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In vivo testing of functional properties of three selected probiotic strains

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Summary

Lactobacillus acidophilus M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 were previously selected as probiotic strains on the base of *in vitro* selection criteria. To investigate functional properties of these three probiotic strains *in vivo*, Swiss albino mice were used as animal model. Survival, competition, adhesion and colonization were monitored in the gastrointestinal tract, as well as the immunomodulating capability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3. During the feeding of mice with probiotic strains with daily dose of 2×10^{10} rifampicin-resistant cells, the number of lactic acid bacteria in the faeces increased and reduction of enterobacteria and sulphite-reducing clostridia was observed. Rifampicin-resistant colonies of probiotic strains could be reisolated from the faeces of mice fed with the rifampicin-resistant cells. The similar results were obtained in homogenates of small and large intestine of mice on the first and fourteenth days after feeding with *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3. The adherence of the probiotic strains obtained *in vitro* correlated with their capability to adhere to mouse ileal epithelial cells *in vivo*. After oral immunization of mice with viable cells of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 with a daily dose of 2×10^{10} cells, the concentrations of serum IgA, IgG and IgM antibodies from all groups of mice were significantly higher in comparison to the control.

Introduction

Modern consumers are increasingly interested in their personal health, and expect their food to be healthy or even capable of preventing illness. Gut health in general has shown to be the key sector for functional foods in Europe (Mattila-Sandholm *et al.* 2002). A major development in functional foods pertains to foods containing probiotics. An Expert Committee defined the term probiotics, popularized by Roy Fuller in 1989, as 'Living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition' (Guarner & Schaafsma 1998). Several aspects, including general, functional and technological characteristics have to be taken into consideration in the selection process of probiotic strains. Functional aspects include survival and adhesion to intestinal epithelium in the gastrointestinal tract, immunomodulation, antagonistic activity and influence on metabolic activities (Sanders & Huis in't Veld 1999). In order to survive in and colonize the gastrointestinal tract, probiotic bacteria need to express high tolerance to acid and bile and to have the ability to adhere to intestinal surfaces (Lee & Salminen 1995; Fujiwara *et al.* 2001; Šušković *et al.* 2001). Adhesion is considered important for immune modulation (the intestine is the largest immune organ of the body), pathogen exclusion, enhanced healing of damaged mucosa and prolonged transient colonization (Guarner & Malagelada 2003). Immunomodulation in humans by orally administered microorganisms, particularity by those considered probiotic, has received increasing attention. Various immune responses were influenced by probiotics and these immunomodulatory effects have been proposed to include cure of infections like diarrhoea as is well demonstrated in several publications (Kirjavainen *et al.* 1999; Ouwehand *et al.* 2002).

In our laboratory, three potential probiotic strains were previously selected, on *in vitro* selection criteria basis: *Lactobacillus acidophilus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3. These three strains have shown ability to survive simulated conditions in the gastrointestinal tract, are bile resistant, have antibacterial activity against some enteropathogenic and spore-forming bacteria, and are, as such, potential candidates for probiotics (Šušković 1996; Kos *et al.* 2000; Šušković *et al.* 2000). Furthermore, *in vitro* studies have shown that the three selected strains assimilate cholesterol in the presence of bile, so it is postulated that these strains might help in lowering serum cholesterol

in vivo (Kos 2001). All three strains adhere to porcine ileal epithelial cells *in vitro* (Kos *et al*. 2003). The results on the aggregation and adhesion of *L. acidophilus* M92 suggested that these processes are mediated by proteinaceous components (S-layer) on the cell surface (Frece 2003; Frece *et al*. 2005). Preliminary results from the technological point of view have shown activity and viability of the selected strains at high population level during freezing, freeze-drying and storage at different temperatures (unpublished data). Strains *L. plantarum* L4 and *E. faecium* L3 were successfully applied as starter cultures for silage fermentation (Runjić-Perić 1996), and strain *L. plantarum* L4 as starter culture for white cabbage fermentation (unpublished data). *L. acidophilus* M92 especially has a great potential as probiotic strain for fermented milk products because of the protective role of S-layer proteins during transit through the gastrointestinal tract and also during processing of the culture for probiotic products (Frece *et al*. 2005).

