32         | 512          | 32| 128<sup>a</sup>| 256| <1| 16| 1 |   |
| *Lb. plantarum* T48         | 512          | 8 | 32| 16| <1| 16| 1 |   |
| *Lb. plantarum* T71         | ≥ 1024        | 32| 64| 256| <1| 128<sup>a</sup>| 1 |   |
| *Lb. plantarum* T73         | ≥ 1024        | 32| 32| 256| <1| 16| 1 |   |
| *Lb. plantarum* L79         | 512          | 4 | 32| 32| <1| 8 | 1 |   |
| *Lb. plantarum* L119        | ≥ 1024        | 4 | 16| 256| <1| 128<sup>a</sup>| 1 |   |
| *Lb. plantarum* L125        | ≥ 1024        | 8 | 32| 64| <1| 32| 2 |   |
| *Lb. plantarum* L132        | 512          | 32| 128<sup>a</sup>| 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| 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| 64| <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<sup>a</sup>| 1 |   |
| *Lb. casei* Shirotia        | ≥ 1024        | 16| 4 | 128<sup>a</sup>| 2<sup>a</sup>| 16<sup>a</sup>| 8<sup>a</sup> |   |
| *Lb. rhhamnosus* GG         | ≥ 1024        | 16| 325<sup>a</sup>| 32| <1| 2 | 4 |   |

<sup>a</sup>Resistant according to the EFSA's breakpoints [54]; V: vancomycin, G: gentamycin, K: kanamycin, S: streptomycin, E: erythromycin, T: tetracycline, C: chloramphenicol. **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].
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 1: Cluster analysis of PFGE results. SmaI 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 (%).](image)
value encountered in the stomach, it guarantees 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, Lb. curvatus L363, Lb. casei group T26, Lb. plantarum L132 and Lb. paraplanterum 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/acid, 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 hypcholesterolemic 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 gentamicin 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 MICs 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.

Acknowledgements

This work has been co-financed by the European Regional Development Fund (ERDF) of the EU and by National Resources under the Operational Program Competitiveness and Entrepreneurship (EPAN II), Action “COOPERATION 2011”. Project “ProbioDairyMeat” (Project No. 115YN_2_571) and by the Hellenic Agricultural Organization-DEMETER, Project “Research and evaluation of quality milk characteristics at responsibility Regions of Western Greece and Peloponnesse”.

Conflicts of interest

The authors declare no conflict of interest.

References

1. Fijan S (2014) Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. Int J Environ Res Public Health 11: 4745-4767.
2. Chaucheyras-Durand F, Durand H (2010) Probiotics in animal nutrition and health. Benef Microbes 1: 3-9.
3. FAO/WHO (2001) Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Cordoba, Argentina.
4. Delorme C (2008) Safety assessment of dairy microorganisms: Streptococcus thermophilus. Int J Food Microbiol 126: 274-277.
5. Ogier JC, Serror P (2008) Safety assessment of dairy microorganisms: The Enterococcus genus. Int J Food Microbiol 126: 291-301.
6. Salminen S, von Wright A, Morelli L, Marteau P, Brassard D, et al. (1998) Demonstration of safety of probiotics - A review. Int J Food Microbiol 44: 93-106.
7. LeBlanc JG, Lain JE, del Valle MJ, Vannini V, van Sinderen D, et al. (2011) B-group vitamin production by lactic acid bacteria – current knowledge and potential applications. J Appl Microbiol 111: 1297-1309.
8. Choi EA, Chang HC (2015) Cholesterol-lowering effects of a putative probiotic strain Lactobacillus plantarum EM isolated from kimchi. LWT Food Sci Technol 62: 210-217.
9. He T, Priebe MG, Zhong Y, Huang C, Harmesen HJM (2007) Effects of yogurt and bifidobacteria supplementation on the colon microbiota in lactose-intolerant subjects. J Appl Microbiol 104: 595-604.
10. Rafter J, Bennett M, Caderni G, Clune Y, Hughes R (2007) Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr 85: 488-496.
11. Shraf R, Shah NP (2014) Immune System Stimulation by Probiotic Microorganisms. Crit Rev Food Sci Nutr 54: 938-956.
12. Whelan K, Quigley EMM (2013) Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease. Curr Opin Gastroenterol 29: 184-189.
13. Kim SE, Choi SC, Park KS, Park MI, Shin JE (2015) Change of Fecal Flora and Effectiveness of the Short-term VSL#3 Probiotic Treatment in Patients With Functional Constipation. J Neurogastroenterol Motil 21: 111-120.
14. McFarland LV (2007) Meta-analysis of probiotics for the prevention of traveler's diarrhea. Travel Med Infect Dis 5: 97-105.
15. Vasiljevic T, Shah NP (2008) Probiotics-From Metchnikoff to bioactive. Int Dairy J 18: 714-728.
16. Saad N, Deliatte C, Urduaci M, Schmitter JM, Bressollier P (2013) An overview of recent advances in probiotic and probiotic food. LWT Food Sci Technol 50: 1-16.
17. Losio MN, Bozzo B, Galuppini E, Martella V, Bertasi B, et al. (2015) Silter Cheese, a Traditional Italian Dairy Product: A Source of Feasible Probiotic Strains. Int J Food Prop 18: 492-498.
18. Zago M, Fornasaria ME, Caminatia D, Burns P, Suarez V, et al. (2011) Characterization and probiotic potential of Lactobacillus plantarum strains isolated from cheeses. Food Microbiol 28: 1033-1040.
