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ORIGINAL PAPER

Functionality of the S-layer protein from the probiotic strain *Lactobacillus helveticus* M92

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Abstract The objective of this study was the characterisation of the S-layer protein (SlpA) and its functional role in the probiotic activity of *Lactobacillus helveticus* M92. SlpA was isolated and identified by SDS-PAGE LC-MS/MS analysis. The *slpA* gene encoding the SlpA from *L. helveticus* M92 was sequenced and compared with other well characterised *slpA* genes. Sequence similarity searches revealed high homology with the SlpA of *Lactobacillus* strains. Purified SlpA showed significantly better immunomodulatory effects in orally immunised mice than *L. helveticus* M92 cells after SlpA removal. SlpA is involved in the autoaggregation of *L. helveticus* M92 cells and coaggregation of *L. helveticus* M92 with *S.* Typhimurium FP1 as these processes were negatively affected after SlpA removal from the cell surface. Therefore, the influence of oral treatment with *L. helveticus* M92 on an oral infection of mice by *S.* Typhimurium FP1 was investigated. Following the oral immunization of mice, with viable *L. helveticus* M92 and *S.* Typhimurium FP1 cells, the concentration in the luminal contents of total S-IgA and specific anti-*Salmonella* S-IgA antibodies, from all immunized mice was significantly higher compared to the control group or a group of mice infected only with *S.* Typhimurium FP1. These results demonstrate that the observed reduced infection by *S.* Typhimurium FP1 in mice with *L. helveticus* M92 is associated with competitive exclusion in the intestinal tract and enhanced immune protection conferred by the *L. helveticus* M92 and its SlpA.

Keywords *Lactobacillus helveticus* · Probiotic · S-layer protein · *Salmonella*

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Introduction

Among lactic acid bacteria many *Lactobacillus* strains have been characterised as probiotics. These strains were reported to exert health benefits such as protection against infection, e.g. by modulating the immune system. Immunostimulation and the ability to colonize mucosal surfaces have prompted efforts aimed at the use of these strains as vaccine delivery vehicles for oral immunization. Although the molecular basis of these probiotic activities are not well understood, several mechanisms have been proposed: contribution to mucosal barrier function, coaggregation with pathogens, competitive exclusion,

modulation of the immune response, decreasing of the luminal pH and secretion of specific compounds such as bacteriocins (Coconnier et al. 2000; Fayol-Messaoudi et al. 2005; Šušković et al. 2010). Still, adhesion of the probiotics to the mucosa is considered a main prerequisite for their survival and establishment in the gastrointestinal tract (GIT) where their health benefits are expected. Ability to temporarily colonize the intestinal epithelia allows probiotics to exert their beneficial effects longer (Servin and Coconnier 2003). Surface-located molecules such as lipoteichoic acid, lectin-like molecules and proteins have been identified as adhesins which specifically interact with different receptor moieties in the intestinal tissue (Martinez et al. 2000; Beganović 2008; Beganović et al. 2010).

Several species of the genus *Lactobacillus* possess surface S-layer protein (SlpA). Due to their structural regularity and the unique self-assembling properties S-layers have potential for many biotechnological applications (Åvall-Jääskeläinen and Palva 2005; Åvall-Jääskeläinen et al. 2008). Although the functional significance of *Lactobacillus* SlpA is not completely elucidated, these proteins are assumed to have an important role in bacteria, because a substantial part of the synthetic capacity of the cell is used for their production. The following biological functions have been shown or presumed for S-layers: (i) protective barrier against environmental hazards, (ii) control of the transfer of nutrients and metabolites, (iii) maintenance of cell shape and envelope rigidity, and (iv) promoter for cell adhesion and surface recognition (Vidgrén et al. 1992; Buck et al. 2005).

Strain *Lactobacillus helveticus* M92 was defined as probiotic according to proposed probiotic selection criteria (Kos et al. 2000; Šušković et al. 2000; Kos et al. 2003; Beganović 2008; Frece et al. 2009; Leboš Pavunc et al. 2010). This strain has the ability to survive simulated GIT conditions, is bile resistant, has antibacterial activity against some enteropathogenic and spore-forming bacteria, adheres to porcine ileal epithelial cells ex vivo, and as such is a potential candidate probiotic (Kos et al. 2000; Šušković et al. 2000; Kos et al. 2003). Furthermore, in vitro studies have shown that *L. helveticus* M92 assimilated cholesterol in the presence of bile, so it is postulated that this strain might help in lowering serum cholesterol in vivo (Šušković et al. 2000, 2001).

