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ORIGINAL PAPER

Characterization of the three selected probiotic strains for the application in food industry

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Abstract Previously selected bacterial probiotic strains *Enterococcus faecium* L3, *Lactobacillus plantarum* L4 and *Lactobacillus acidophilus* M92 have shown their potential as functional starter cultures in silage, white cabbage and milk fermentation. Therefore, the phenotypic and genotypic characteristics important for their application in food industry were investigated. Pulsed-field gel electrophoresis (PFGE) of *Not*I digested genomic DNA, in combination with physiological traits determined by API tests, made a useful tool for identification of these probiotic strains and differentiation among them. Lyophilized probiotic cells remained viable during 75 days of storage at −20, +4 and +15°C, while fresh concentrated cells remained viable only at −20°C with addition of glycerol as cryoprotectant. After the lyophilization with addition of skim milk as lyoprotectant, the viability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 was reduced by only 0.37, 0.44 and 0.50 log, respectively. Furthermore, probiotic strains *L. acidophilus* M92, *L. plantarum* L4, and *E. faecium* L3, demonstrated anti-*Salmonella* activity, and *L. acidophilus* M92 having also antilisterial activity demonstrated by in vitro competition test. Overnight cultures and cell-free supernatants of the three probiotic strains exerted also an antagonistic effect against the Gram-positive and Gram-negative test microorganisms examined, demonstrated by the agar-well diffusion test. The inhibition of *Listeria monocytogenes*, *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Acinetobacter calcoaceticus* obtained, achieved by the neutralized, 5-fold concentrated supernatant of*L. plantarum* L4, may be the result of its bacteriocinogenic activity. On the basis of these results, the application of the three examined probiotic strains may become a point of great importance in respect of food safety.

Keywords Antagonistic effect · *Enterococcus faecium* · *Lactobacillus acidophilus* · *Lactobacillus plantarum* · Lyophilization · Probiotics · Pulsed-field gel electrophoresis (PFGE)

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Introduction

Food fermentation has been shown to have not only preservative effects and the capability of aiding the modification of physico-chemical properties of various foods, but also the capability to provide significant impact on the nutritional quality and functional performance of the raw material (Knorr 1998). This offers a possibility to explore the use of probiotics as functional starter cultures for the manufacture of fermented foods. Functional starter cultures are defined as starters that possess at least one inherent, functional property, aimed at improving the quality of the end product (De Vuyst 2000). According to the definition of the World Health Organization, probiotics are living microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Gilliland et al. 2001). The increasing application of probiotic cultures in food products underscores the need to

properly identify and distinguish these beneficial bacteria among the originally presented microbial population. Moreover, certain probiotic activities are strain-specific and thus, identification of probiotics to the strain level is necessary. Current strain-specific techniques used for probiotics, comprise multiple DNA-based methods such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) PCR, ribotyping and protein-based methods such as SDS-PAGE (Yeung et al. 2004; Klaenhammer et al. 2005; Zoetendal and Mackie 2005). Characterization of bacteria below the strain level could be used to estimate the microbial diversity present in natural populations, and to determine the technological contribution of individual strains or biotypes during the manufacture and ripening processes.

One of the primary benefits associated with probiotic bacterial cultures, is the exclusion of pathogenic bacteria in the small and large intestine. Furthermore, inactivation of undesirable microorganisms during fermentation is an essential part of food preservation. On the other hand, fermentation which is a process to improve the digestibility, quality, safety and physico-chemical properties of the raw material, can be counterproductive to the viability of microorganisms (Knorr 1998; Brul 2005). Technological challenges include the necessity to obtain high productivity and viability of starter cultures, probiotic strains and functional starter cultures.

Several aspects, including general, functional and technological characteristics, have to be taken into consideration while selecting probiotic strains (Sanders and Huis in't Veld 1999; Šušković et al. 2001). Based on in vitro selection criteria, three potential probiotic strains were selected in advance: *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3. These three strains have been shown to have the ability to survive conditions mimicking those in the gastrointestinal tract. Because of their bile resistance and cholesterol assimilation in the presence of bile, it is postulated that these strains might help in lowering serum cholesterol in vivo (Šušković 1996; Kos et al. 2000; Šušković et al. 2000). The probiotic strains examined stimulated humoral immune response, and have the ability to survive and adhere in the mouse intestinal tract, and operate as effective probiotics that positively influence the intestinal microflora of the host (Kos et al. 2003; Frece et al. 2005a). The results obtained on the aggregation and adhesion of *L. acidophilus* M92, suggested that these processes are mediated by proteinaceous components (S-layer) of the cell surface (Frece et al. 2005b). Furthermore, *L. plantarum* L4 and *E. faecium* L3 strains were successfully applied as starter cultures for silage fermentation (Runjić-Perić 1996), while *L. plantarum* L4 successfully fulfilled the role of the starter culture in the process of white cabbage fermentation (Beganović et al. 2005). Especially, *L. acidophilus* M92 has a great potential as probiotic strain for fermented milk products, because of the protective role of S-layer proteins, exhibited during the transit through the gastrointestinal tract (Frece et al. 2005a).