The aim of this study was to investigate functional properties of the three selected strains *in vivo*: the survival and adhesive properties of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 in the intestinal tract of mice, their effects on the intestinal and faecal microflora and immune system modulation. Using rifampicin-resistant variants of probiotic strains, we were able to monitor the survival and persistence of these three strains in the gastrointestinal tract of mice.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus acidophilus M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 are from the culture collection of the Department of Biochemical Engineering, Laboratory of Antibiotic and Enzyme Production, University of Zagreb. These strains were stored at −70 °C in the De Man–Ragosa–Sharpe (MRS) broth (Difco, Detroit, MI, USA) with 30% (v/v) glycerol. Before experimental use, cultures were subcultured twice in MRS broth (Difco).

Animals

Four months old female Swiss albino mice were used after a month quarantine period. Animals were housed four per cage and cages were changed twice a week. Water was available continuously from bottles. All mice were fed *ad libitum* with standard rodent feed (Lab-feed, Sydney, Australia). For examination of organs and sampling, mice were killed by carbon dioxide anaesthesia followed by cervical dislocation.

Rifampicin marking

L. acidophilus M92, *L. plantarum* L4 and *E. faecium* L3 were cultured aerobically in MRS-broth at 37 °C for 18 h. The cultured cells were added to MRS plates containing 100 μg/ml rifampicin (Sigma Chemical Co., St. Louis, MO, USA) and incubated for 2 days at 37 °C. The selected antibiotic-resistant strains were isolated and used for monitoring survival and persistance of these three strains in the gastrointestinal tract of mice. The rifampicin resistance of probiotic strains did not change even after seven passages in rifampicin-free MRS medium.

Mouse feeding and faecal sampling

Rifampicin-resistant *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 cells were cultured aerobically in MRS-broth at 37 °C for 18 h. Cells were removed by centrifugation at $10{,}000 \times g$ for 2 min, washed three times and resuspended in sterile 0.5% NaCl solution to final concentration of 1×10^{11} viable bacterial cells per ml. After mice were fed with 200 μl of these suspensions, survival of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 during transit through the gastrointestinal tract was determined in faecal samples. Samples (1 g wet weight) were homogenized in 1 ml sterile 0.5% NaCl solution and serially diluted before plating in non-selective medium (Peptone yeast extract glucose agar, Biolife, Milano, Italy) and selective media: MRS-agar for lactic acid bacteria count and MRS-agar with rifampicin (100 μg/ml), Violet red bile glucose agar (Biolife) for *Enterobacteriaceae* count and Sulfite agar (Difco) for sulphite-reducing clostridia count. The plates were incubated for 48 h at 37 °C under aerobic condition. Additionally, plates with non-selective medium and Sulfite agar were incubated under anaerobic conditions, obtained by placing one activated Anaerocult A gas pack (Merck, Darmstadt, Germany) in jar (Oxoid Ltd, Hampshire, UK). Lactic acid bacteria, *Enterobacteriaceae* and sulphite-reducing clostridia were identified on the basis of colony morphology, Gram stain, cell morphology and the catalase reaction.

In vitro adhesion test

The *in vitro* adhesion test was performed using the method of Mäyra-Mäkinen *et al*. (1983) with modifications. Ileal samples were collected from 4-month-old Swiss albino mice. The tissues were held in sterile 0.5% NaCl solution at 4 °C for 30 min to lose surface mucus and then washed three times with sterile saline solution. The adhesion test was performed by incubating tissue samples (1 cm^2) in bacterial suspensions (10^9 cells ml^{-1} sterile 0.5% NaCl solution) at 37 °C for 30 min. Samples of ileum were fixed in 10% formalin, dehydrated by increasing concentrations of ethanol and embedded in paraffin. Serial sections (5 μm) were cut, mounted on standard microscope slides and stained for identification of Gram-positive and Gram-negative bacteria, according to Brown and Brenn (Švob 1974). Slides were examined and photographed using Nikon Mikrophot-FXA light microscope (Nikon, Tokyo, Japan).

In vivo adhesion test

Adhesion ability of examined probiotic strains was determined in homogenates of small and large intestine of Swiss albino mice on days 1 and 14 after feeding with *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 with a daily dose of 2×10^{10} of rifampicin-resistant cells for eight consecutive days. The lengths (5 cm) of small and large intestine were gently rinsed with sterile 0.5% NaCl solution and homogenized using a Teflon homogenizer (1 g of tissue samples per ml of sterile 0.5% NaCl solution), and serially diluted before plating in non-selective and selective media as it was described in the section *Mouse feeding and faecal sampling*.