Lactobacillus helveticus M92 possesses an SlpA. A gene coding for the SlpA protein from *L. helveticus* M92 was detected by Southern blot hybridization (Frece et al. 2005a). Various data suggested that some of the *L. helveticus* M92 probiotic traits could be mediated by its SlpA, notably data concerning strain adhesion to the host cells. Kos et al. (2003) and Frece et al. (2005a) have shown a protective role of S-layers during transit through the GIT and during freeze-drying of cultures for probiotic applications. The role of the S-layer in the adherence of *L. helveticus* M92 to mouse and pig intestinal epithelial cells was demonstrated (Kos et al. 2003; Frece et al. 2005a). Adhesion is believed to be a requirement for the realisation of probiotic effects, such as pathogen exclusion and immunomodulation (Buck et al. 2005; Lebeer et al. 2008). Indeed, S-layers of *Lactobacillus* species have been shown to interact with the receptors on the host epithelial cells, thereby blocking receptor sites on the mucosal surfaces for the adherence of pathogenic species (van der Mei et al. 2003; Liu et al. 2010).

In the present study, the main objective was to characterise the SlpA and its functional role in the probiotic activity of *L. helveticus* M92. Previous research in our laboratory showed that oral administration of *L. helveticus* M92 can enhance immune functions in mice by increasing the concentrations of serum IgA, IgG, and IgM antibodies (Frece et al. 2005b). Hence, the possibility of inducing an immunogenic response by using purified SlpA in mice was investigated. In addition, the role of the *L. helveticus* M92 in enhanced protection of mice against oral challenge infection by *Salmonella enterica* serovar Thyphimurium FP1 was studied.

Materials and methods

Bacterial strains and growth conditions

Strains *L. helveticus* M92, *Lactobacillus fermentum* A8 and *S. enterica* serovar Typhimurium FP1 were obtained from the culture collection of the Department of Biochemical Engineering, Laboratory for Antibiotic, Enzyme, Probiotic and Starter cultures Technology, Faculty of Food Technology and Biotechnology, University of Zagreb. *L. helveticus* M92 and *L. fermentum* A8 were stored at −80°C in MRS

broth (Difco, Detroit, MI, USA) containing 30% (v/v) glycerol. In order to distinguish and monitor the survival of *L. helveticus* M92 in the GIT of the mice, rifampicin marking of the strain was performed according to Frece et al. (2005b). This was performed just for the purpose of the present research because a rifampicin-resistant variant of *L. helveticus* M92 is not applicable in food. *S.* Typhimurium FP1 was stored at −80°C in the nutrient broth (Biolife, Milano, Italy) with 30% (v/v) glycerol.

Extraction of the *L. helveticus* M92 SlpA

An overnight culture of *L. helveticus* M92 grown in MRS broth was used to inoculate 400 ml of MRS broth, to an optical density of 0.05 at 600 nm (UVIKON 931 spectrophotometer, KONTRON Instruments) and then cultivated at 37°C until the exponential phase of growth ($OD_{600\ nm}$ of 0.7). The cells were washed twice with an equal volume of ice-cold water and resuspended in 10 ml of 5 M LiCl and incubated for 30 min at room temperature. SlpA from *L. helveticus* M92 was LiCl extracted and extensively purified by dialysis using a method described by Frece et al. (2005a). After the freeze-drying of the dialysed S-layer (CHRIST Alpha 1-2 LDplus freeze-dryer, SciQuip, Shropshire, UK), protein concentration was determined by the Bradford method (Bradford, 1976) and the purity of the preparation was analysed by denaturing SDS-PAGE on 4–12% polyacrylamide minigels in MES buffer (200 V, 110 mA for 45 min). The gel was stained with Blue safe stain (Invitrogen, Carlsbad, CA) while shaking on an orbital shaker for 60 min after which the gel was washed twice with 100 ml of Milli-Q water.