The main objectives of this study were phenotypic and genotypic characterization, the estimation of viability exhibited during culture production and storage, and the estimation of antagonism to pathogens, carried out by the three selected probiotic strains.

Materials and methods

Bacterial strains and growth conditions

The three selected probiotic strains, *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4, and *Enterococcus faecium* L3 were from the culture collection of the Department of Biochemical Engineering, Laboratory of Antibiotic, Enzyme, Probiotic and Starter Cultures Production, University of Zagreb. *Lactobacillus acidophilus* ATCC 4356 and *Leuconostoc mesenteroides* LMG 7954 strains were also used. All of these strains were stored at −70°C in the DeMann Ragosa Sharpe (MRS) broth (Difco) with 30% (v/v) glycerol. Before the experimental use, these cultures were sub-cultured twice in the MRS broth.

The three selected probiotic strains were examined for their antagonistic activities against the following test microorganisms: *Escherichia coli* DH5α, *Salmonella enterica* serovar *typhimurium*, *Acintetobacter calcoaceticus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Gardnerella vaginalis*, *Bacillus cereus*, *Pseudomonas* sp. S12+ and *Vibrio anguillarum*. All test microorganisms were from the Department of Agrobiology and Agrochemistry, Faculty of Agricultural Science, University of Tuscia, Viterbo. Test microorganisms were cultured aerobically at 37°C on the PCA broth and agar (Difco).

Carbohydrate fermentation

The ability of *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4, and *Enterococcus faecium* L3, to ferment various carbohydrates, was determined using the API 50 CH (for lactobacilli) and API 20 Strep (for *Enterococcus*), applied according to the manufacturer's instructions (API systems, BioMérieux).

Pulsed-field gel electrophoresis (PFGE)

Intact genomic DNA was isolated and digested in agarose plugs as described by Yeung et al. (2004), with only minor

modifications. Purified DNA was digested with five restriction enzymes (*Sma*I, *Sfi*I, *Sal*I, *Apa*I, *Not*I), all produced by Fermentas GmbH. *Not*I was found to be the best enzyme, i.e., it yielded reproducible and more informative digestion patterns with complete DNA digestion into a suitable number of fragments, hence it was used for further optimization of the electrophoretic conditions.

The chromosomal DNA digests were separated by PFGE using a CHEF DRIII apparatus (Bio-Rad). Electrophoresis was performed through 1% w/v agarose gels at 14°C and 6 V/cm in 0.5× Tris–Borate–EDTA buffer (TBE). Two pulse range times were applied; 0.5–25 s for 12 h and 25–50 s for 6 h.

Viability of probiotic strains during preparation and storage

The cultures were cultivated overnight in MRS broth. The cells were collected by centrifugation (10,000 rev/min/ 20 min), washed and resuspended in skim milk (10% w/v) for lyophilization or in phosphate buffer saline (PBS) containing 50% (v/v) glycerol. Cells were frozen at −20°C overnight, then lyophilized in bench top freeze-dryer (B. Braun Biotech International, model Christ Alpha 1-4). Lyophilized cells and cells suspended in PBS containing 50% (v/v) glycerol were stored for 75 days at −20, +4 and +15°C. Viable cells recovery was determined by colony formation on MRS agar using the standard pour-plating method.

Antibacterial activity of probiotic strains

Probiotic strains were tested for their antibacterial activity by in vitro competition test and agar-well diffusion test.

In vitro competition test were performed in Erlenmeyer flasks containing 200 ml of PTG broth. The broth media were inoculated with probiotic strain and test microorganism in cell ratio 1:1, 2:1, and 3:1 log units. The test microorganism and the probiotic strain were added in the media at the same time, or, alternatively, the test microorganism was added 2 h after the probiotic strain. The flasks were incubated aerobically for 24 h at 37°C, and the number of viable cells alone or associated within the broth, were determined by the standard pour-plating method using selective media (MRS for lactic acid bacteria, Brilliant green agar for *Salmonella*), or the temperature selective for growth of the particular cells (e.g., 7°C for the growth of *Listeria*).