Immunization

The oral immunization of mice with probiotic bacteria was performed eight times over a period of eight successive days. Each day the mice were given a dose of 2×10^{10} viable bacterial cells in sterile 0.5% NaCl solution, by automatic 200 μl pipette, directly into the mouth. The control group was challenged with 200 μl of sterile 0.5% NaCl solution. During the experiment the mice were fed *ad libitum*. On days 4, 8, 10, 14, 17 and 21 after first immunization the blood samples were collected by bleeding of the tail vein with heparinized capillaries into the tubes, allowed to clot at room temperature for 1 h and left overnight at 4 °C. Tubes were centrifuged at $3000 \times g$ for 20 min. The serum samples were kept at −20 °C until use.

Serum antibody determination (ELISA method)

The total antibody sera titres were determined in polystyrene microtitre plates (NUNC), using the method of Rogelj *et al.* (1999). Plates were first incubated with antimouse IgA (α-chain specific, Sigma M-1272; 400 ng/well), or antimouse IgG (γ-chain specific, Sigma M-1397; 400 ng/well), or antimouse IgM (μ-chain specific, Sigma M-8644; 400 ng/well) overnight at 4 °C. To saturate the remaining binding sites, plates were incubated with 200 μl per well of 3% bovine serum albumin in phosphate-buffered saline (0.01 M, 0.15 M NaCl, pH = 7.2) (PBS) for 2 h at 37 °C. After washing four times for 10 min with 0.05% Tween in PBS (T-PBS) (200 μl/well) the plates were incubated for 2 h at 37 °C with dilutions of the sera (100 μl/well) in T-PBS. The plates were then washed 4 times in 0.05% T-PBS for 10 min and incubated in peroxidase-conjugated goat antimouse IgA (α-chain specific, Sigma A-4789) or antimouse IgG (Fc-specific, Sigma A-9309) or antimouse IgM (μ-chain specific, Sigma A-8786) antibodies diluted 1:10,000 in PBS (100 μl/well) for 2 h at 37 °C. The plates were then washed 4 times with 0.05% T-PBS for 10 min (200 μl/well). The total antibody sera titres were estimated after the reaction with the horse-radish peroxidase substrate *o*-phenylenediamine (ODP) dihydrochloride (Sigma P-8287) diluted in phosphate-citrate buffer (0.05 M, pH = 5) with 0.03% sodium perborate (100 μl/well). After 40 min the reaction was stopped with 0.2 M H_2SO_4 (50 μl/well) and absorption was determined at 450 nm with a Micro plate rider (LKB 5060-006, "GDV", Roma). The control of the peroxidase-conjugated antibodies (PBS instead of serum) and the control of the substrate (PBS instead of serum and peroxidase conjugated antibodies) were also performed.

Results

Survival of probotic strains and influence on faecal microflora of mice

The number of lactic acid bacteria on MRS and MRS with rifampicin, obtained from faeces of mice after receiving probiotic strains, was increased for about 2 log units in comparison to control (Table 1). Furthermore, all of three examined strains positively influenced the faecal microflora by decreasing the number of enterobacteria by ~1 log c.f.u./g and totally reducing the number of sulphite-reducing clostridia (Table 1).

Probiotic adhesion and influence on intestinal microflora of mice

The adhesiveness of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 to intestinal tissue was firstly investigated by an *in vitro* test. Microscopic examinations showed that all three probiotic strains adhered to intestinal epithelium of the mouse (Figure 1). *In vitro* adhesion ability of rifampicin-resistant strains to the intestinal epithelium of mouse were similar to that of the wild type strains (data not shown). *In vivo* adhesion of rifampicin-resistant cells of probiotic strains was moni-

Table 1. Comparison of the bacterial count in faeces of mice before and after feeding with *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3.