Protein identification by mass spectrometry

In- gel digestion with sequencing grade modified trypsin (Promega, Madison, WI, USA) was performed as previously described by Beganović et al. (2010). For liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, tryptic protein digests were resuspended in 25 µl of precolumn loading buffer (0.08% TFA and 2% ACN in water). Tandem mass spectrometry analysis (LC/ESI-MS/MS) was performed on an Ultimate 3,000 LC system (Dionex, Voisins le Bretonneux, France) connected to a linear ion trap mass spectrometer (LTQ, Thermo Fisher, USA) by a nanoelectrospray interface. Peptide samples (4 µl) were loaded at a flow rate of 20 µl/min at precolumn (Pepmap C18; 0.3 × 5 mm, 100 Å, 5 µm; Dionex). After 4 min, the precolumn was connected to the separating nanocolumn Pepmap C18 (0.075 × 15 cm, 100 Å, 3 µm) and the gradient was started at 300 nl/min. All peptides were separated on the nanocolumn using a linear gradient from 2 to 36% of buffer B for 18 min (buffer A: 0.1% formic acid, 2% acetonitrile and eluting buffer B: 0.1% formic acid, 80% acetonitrile). Including the regeneration step, the run length was 50 min. Ionization was performed on the liquid junction with a spray voltage of 1.3 kV applied to a non-coated capillary probe (PicoTip EMITER 10 µm ID; New Objective, USA). Peptides ions were analysed by the Nth-dependent method as follows: (i) full Ms scan (*m/z* 300–2,000), (ii) ZoomScan (scan of the three major ions), (iii) MS/MS on these three ions with classical peptides fragmentation parameters: $Qz = 0.25$, activation time = 30 ms, collision energy = 40%. The time during which the same ion cannot be reanalyzed was set to 30 s.

Protein identification was performed using Bioworks 3.2^{TM} software (Thermo scientific). The Bioworks 3.2^{TM} search parameters included: trypsin specificity allowing one missed cleavage site, oxidation variable of methionine. The mass tolerance was fixed to 1.4 Da for precursor ions and 0.5 Da for fragment ions. The search result was filtered using Bioworks 3.2. using following criteria: Xcorrelation score (X_{corr}) > 1.7, 2.5, and 3.0 for mono-, di-, and tricharged peptides, respectively; peptide probabilities lower than 0.01; ΔCn defined by $[(X_{corr1} - X_{corr2})/X_{corr1}]$ bigger than 0.1 and only the first match result for each identified peptide. Upon completion of the LC/ESI-MS/MS run, the acquired MS/MS spectrum was analysed on LTQ linear ion trap mass spectrometer by SEQUEST protein search algorithm.

Detection of S-protein genes by PCR

SlpA gene-specific oligonucleotides used for PCR for the detection of *slpA* genes are listed in Table 1. Chromosomal DNA was isolated from *L. helveticus* M92 essentially as described by Frece et al. (2005a). A PCR reaction containing 1 µl of diluted template DNA, 0.2 mM deoxynucleoside triphosphate mix, 1 mM $MgCl_2$, 1 pmol/µl each oligonucleotide, and

Table 1 Oligonucleotides used as primers in PCR reactions in this study

Oligonucleotides	Nucleotide sequence (5′→3′)
Oligo-1	CAGATGATATCGCATGCTTAT TCAAAGTTAGCAACCTTAAC
Oligo-2	AACGCGTCGACATGCATCATT ATAGGCTCCTTTCTCATG
F-slp	ATGAAGAAAAATTTAAGAAT
R-slp	CACCGATCTTGTAGTA
R2-slp	CAGTAAGGCTACCTGGGATA
F2-slp	CAGCTAACCCAAATGTAACC