Using the agar-well diffusion test described by Tagg et al. (1976), antibacterial activity of the three probiotic strains was further examined. Overnight cultures and cell-free supernatants of *L. acidophilus* M92, *L. plantarum* L4, and *E. faecium* L3, were used for the examination of their antibacterial activity. The cells were removed by centrifugation (10,000 rev/min at 4°C for 20 min) and the supernatants were filtered through a 0.22 μm Millipore filter. The cell-free supernatant was concentrated in a 50-ml Amicon cell (Amicon, Beverly, USA) equipped with a selective (10,000 Da) membrane. In order to avoid any pH effect, the 5-fold concentrated supernatants were tested for their inhibitory activity at the pH adjusted to 6.5 and using 1 M NaOH solution. Furthermore, catalase was added at the final concentration of 1 mg/ml, to provide against the possible presence of hydrogen peroxide. Briefly, the PCA medium was seeded with the overnight cultures of the test microorganisms at the final concentration of about 10^6 c.f.u./ml. Wells (9 mm) were cut in the solidified agar using a sterile metal cork borer, and filled with 120 μl of the overnight culture, cell-free supernatant and neutralized 5-fold concentrated supernatant. The plates were kept at 4°C for 2 h to allow the diffusion on the assay material, and then incubated at 37°C for 18 h. The diameters of the clear inhibition zones were then measured.

Results

Biochemical and molecular characterization of the three selected probiotic strains

The phenotypic characterization of the probiotic bacteria (to ferment various carbon sources) has been done using the API tests (API 50 CH and API 20 Strep). As a result, the carbohydrate fermentation patterns of *L. acidophilus* M92, *L. plantraum* L4, and *E. faecium* L3, were observed and their identification was confirmed in comparison with the type species from the database of the API systems (BioMérieux) with 78.6, 99.9 and 93.9% similarity, respectively (Tables 1 and 2).

Prior to the evaluation of PFGE patterns, the selection of the restriction enzymes was performed. In order to determine the restriction enzyme which provides suitable fragment patterns, five restriction enzymes were tested (*Sma*I, *Sfi*I, *Sal*I, *Apa*I, *Not*I). It was found that *Not*I could generate clear and easy-to-interpret PFGE patterns of all examined strains (Fig. 1). The PFGE pattern of the strain *L. acidophilus* M92 was similar to those observed for the reference strain *L. acidophilus* ATCC 4356. All other strains produced distinctly different patterns (Fig. 1).

Viability of probiotic strains during preparation and storage

The effect of lyophilization on the viability of the three probiotic strains is shown in Table 3. Although

Table 1 Fermentation patterns of *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 on API 50 CH

Carbohydrates	M92	L4	L3	Carbohydrates	M92	L4	L3
Control	–	–	–	Arbutin	+	+	±
Glycerol	–	–	–	Esculin	±	+	+
Erthritol	–	–	–	Salicin	±	+	±
D-arabinose	–	–	–	Cellobiose	+	+	+
L-arabinose	–	+	+	Maltose	+	+	+
Ribose	–	+	+	Lactose	±	+	+
D-xylose	–	–	–	Melibiose	–	+	±
L-xylose	–	–	–	Saccharose	+	+	+
Adonitol	–	–	–	Trehalose	±	+	+
β-methyl-xyloside	–	–	–	Inulin	–	–	–
Galactose	+	+	+	Melezitose	–	+	–
D-glucose	+	+	+	D-raffinose	–	+	–
D-fructose	+	+	+	Amidon	–	–	–
D-mannose	+	+	+	Glycogen	–	–	–
L-sorbose	–	–	–	Xylitol	–	–	–
Rhamnose	–	–	–	β-gentiobiose	±	+	±
Dulcitol	–	–	–	D-turanose	±	+	+
Inositol	–	–	–	D-lyxose	–	–	–
Mannitol	±	+	+	D-tagatose	–	–	+
Sorbitol	–	+	–	D-fucose	–	–	–
α Methyl-D-mannoside	–	+	–	L-fucose	–	–	–
α Methyl-D-glucoside	–	–	–	D-arabitol	–	–	–
N-acetylglucosamine	+	+	±	L-arabitol	–	–	–
Amygdalin	+	+	±	Gluconate	–	+	–
2-ketogluconate	–	–	–	5-keto-gluconate	–	–	–

–, Negative reaction, the color did not change; +, positive reaction, the color changed to yellow in 48 h; ±, the color ranged between green and yellow