Growth media	Log c.f.u./g faeces			
	Control	M92	L4	L3
A	9.68 ± 0.14	9.23 ± 0.11*	8.74 ± 0.16*	8.65 ± 0.24*
B	9.81 ± 0.19	9.69 ± 0.16*	9.76 ± 0.14*	9.71 ± 0.23*
C	7.95 ± 0.29	10.25 ± 0.14	9.96 ± 0.14	9.61 ± 0.21
D	1.17 ± 0.21	3.15 ± 0.22	2.95 ± 0.18	2.80 ± 0.23
E	5.79 ± 0.36	4.58 ± 0.17	4.65 ± 0.21	4.54 ± 0.20
F	3.06 ± 0.19	–	–	–

Total aerobic (A) and anaerobic bacteria (B) on peptone yeast extract glucose agar; total lactic acid bacteria (C) and rifampicin-resistant lactic acid bacteria (D) on MRS-agar; E – *Enterobacteriaceae* on violet red bile glucose agar; F– sulphite-reducing clostridia on sulfite agar.Mean (± standard deviations) of results from three separate experiments. (–) colonies are not detected. Values marked with asterisks are not significantly different from the control group, according to the student's test ($P \leq 0.01$).

tored by determination of the microflora in homogenates of small and large intestine. The first day after oral administration of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3, the number of lactic acid bacteria (LAB) in small intestine of mice was increased by ~1.8 log c.f.u./g, ~0.8 log c.f.u. /g and ~0.5 log c.f.u./g, respectively, in comparison to control group (Figure 2 a, b, c). The higher number of LAB than in small intestine of control mice was also detected on day 14 after administration of probiotic bacteria. The number of enterobacteria was decreased in comparison to the control group by ~1.0 and 0.5 log units, on days 1 and 14, respectively. Furthermore, each probiotic strain reduced the number of sulphite-reducing clostridia in the small intestine (Figure 2 a, b, c). Similar results were obtained in the large intestine of mice after administration of probiotic strains. The number of enterobacteria was decreased and reduction of sulphite-reducing clostridia was observed (Figure 3 a, b, c). Also, the number of LAB increased in comparison to control groups, but their number in the large intestine was higher than in the small intestine by ~0.5 log c.f.u./g (Figures 2 and 3).

Probiotic modulation of mouse immune responses

Oral immunization of mice with viable cells of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 stimulated the humoral immune response (Figure 4). The levels of serum IgA, IgG and IgM antibodies from all groups of mice were significantly higher in comparison to control groups. The highest level of serum antibodies was observed after oral immunization of mice with viable cells of *L. acidophilus* M92 (Figure 4).

Discussion

In vitro screening of candidate probiotic strains is thought to provide some insight into wise choices for *in vivo* functionality. The article presents results of some *in vivo* preselective studies on *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 since these three selected probiotic strains fulfil *in vitro* selection criteria and exert inhibitory activity against a wide range of bacteria including some pathogens (Kos *et al.* 2000; Šušković *et al.* 2000; Frece *et al.* 2005). It is also important that these three strains have a great potential to be applied as starter cultures for fermented products. Preliminary results have shown successful application of *L. acidophilus* M92 in probiotic whey drink production, *E. faecium* L3 together with *L. plantarum* L4 has desirable traits for silage production, and *L. plantarum* L4 with *Leuconostoc mesenteroides* LMG7954 shortens duration of white cabbage fermentation and lowers biogenic amine content (data not shown).

From a functional point of view, in the selection of probiotic microorganisms it is essential to determine if