0.05 U/μl *Taq* polymerase was prepared and amplified under the following conditions: 94°C for 5 min followed by 25 cycles of 1 min at 94°C, 1 min at the oligonucleotide-specific annealing temperature (Ta) (Table 1), and 2 min of extension at 72°C, and then a hold at 72°C for 8 min. The negative control consisted of 1 μl sterile MilliQ H_2O and 1 μl diluted (1/20) DNA from *L. fermentum* A8 (an SlpA-negative strain). The presence or absence of PCR products and the sizes of the fragments from positive PCR reactions were analyzed using a 1% agarose gel. Nucleotide sequencing was performed with an ABI PRISM 310 Genetic Analyser-Bioscreen (PE Biosystems, USA) and sequence editing was performed with the Sequencher (version 3.0) software (Gene Codes Corporation, Ann Arbor, MI). Homology searches of the databases were done with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Anti-*Salmonella* effect of *L. helveticus* M92 in vitro and in vivo in experimental mice

Autoaggregation assay and coaggregation with *S.* Typhimurium FP1 were performed according to Kos et al. (2003) to explore the anti-*Salmonella* effect of *L. helveticus* M92 in vitro as salmonellosis is among the most common causes of foodborne human gastroenteritis worldwide (Golowczyc et al. 2007). Swiss albino mice have been used as a suitable animal model to study the events following experimental administration of probiotic strains (Frece et al. 2005a, b; Racedo et al. 2006; Frece et al. 2009; Hajduk et al. 2009). In the present study, we used this mouse model to study the dynamics of antibody responses to *L. helveticus* M92 expressing SlpA. Hence, female Swiss albino mice (four mice per group) for the in vivo experiments were treated as described previously (Frece et al. 2009). Rifampicin-resistant *L. helveticus* M92 cells and *S.* Typhimurium FP1 cells were centrifuged at 10,000 *g* for 2 min, washed 3 times and resuspended in sterile 0.5% NaCl solution to reach final concentration of ca. 10^{11} CFU/ml for *L. helveticus* M92, and ca. 10^3 CFU/ml for *S.* Typhimurium FP1, respectively, using standard curves for each bacterium. The concentration of probiotic cells corresponds to recommended daily probiotic dose, while the concentration of *Salmonella* cells represents a possible infective dose. Two groups of mice were orally treated daily with 200 μl of prepared suspension of probiotic cells during seven consecutive days. On the 3rd day, mice in one group (M92+S) were challenged by single oral infection with *S.* Typhimurium FP1, whereas the second group (M92) were not. A third group of mice (S) was infected with 200 μl of prepared suspension of *S.* Typhimurium FP1 cells at 3 days. A fourth (control) group of mice was fed only with standard rodent feed and received no bacterial infection. The group of mice treated daily with 200 μl of prepared suspension of *L. helveticus* M92 cells was used as negative control (M92 group). Each group of experimental animals consisted of four mice. In vivo adhesion test was carried out as described by Frece et al. (2005b).

Oral immunization of mice with purified SlpA, whole *L. helveticus* M92 cells or *L. helveticus* M92 without S-layer, as well as treatment with *L. helveticus* M92 in combination with *S.* Typhimurium FP1 infection and *S.* Typhimurium FP1 alone was performed as described previously (Frece et al. 2005b). The total S-IgA and specific S-IgA antibodies against *S.* Typhimurium FP1 were determined in polystyrene microtiter plates (NUNC) according to the method described by Frece et al. (2005b). All animal studies were performed according to ethical procedures set in the "Guide for the Care and Use of Laboratory Animal's of the National Research Council'' (1996).

Statistical analysis

Data were expressed as means of three independent trials ± standard deviation (SD). Data were subjected to a one-way analysis of variance. Statistical analysis was made by Statistica 9.0 software (StatSoft Inc.,

Tulsa, OK). A P value of <0.05 was considered to indicate a significant difference.

Results

Characterisation of *L. helveticus* M92 SlpA

A generally employed method for the removal of SlpA from the cell surfaces, LiCl extraction, was applied for the isolation of SlpA from *L. helveticus* M92. An identification of the *L. helveticus* M92 S-layer was performed by SDS-PAGE coupled to LC/ESI–MS/MS, which revealed that the molecular mass of the SlpA is 46541.9 Da (Table 2). BLASTP analysis of *L. helveticus* M92 SlpA sequence, obtained by mass spectrometry analysis, showed that this protein shared a high sequence identity to related SlpA of other *L. helveticus* and *Lactobacillus acidophilus* strains (Fig. 1).