Table 2 Reactions of *Enterococcus faecium* L3 in API 20 Strep

Test	Reactions	Test	Reactions
Voges-Proskauer	+	*Fermentation of:*	
Hippurate hydrolysis	–	Ribose	+
Esculin hydrolysis	+	Arabinose	+
Pyrrolidonyl arylamidase	±	Mannitol	+
α-galactosidase	–	Sorbitol	–
β-glucuronidase	–	Lactose	+
β-galactosidase	+	Trehalose	+
Alkaline phosphatase	–	Inulin	–
Leucine aminopeptidase	+	Raffinose	–
Arginine dihydrolase	+	Starch	+
		Glycogen	–

+, Positive reaction; –, negative reaction

lyophilization had a deleterious effect on the viability of probiotic microorganisms, they had shown high a survival rate in the presence of skim milk as lyoprotectant. The viability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3, was reduced after lyophilization by only 0.37, 0.44 and 0.50 log, respectively (Table 3). Furthermore, the viable cell counts of the three probiotic strains remained constant during 75 days of storage at −20, +4 and +15°C (data not shown). When the experiment was performed with concentrated fresh cells instead of the lyophilized ones, the recovery of viable cells during 75 days of storage with glycerol as cryoprotectant, was revealed to be the highest at −20°C, which goes for all of the three tested strains (Fig. 2). The number of *E. faecium* L3 cells remained constant at +4 and +15°C during 15 days of storage, but *L. plantarum* L4 cells remained viable in the same period only when stored at +4°C. The greatest survival at +4°C had been shown by *E. faecium* L3, with the viable cell counts of about 7 log units after 75 days of storage (Fig. 2). At the end of the storage period, the viable cell counts of *L. plantarum* L4 and *E. faecium* L3, steadily approximated 10^4 cells/ml at +15°C (Fig. 2).

Antagonistic activity of probiotic strains

Two different methods were used for the examination of antibacterial activity of the three probiotic strains. Antagonistic activity of the probiotic strains was first tested by in vitro competition test, using PTG broth inoculated with

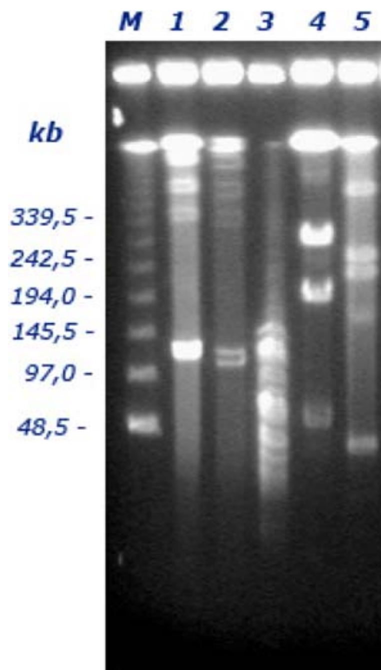

Fig. 1 Electrophoretic karyotype of *Not*I-digested genomic DNA of the lactic acid bacteria examined. Lane M, Lambda-DNA PFGE molecular mass markers; lane 1, *Lactobacillus acidophilus* ATCC 4356; lane 2, *Lactobacillus acidophilus* M92; lane 3, *Lactobacillus planatarum* L4; lane 4, *Enterococcus feacium* L3; lane 5, *Leuconostoc mesenteroides* LMG 7954

both the probiotic strain and the test microorganism (*Salmonella typhimurium* or *Listeria monocytogenes*). When the probiotic strain and the test microorganism were inoculated into the growth medium at the same time (with the ratio of the cell counts 3:1 log units, respectively), the inhibition of *S. typhimurium* occurred after 12 h of incubation with *L. plantarum* L4 (Fig. 3A). However, by inoculating the test microorganism 2 h after the probiotic strain, *S. typhimurium* was inhibited almost immediately (Fig. 3B). Similar results were obtained by *E. faecium* L3 and *L. acidophilus* M92 against *S. typhimurium* (data not shown). With the inoculation of the lower initial number of probiotic bacteria *L. acidophilus* M92 (with the ratio of cell

Table 3 Survival of potential probiotic strains after lyophilization with skim milk as lyoprotector

Microorganisms	Viable count (log_{10}) c.f.u./ml of probiotic cultures[a]	
	Before lyophilization	After lyophilization
Lactobacillus acidophilus M92	8.87 ± 0.23	8.50 ± 0.17
Lactobacillus plantarum L4	8.71 ± 0.31	8.27 ± 0.28
Enterococcus faecium L3	8.89 ± 0.29	8.39 ± 0.20

[a] Measurements are expressed as means ± standard error of the three replicates of the each individual strain