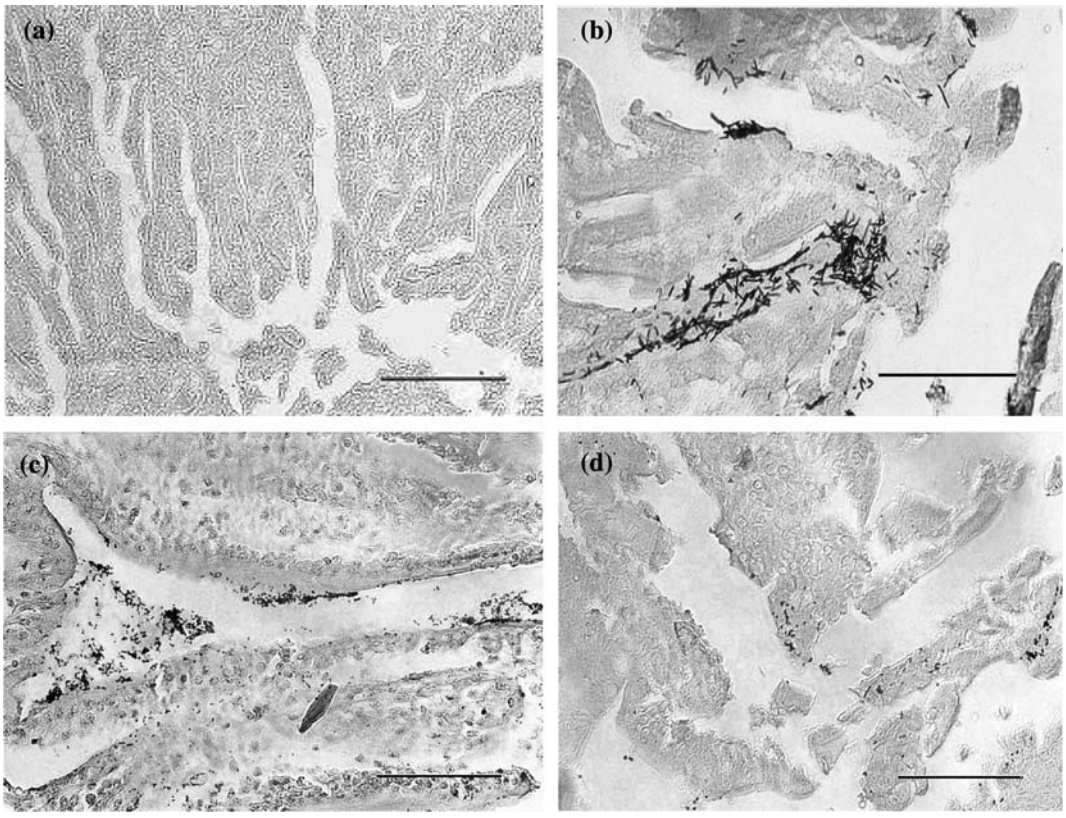

Figure 1. In vitro adhesion test performed on the intestinal epithelium of mouse before (a) and after treatment with cell suspension of *L. acidophilus* M92 (b), *L. plantarum* L4 (c) and *E. faecium* L3 (d). Magnification, 40 × 2.5. Bars represent 50 μm.

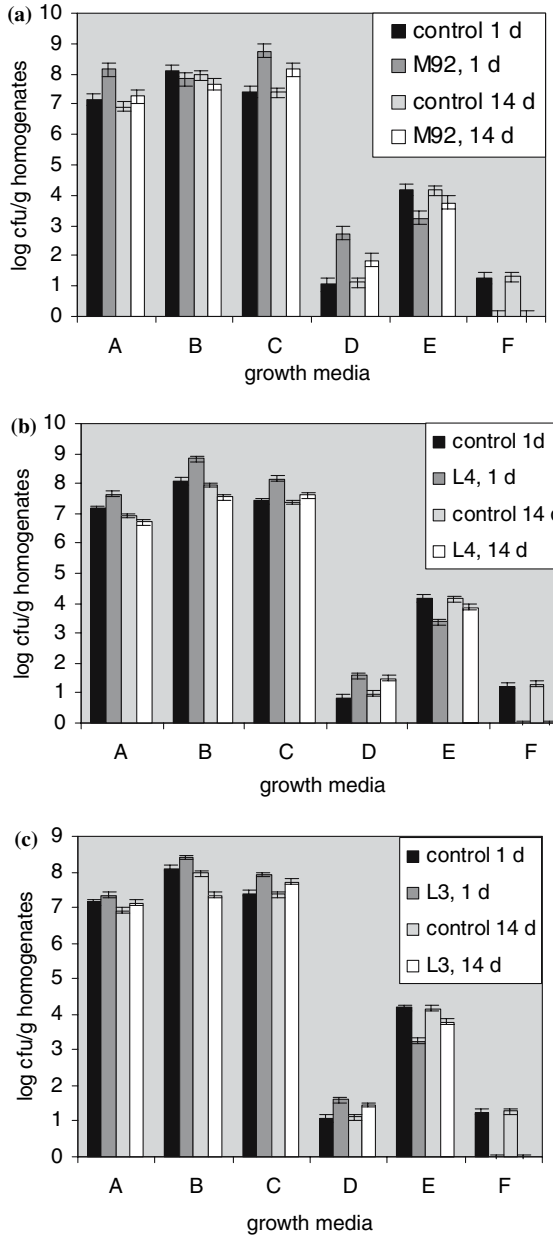

Figure 2. Bacterial count in small intestine of mice on days 1 and 14 after feeding with *L. acidophilus* M92 (a), *L. plantarum* L4 (b) and *E. faecium* L3 (c). A–F, for the legend see Table 1. Error bars represent standard deviations of the mean values of results from three replicates.