PCR amplification with specific primers, designed from the protein sequence obtained by mass spectrometry analysis, was used to amplify the *slpA* gene from the genome of *L. helveticus* M92. A single 1.2 kbp PCR product was obtained from *L. helveticus* M92 chromosomal DNA, while DNA from *L. fermentum* A8 was used as negative control (Fig. 2). The nucleotide sequence of the *slpA* gene of *L. helveticus* M92 revealed an ORF (open reading frame) of 1,439 bp and is deposited in the GenBank database under the accession number HM140425 and needs to be processed for further annotation. A similarity search using the deduced nucleotide sequence of the *L. helveticus* M92 *slpA* with the algorithm BLASTN revealed a high level of sequence homology to the other *Lactobacillus* S-layer genes, showing between notably with *L. helveticus* (98% sequence identity over >1,330 nucleotides with GenBank accession no. AJ388558, X91199), *Lactobacillus crispatus* (85% sequence identity over 492 nucleotides with GenBank accession no. AY941197) and *L. acidophilus* (79% sequence identity over 1,415 nucleotides with GenBank accession no. X71412).

Functional role of SlpA in the probiotic activity of *L. helveticus* M92

To asses the functional role of the identified SlpA, the influence of S-layer removal from the surface of *L. helveticus* M92 cells on autoaggregation and coaggregation with the enteropathogen *S.* Typhimurium FP1 was investigated. These two characteristics were markedly affected by treatment of *L. helveticus* M92 cells with LiCl (which resulted in ca. 10% lower autoaggregation and ca. 16% lower coaggregation). This suggested that the SlpA is involved in autoaggregation as well as in coaggregation with *S.* Typhimurium FP1 (Table 3). Coaggregation is a part of competitive exclusion mechanism which, coupled with the antimicrobial activity of the probiotic strain, enables a decrease of the pathogenic load during infections. Therefore, an antagonistic activity of *L. helveticus* M92 against *S.* Typhimurium FP1 and its influence on the composition of the intestinal microflora was tested in vivo on Swiss Albino mice. Seven days after the oral administration of *L. helveticus* M92 in combination with *S.* Typhimurium FP1 challenge, enterobacterial counts as well as *Salmonella* sp. counts decreased by ca. 2 log units compared to the enterobacteria and *Salmonella* sp. counts determined in the group of mice infected only with *S.* Typhimurium FP1 (Fig. 3). Additionally, the LAB and rifampicin-resistant LAB (representing the probiotic strain administered) counts in the small and large intestines

Table 2 *L. helveticus* M92 SlpA identified by tandem mass spectrometry

Protein	Peptide sequence	ΔCn	Xcorrelation score	Molecular mass (Da)
S-layer	K.APHTFTVNVK.A	0.54	50.21	46541.9
	K.YFAAQYDKKQ.L	0.57		
	K.SDTMPAIPGL.S	0.56		
	K.VSNLNVGLLVL.A	0.43		
	K.RYNSVSVL.P	0.69		

Protein is given by delta-correlation scores (ΔCn), Xcorrelation score and molecular weight theoretical value for protein obtained from NCBI database