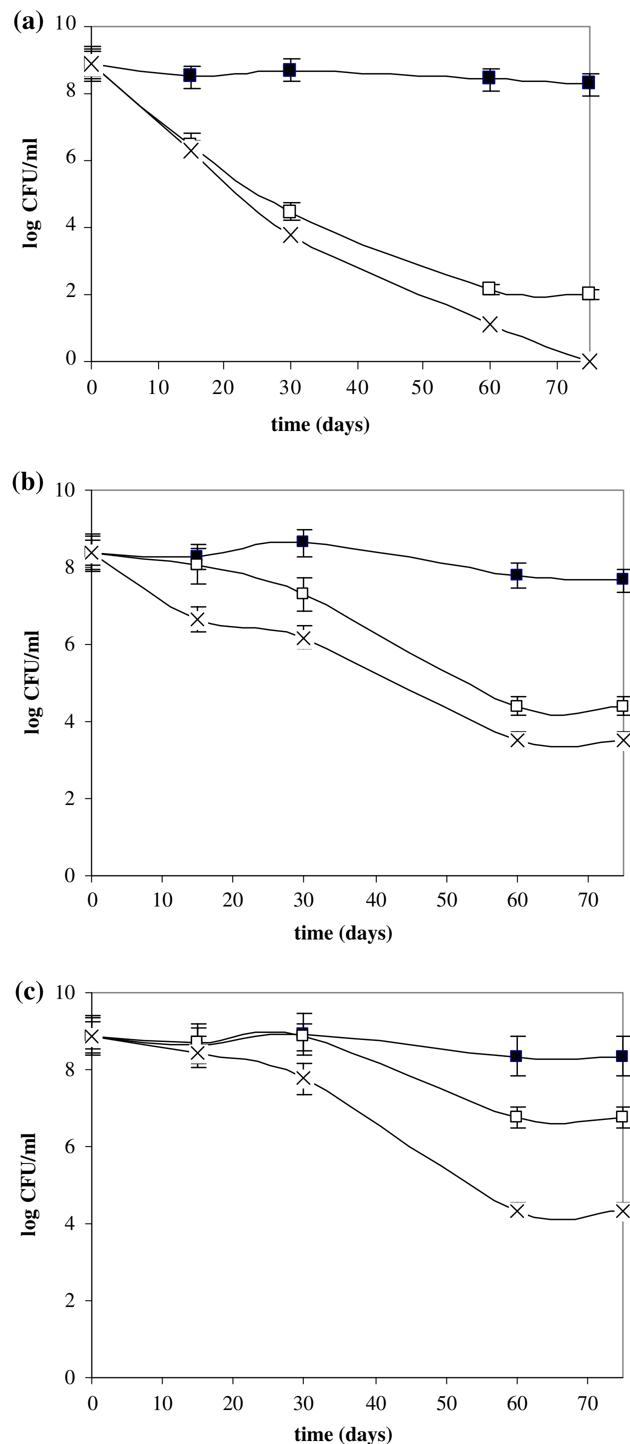

Fig. 2 Survival of the concentrated cultures of *Lactobacillus acidophilus* M92 (**a**), *Lactobacillus plantarum* L4 (**b**), and *Enterococcus faecium* L3 (**c**), stored with glycerol as protector at −20°C (■), +4°C (□) and +15°C (x)

counts 2:1 log units instead of 3:1 log units), the inhibition started after 2 h of incubation, and *S. typhimurium* was completely inhibited after 10 h of incubation with the probiotic strain *L. acidophilus* M92 (Fig. 4). Furthermore,