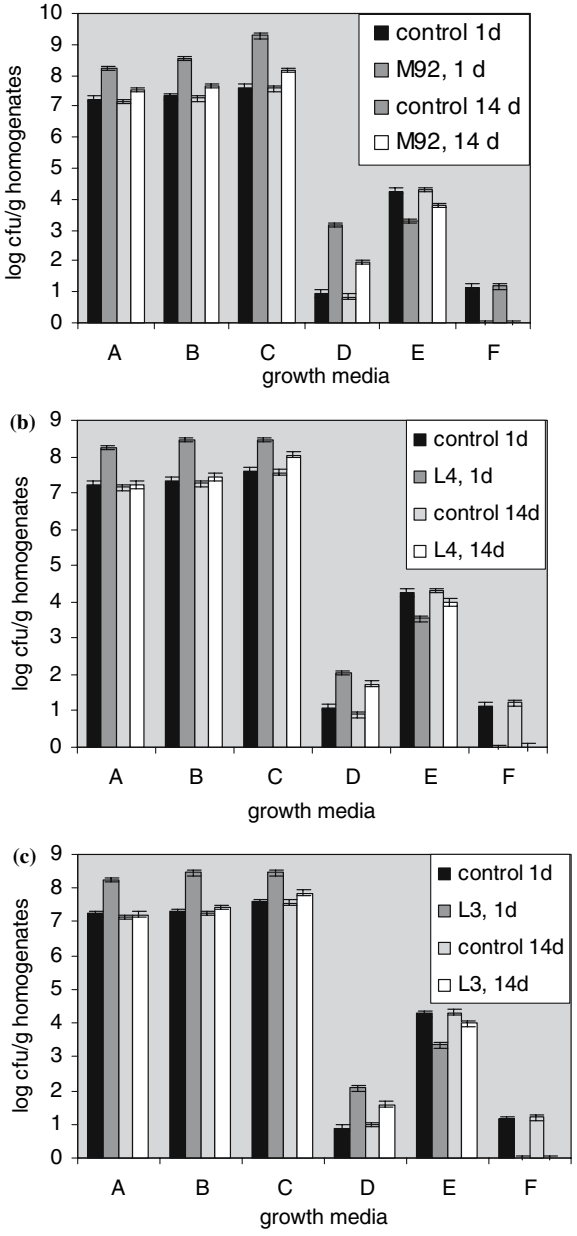

Figure 3. Bacterial count in large intestine of mice on days 1 and 14 after feeding with *L. acidophilus* M92 (a), *L. plantarum* L4 (b) and *E. faecium* L3 (c). A–F, for the legend see Table 1. Error bars represent standard deviations of the mean values of results from three replicates.

the probiotic strain survives gastrointestinal transit and adheres to the mucosa of the gut. Cell adhesion is a multistep process involving contact of the bacterial cell wall membrane and interacting surfaces (Pelletier *et al.* 1997; Del Re *et al.* 2000). *In vitro* adhesion ability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 was studied as a prerequisite for *in vivo* adhesion of these strains in Swiss albino mice, and the results have shown adhesivity of examined strains to intestinal epithelial cells of mice. Apparent better adhesion of *L. acidophilus* M92 in comparison to other two strains examined can be explained by the presence of S-layer proteins on the surface of *L. acidophilus* M92. Several authors have investigated the composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells. It has been proposed that S-layer proteins are involved in cell protection and surface recognition and that they could be potential mediators in the initial steps involved in adhesion (Schneitz *et al.* 1993; Green & Klaenhammer 1994; Mukai & Arihara 1994). Recently, in *Lactobacillus brevis* the S-layer has been demonstrated to function as an adhesin to human epithelial cells (Hynonen *et al.* 2002). The *in vitro* adhesion of *L. acidophilus* M92 to porcine ileal epithelial cells demonstrated that these processes are mediated by S-layer protein (Kos *et al.* 2003; Frece *et al.* 2005).