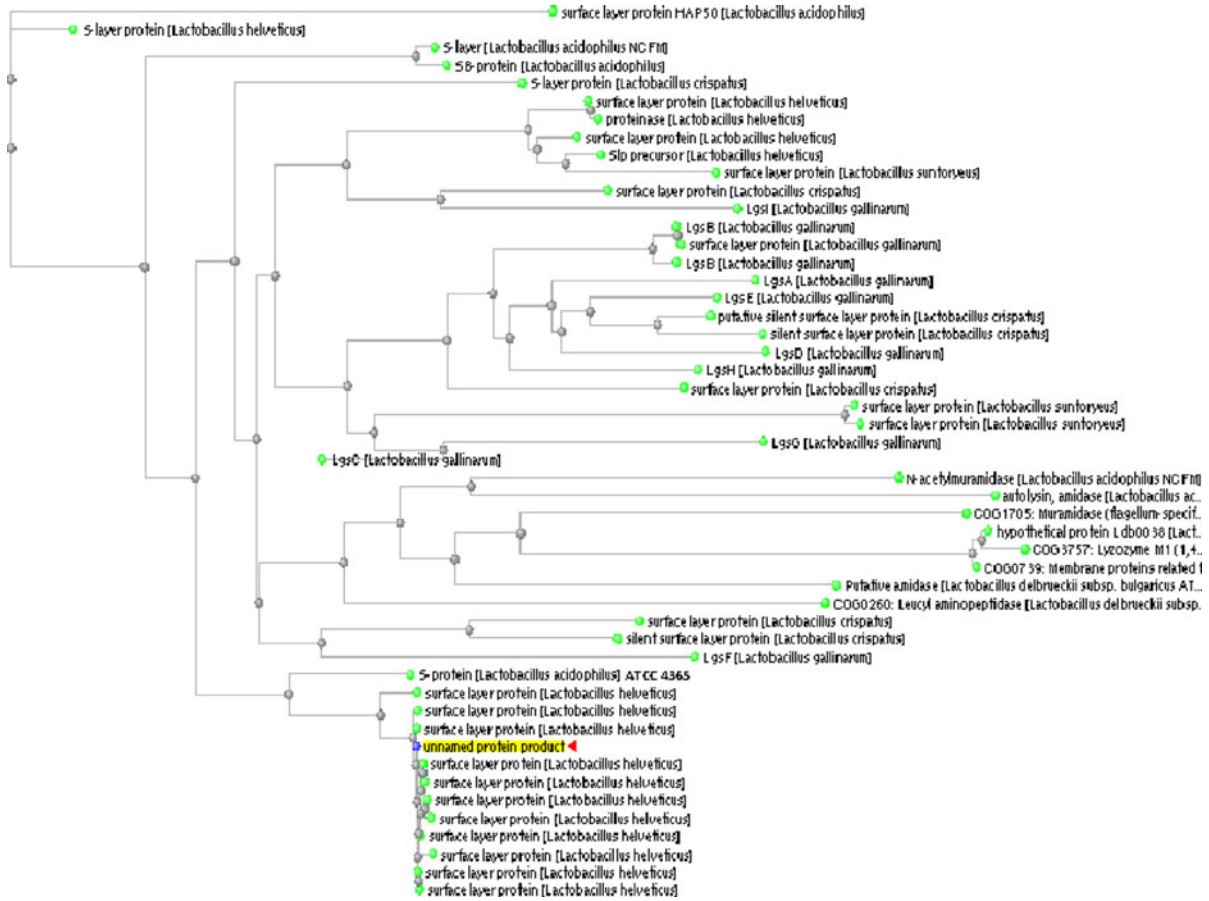

Fig. 1 Dendrogram depicting the relationships among SlpA proteins from strains belonging to *Lactobacillus* genus. The tree was constructed by BLAST pairwise alignments, max sequence distance 0.75

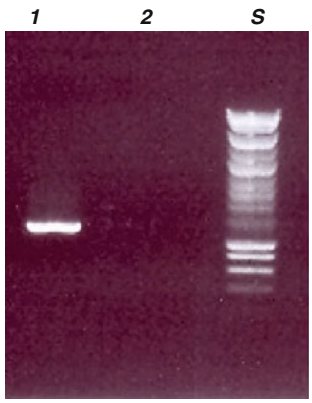

Fig. 2 PCR detection of S-protein gene. PCR analysis was performed with the specific primers ATGAAGAAAAATTTA AGAAT and CACCGATCTTGTAGTA. *Lane 1 L. helveticus* M92, *Lane 2 L. fermentum* A8, *Lane S* DNA molecular mass standard

of mice were significantly increased compared to the control group by ca. 1.5–2 log units, respectively (Fig. 3).