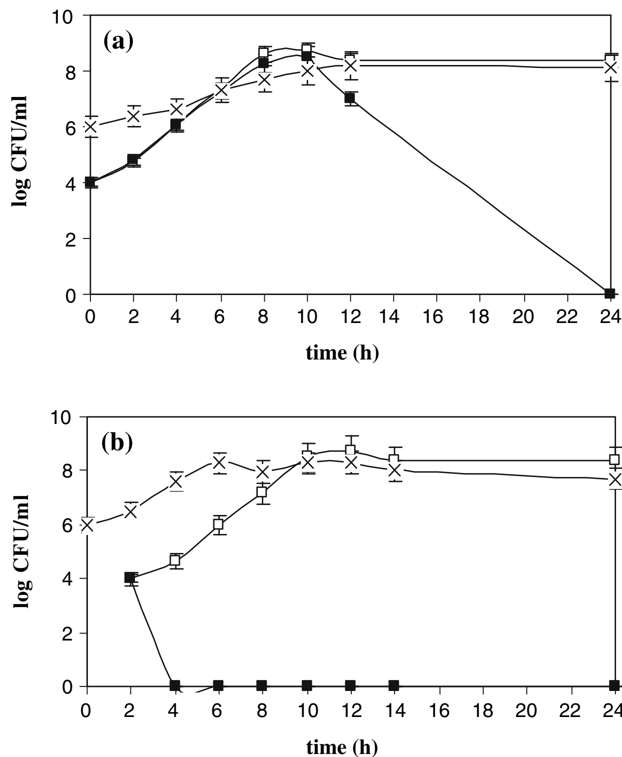

Fig. 3 Inhibition of *Salmonella typhimurium* when grown in association with *Lactobacillus plantarum* L4 in PTG broth at 37°C. *S. typhimurium* was inoculated at the same time (**a**) and 2 h after probiotic strain *L. plantarum* L4 (**b**). Ratio of cell numbers *Lactobacillus*:*Salmonella* was 3:1 log units. ■, log *N* of *S. typhimurium* associated; □, log *N* of *S. typhimurium* alone; x, log *N* of *L. plantarum* L4 alone and associated

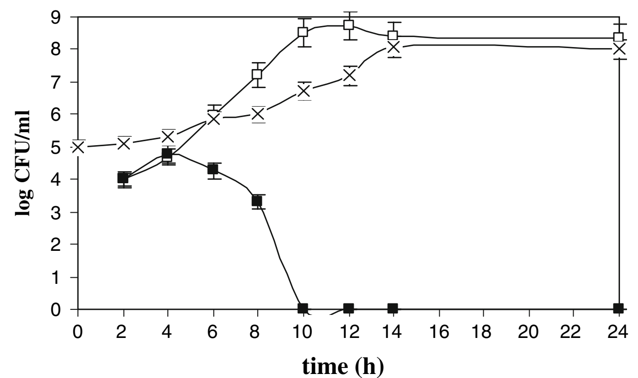

Fig. 4 Inhibition of *Salmonella typhimurium* when grown in association with *Lactobacillus acidophilus* M92 in PTG broth at 37°C. Ratio of cell numbers *Lactobacillus*:*Salmonella* was 2:1 log units. ■, log *N* of *S. typhimurium* associated; □, log *N* of *S. typhimurium* alone; x, log *N* of *L. acidophilus* M92 alone and associated

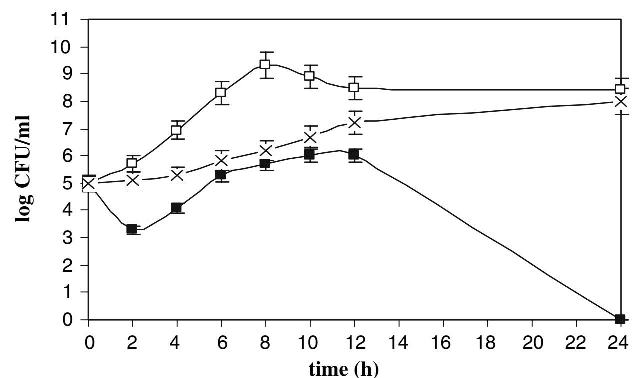

Fig. 5 Inhibition of *Listeria monocytogenes* when grown in association with *Lactobacillus acidophilus* M92 in PTG broth at 37°C. Ratio of cell numbers *Lactobacillus*: *Listeria* was 1:1 log units. ■, log *N* of *L. monocytogenes* associated; □, log *N* of *L. monocytogenes* alone; x, log *N* of *L. acidophilus* M92 alone and associated

after inoculation of the same initial number of the probiotic strain and the test microorganism, during the first 12 h of incubation with *L. acidophilus* M92, the number of viable *L. monocytogenes* decreased for 2 log-values in comparison to the control, and was completely inhibited after 24 h of incubation (Fig. 5).

When the overnight cultures and cell-free supernatants were examined by the agar-gel diffusion test, the probiotic strains exerted antibacterial activity against different Gram-positive and Gram-negative test microorganisms (Table 4). Furthermore, positive results were obtained with the usage of the neutralized, 5-fold concentrated supernatant of *L. plantarum* L4 against *Listeria monocytogenes, Salmonella typhimurium*, *Yersinia enterocolitica*, and *Acinetobacter calcoaceticus*.