It was generally accepted that mouse is a good animal model for studying interactions between the gut microbes and the host, since it is the best studied of the intestinal

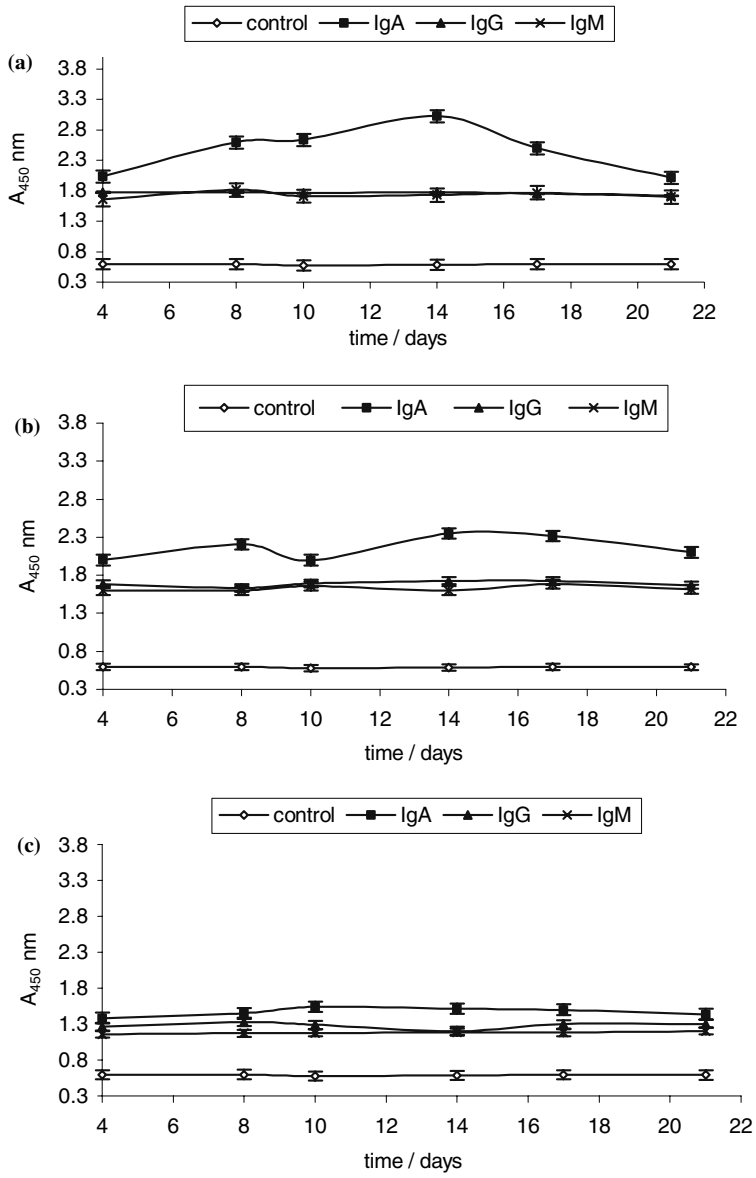

Figure 4. Determination of IgA, IgG and IgM antibodies in sera diluted 1:100 after oral imunization of mice with viable cells of *L. acidophilus* M92 (a), *L. plantarum* L4 (b), *E. faecium* L3 (c) by ELISA method. Error bars represent standard deviations of the mean values.

ecosystems of monogastric animals. Although there are some anatomical differences in the gastrointestinal tracts of mice and men, the faecal bacterial populations of the major groups of bacteria were similar (Tannock 1999) and hence it is suggested that the mouse could be considered as an animal model to study the dietary impact on the population of colonic bacteria (Wang *et al*. 2002). Survival of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 during transit through gastrointestinal tract of mice was determined in samples of faeces as rifampicin-resistant strains. The number of lactic acid bacteria (LAB) was increased and reduction of enterobacteria and sulphite-reducing clostridia was observed. These results suggested the capability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 to survive transit through the gastrointestinal tract of the mouse and to interact and compete with other microorganisms within the gut environment. Other workers have also showed that administration of certain strains of lactic acid bacteria can decrease the numbers of faecal *Escherichia coli*, anaerobic cocci and sulphite-reducing clostridia (Lund *et al*. 2002; Marquina *et al*. 2002), but our three selected strains have shown very strong and unusual inhibition against clostridia. This trait will be important in application of *L. plantarum* L4 and *E. faecium* L3 as starter cultures for silage and sauerkraut production, where species from the genus *Clostridium* frequently cause undesirable flavour by production of butyric and propionic acids. Besides the antibacterial activity, the advantage of *L. acidophilus* M92 strain is that it contains surface (S-layer) proteins responsible for better survival of this strain in the gastrointestinal tract of mice in comparison with *L. plantarum* L4 and *E. faecium* L3. These proteins have been shown to provide resistance to pepsin and pancreatic juice (Frece *et al*. 2005).