Furthermore, the oral immunization of mice with purified *L. helveticus* M92 *slpA* protein and with *L. helveticus* M92 cells without SlpA stimulated the immune response in mice (Figs. 4a–c, 5b). After the oral immunisation of mice with purified SlpA, the levels of serum IgA, IgG, and IgM antibodies were significantly higher in comparison to the levels of these antibodies in the control group of mice and in the group of mice immunized with *L. helveticus* M92 cells either with or without SlpA (Fig. 4a–c). The highest luminal content of anti-*S.* Typhimurium S-IgA antibody was detected in the group of mice infected with *S.* Typhimurium FP1 in combination with *L. helveticus* M92 (Fig. 5a). Total secretory

Table 3 Aggregation percentage and coaggregation of probiotic strains and *S.* Typhimurium FP1 after 5 h of incubation in PBS (pH = 7.2)

Autoaggregation	(%)
L. helveticus M92	70.29 ± 5.23
L. helveticus M92 without S-protein	53.73 ± 4.63
S. Typhimurium FP1	5.46 ± 1.78
L. fermentum A8	60.9 ± 3.91
Coaggregation with *S.* Typhimurium FP1	(%)
L. helveticus M92	31.43 ± 3.18
L. helveticus M92 without S-protein	23.91 ± 1.92
Purified S-protein	8.76 ± 1.25
L. fermentum A8	7.53 ± 2.71

S-IgA antibody levels were the highest after the oral administration of mice with *L. helveticus* M92 alone and were higher in combination with *S.* Typhimurium FP1, than in the control group of mice (Fig. 5b).

Discussion

Probiotics exert several beneficial effects on human health, including interaction with the immune system, production of antimicrobial substances, enhancement of the mucosal barrier function and competition with enteropathogens for adhesion sites (Boesten and de Vos 2008). Although the molecular mechanisms by which probiotic bacteria exert health benefits to the host are largely unknown, it has been accepted that surface molecules, mostly proteins, are involved in their adhesion and colonization in intestinal tract, which are correlated with their probiotic activity (Åvall-Jääskeläinen and Palva 2005). This research is aimed to elucidate if there is a relationship between some important probiotic traits of *L. helveticus* M92, such as adhesion ability, *Salmonella* exclusion and immunomodulation in mice intestinal tract, and its SlpA. The *slpA* gene of *L. helveticus* M92 was originally identified by Southern blot hybridization (Frece et al. 2005a). Here, the *slpA* gene was sequenced and sequence similarity searches revealed high homology with the other SlpAs of *Lactobacillus* strains. The identification of the *L. helveticus* M92 surface paracrystalline SlpA, encoded by *slpA* gene, was also achieved by means of mass spectrometry analysis, SDS-PAGE coupled to LC-MS/MS. Previously Kos et al. (2003) suggested that this poorly soluble SlpA could be responsible for the hydrophobicity of *L. helveticus* M92 cells. Kos et al. (2003) and Frece et al. (2005a) demonstrated, in ex vivo experiments, that SlpA was involved in *L. helveticus* M92 adhesion to the intestinal epithelial cells of a pig and a mouse. Hence, it was postulated that the SlpA could be responsible for the interactions with intestinal epithelial cells in vivo and for the autoaggregation ability of this strain. The results of the present study demonstrated that the autoaggregation percentage determined for *L. helveticus* M92 was significantly lower after the removal of S-layer from the bacterial surface. These results support the hypothesis that the S-layer from *L. helveticus* M92, through

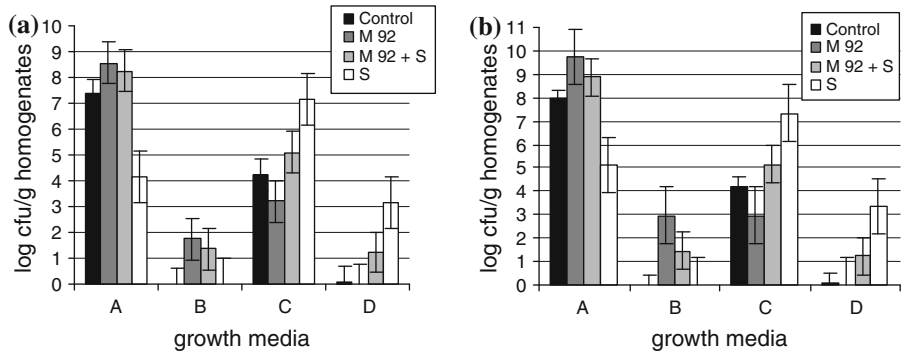

Fig. 3 Bacterial viable cell count determined in the small intestine (**a**) and large intestine (**b**) of mice, 7 days after the oral treatment with *L. helveticus* M92, *L. helveticus* M92 in combination with *S.* Typhimurium FP1, or after the challenge with *S.* Typhimurium FP1. Total LAB (*A*) and rifampicin-resistant LAB (*B*) on MRS-agar; *Enterobacteriaceae* (*C*) on Violet red bile glucose agar, *Salmonella* sp. (*D*) on Brilliant green violet agar. Error bars represent standard deviations of the mean values of results from three replicates