Discussion

Lactic acid bacteria have a worldwide industrial use as starter cultures in the manufacturing of different fermented products. Their confirmed probiotic properties are of great importance for the application of the three selected strains, as functional starter cultures for milk (*L. acidophilus* M92) and vegetable (*L. plantarum* L4 and *E. faecium* L3) fermentations. These strains fulfill in vitro and in vivo selection criteria, such as survival, competition, adhesion and colonization in the gastrointestinal tract, as well as immune-modulating capability (Šušković et al. 2000; Kos et al. 2003; Frece et al. 2005b). However, increasing application of probiotics in food products underscores the need to properly identify these beneficial bacteria and distinguish them from among other microorganisms present in the products. Using strain-specific patterns to verify phenotypic and genotypic characteristics of the applied probiotic bacteria represents a necessary approach as to provide quality control of probiotic products. The carbohydrate fermentation analysis performed by the API system was first carried out as the primary method for phenotypic

Table 4 Antibacterial activity of the overnight culture, cell-free supernatant and neutralized 5-fold concentrated supernatant of *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3, obtained by agar-well diffusion test

Test microorganisms	Zones of inhibition (mm) obtained by								
	Overnight culture			Cell-free supernatant			Neutralized concentrated supernatant		
	M92	L4	L3	M92	L4	L3	M92	L4	L3
Escherichia coli DH5α	25	18	18	17	19	15	–	–	–
Salmonella typhimurium	29	27	22	18	20	15	–	12	–
Acintetobacter calcoaceticus	15	22	14	15	17	13	–	10	–
Listeria monocytogenes	21	22	15	15	14	10	–	11	–
Yersinia enterocolitica	24	28	22	17	28	12	–	10	–
Gardnerella vaginalis	25	22	19	14	14	12	–	–	–
Bacillus cereus	27	27	20	19	17	14	–	–	–
Pseudomonas sp. S12+	22	23	17	14	15	11	–	–	–
Vibrio anguillarum	26	22	15	15	13	12	–	–	–

characterization of the three probiotic strains. High similarity of the carbohydrate utilization profile between the probiotic strains examined and the corresponding type-strains originating from the API system database (bioMérieux), was demonstrated. The results obtained could be useful in the application of the three strains as functional starter cultures for different fermented products. However, the carbohydrate fermentation pattern can be affected by experimental conditions such as incubation time and temperature, and limited in terms of its discriminating ability and accuracy. In contrast, molecular methods such as the PFGE, offer the advantage of having a good level of taxonomic resolution at species or subspecies level, so being able to distinguish added probiotic cultures among microbial population originally present in the functional food products (Yeung et al. 2004). PFGE patterns of *Not*I-digested genomic DNA of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 have shown that this method can be successfully applied for the identification of these strains and the differentiation among them. A highly reproducible method of characterizing and distinguishing closely-related strains is the PFGE performed by infrequently cutting endonucleases (Pepe et al. 2004). Namely, based on the DNA–DNA hybridizations, six different species were identified within the *L. acidophilus*-group, and two of them (*L. acidophilus* and *L. johnsonii*) are mainly used in fermented dairy products and show nearly identical phenotypical properties (Fujisawa et al. 1992; Reuter et al. 2002). Genetic differentiation of strains of several lactic acid bacterial species has been successfully performed by PFGE, and has revealed that bacterial isolates of the same species can exhibit closely related, but not identical patterns (Yeung et al. 2004). This is confirmed by the similar, but not identical restriction fragments obtained by the PFGE as regards *L. acidophilus* M92 and type strain *L. acidophilus* ATCC 4356. A remarkable genetic restriction polymorphism was obtained by the PFGE after the digestion of the genomic DNA of the 30 *L. plantarum* strains by *Not*I (Pepe et al. 2004). The PFGE profiles have shown the large number of restriction fragments ranging between 9 and 150 kb in size, similar to those obtained with the examined probiotic strain *L. plantarum* L4. Furthermore, *Not*I digestion pattern of the probiotic strain *E. faecium* L3, was similar to those obtained for *E. faecium* ATCC 19436. It yielded five DNA bands in the range of 10–1,000 kb (Oana et al. 2002). Very clear, reproducible restriction patterns of the three probiotic strains examined were obtained, allowing monitoring of these strains during food processing.

The viability and activity of probiotic bacteria during preparation and storage, is also very important for their industrial application. They can be added to probiotic products as fresh or lyophilized cells. High population levels, between 10^6 and 10^8 microbial cells per ml, should be present in probiotic products (Sanders and Huis in't Veld 1999). However, during preparation for industrial application, bacteria are subjected to stress conditions such as freezing, drying and concentration stress, which diminish cell viability. The frequency of cell death is usually correlated with cell membrane damage. Cryoprotectants (for freezing) and lyoprotectants (for lyophilization) are usually used for membrane stabilization. These are small molecules with osmotic behavior, or polymers, which promote the formation of amorphous or "glassy" solids and reduce ice formation that can be cell-damaging (Conrad et al. 2000; Capela et al. 2006). For protection of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 cells, glycerol as cryoprotectant and skim milk as lyoprotectant have been used. All of the three strains examined had shown the ability to withstand the stresses associated with lyophilization, and to retain their viability during a 75 day-storage period in skim milk at −20, +4 and +15°C.