In vivo adherence ability of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 was determined in

homogenates of small and large intestine of Swiss albino mice. The increased number of LAB in the small and large intestines pointed out their adherence ability. All three strains examined showed better affinity to the large, than to the small intestinal epithelial cells of mice. The number of enterobacteria and sulphite-reducing clostridia in the small and large intestine of mice was reduced by the administered probiotic strains. Furthermore, the increased number of LAB in small and large intestine was detected even on day 14 after *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 administration, and the number of enterobacteria and sulphite-reducing clostridia in the small and large intestines of mice stayed decreased. This result could be a consequence of lactic acid and bacteriocin production which has been shown for all three probiotic strains examined. Their antibacterial activities were confirmed against some enteropathogenic bacteria *in vitro* (Šušković 1996; Kos 2001). The good preliminary results of silage and sauerkraut production with *E. faecium* L3 and *L. plantarum* L4 confirmed the importance of antibacterial activity of these strains, among other desirable traits like high growth rate and fast lactic acid production (Runjić-Perić 1996).

However, the number of probiotic bacteria detected 14 days after the administration of probiotic strains was lower than on the first day after administration of these strains. This observation is supported by the results of a human study completed by Goldin *et al.* (1992) where it was demonstrated that 60–80% of individuals consuming *L. rhamnosus* GG excreted this strain for 3–4 days, but only 33% of the population after 7 days. Lund *et al.* (2002) observed that the total amount of *E. faecium* increased significantly in human subjects during probiotic administration, but three weeks after cessation of probiotic intake, the *E. faecium* strain could not be detected in any of the subjects suggesting that this strain was either washed out or stayed in numbers below the detection limit. Therefore, it appears likely that daily administration is necessary for maintenance of high probiotic levels.

The possible competitive exclusion mechanisms of probiotic action include direct attack of probiotic cells by production of antibacterial substances, competition for nutrients and receptors on the gut enterocytes and also immune stimulation of the non-specific immune system. Therefore, the immunomodulating capacity of *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3, which means induction of antibody production in Swiss albino mice by these strains, was examined. After oral immunization of mice with viable probiotic cells, the levels of serum IgA, IgG and IgM antibodies from all groups were significant in addition to the control groups and the levels of total IgA antibodies were the highest. A significant increase in intestinal IgA antibody is an important result since IgA is the predominant mucosal antibody and plays an important role in protection against intestinal pathogens (Shu & Harsharnjit 2001). More recently, oral ingestion of cytoplasm fractions of probiotic bacteria has resulted in enhancement of IgA responses; this finding is similar to findings obtained after oral supplementation with live probiotic bacteria (Kaila *et al.* 1995; Takahashi *et al.* 1998). The highest immune response caused by *L. acidophilus* M92, could be a consequence of the immunomodulatory effect of its surface S-layer proteins. Results of Maldonado & Perdigon (2004) demonstrated that only small antigenic particles, but not the whole bacteria, could penetrate epithelial cells and make contact with immune cells. Therefore, there is the potential of S-layer proteins as immunomodulators, which will be investigated in future studies.

In conclusion, our findings indicate that *L. acidophilus* M92, *L. plantarum* L4 and *E. faecium* L3 are immunomodulators, because they stimulated the humoral immune response and have ability to survive and adhere in the intestinal tract of mouse and operate as effective probiotics that positively influence the intestinal microflora of the host. Confirmed probiotic properties together with technological properties are of great importance for their application in fermented foods as functional starter cultures for silage (*L. plantarum* L4, *E. faecium* L3), sauerkraut fermentation (*L. plantarum* L4) and fermented dairy products (*L. acidophilus* M92). However, further investigations are needed to determine the influence of food matrix and applied process technology on the functionality of these probiotic strains.

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