Higher survival rates of *L. acidophilus* M92, exhibited during lyophilization, may be attributed to its specific cell surface composition. Namely, *L. acidophilus* M92 possesses surface layer (S-layer) proteins protective for this strain during its transit through the intestinal tract of mice, which also mediate adhesion (Frece et al. 2005b). There exists an increasing amount of evidence that S-layer-carrying bacteria may use S-layer protein genes for the adaptation to different stress factors, such as drastic changes in environmental conditions (Jakava-Viljanen et al. 2002). Furthermore, fresh concentrated cells of the three probiotic strains, can be stored at −20°C for 75 days without any loss of viability. Additionally, *E. faecium* L3 cells retained their high viability (10^7 cells/ml) even at +4°C for 75 days of storage.

Another functional, strain-specific property of probiotic strains is their antagonism to pathogens. The antimicrobial activity of probiotic strains in the variety of food products, may contribute to an improvement in the quality of fermented foods, achieved through the control of spoilage and pathogenic bacteria, extending shelf-life and improving sensory quality (Wei et al. 2006; Siripatrawan and Harte 2007). Two common pathogens, *Salmonella typhimurium* and *Listeria monocytogenes*, were used for the investigation antagonistic activity of the probiotic bacteria examined, carried out via the in vitro competition test. All of the three strains have shown strong antibacterial activity against *S. typhimurium*. Additionally, *L. acidophilus* M92 exerted anti-listerial bactericidal activity which could be of great technological importance, since *L. monocytogenes* is able to survive milk fermentation conditions as well as cheese manufacturing, within the frame of which *L. acidophilus* M92 can be applied as probiotic strain. Based on the suggestion that high competitive power is an essential property of a protective culture (Wei et al. 2006), the obtained results indicated that both early and late contaminations with the examined pathogens could be combated by application of probiotic strains *L. acidophilus* M92, *L. plantarum* L4, or *E. faecium* L3. On the occasion of the agar-well diffusion test, overnight cultures of these three strains and their cell-free supernatants also exhibited antibacterial activity against a wide range of test microorganisms. By means of neutralization of concentrated cell-free supernatants, the antimicrobial activity of lactic acid was eliminated. The inhibition of some pathogens obtained attained by *L. plantarum* L4 supernatants, may be the result of its bacteriocinogenic activity. Furthermore, the antibacterial activity of the supernatants against *L. monocytogenes* was eliminated or reduced by their neutralization. As *L. monocytogenes* tolerates low pH environments, its inhibition attained by overnight cultures and supernatants could be the result of bacteriocinogenic activity of the examined probiotic strains. However, the activity of the produced bacteriocins could be affected by the change in pH value of the medium, which could explain the reduction of *L. monocytogenes* inhibition. Namely, bacteriocins are ribosomally synthesized antimicrobial peptides produced by one bacterium, active against other bacteria, either in the same species (narrow spectrum) or across genera (broad spectrum). They are produced by food-grade bacteria, are usually heat-stable, and can inhibit a number of primary pathogenic and spoilage organisms that cause problems in minimally processed foodstuffs. Although bacteriocins characterized by a wide spectrum of activity, are usually those most sought after, other factors including pH optima, solubility and stability are as important and are major considerations in choosing bacteriocins. However, up to now, only nisin and pediocin PA1/AcH have found widespread use in the food domain. Bacteriocins can have implications on the development of desirable flora in fermented food, and can be produced in situ by bacterial cultures that substitute for all, or part of the starter cultures (Cotter et al. 2005). A wide range of the inhibitory spectrum of bacteriocins produced by *L. plantarum* strains have been reported included anti-listerial bactericidal activity (Messi et al. 2001; Maldonado et al. 2003; Bernbom et al. 2006). Interestingly, bactericidal activity of the concentrated neutralized supernatant of *L. plantarum* L4 was observed with Gram-negative strains, and was best-achieved against *Y. enterocolitica*. Activity against Gram-negative strains exhibited by Gram-positive bactreriocin producers, has rarely been reported (Messi et al. 2001; Elgado et al. 2004). Further characterization of antibacterial substances produced by *L. plantarum* L4 will be performed.

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