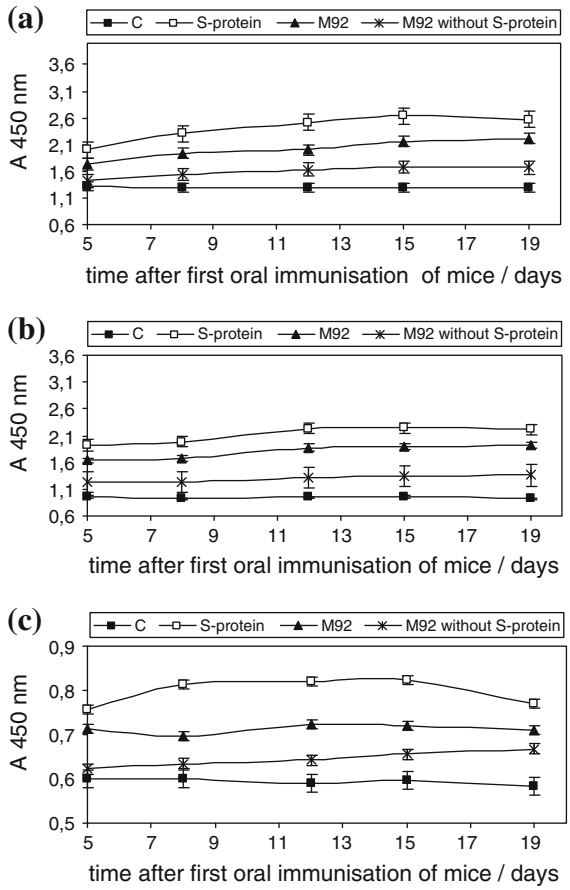

Fig. 4 Total **a** IgA, **b** IgG, and **c** IgM antibodies in sera, determined during and after oral immunisation of mice with purified S-protein, with viable *L. helveticus* M92 cells and with viable *L. helveticus* M92 cells after SlpA removal, by ELISA method (C–control). Sampling started on 5th day after the first oral immunization that was performed during seven consecutive days

autoaggregation, could be involved in adhesion, which is known to be a prerequisite for the colonization of the GIT by probiotic strains in high viable cell count (Kos et al. 2003). This is in agreement with Mobili et al. (2009) who showed a correlation between the structure of SlpAs from different *L. kefir* strains and aggregation properties of whole bacterial cells.

In addition to the above, coaggregation of probiotic strains with pathogens, as well as their ability to displace pathogens through antimicrobial activity, is of importance for the therapeutic manipulation of an aberrant intestinal microbiota (Servin and Coconnier 2003). Interestingly, coaggregation was significantly reduced when *L. helveticus* M92 cells were lacking SlpA compared to the results obtained with whole *L. helveticus* M92 cells, again implicating the importance of the S-layer in this process. Coaggregation, which is thought to facilitate the clearance of pathogens during mucus flushing, is described as an additional mechanism to decrease the pathogenic load during infections. Moreover, adhesion to epithelial cells and mucus mediates colonisation of the GIT by lactobacilli and may be prerequisite for competitive exclusion of enteropathogenic bacteria and immunomodulation of the host (Perdigón et al. 2003). Johnson-Henry et al. (2007) reported that SlpA extracts from *L. helveticus* had inhibited enterohaemorrhagic *Escherichia coli* adhesion to host epithelial cells, while Buck et al. (2005) and Frece et al. (2005a) demonstrated a decrease of *L. crispatus* and *L. helveticus* M92 ability to bind to intestinal epithelial cells in vitro after the removal or disruption of SlpAs. A complementary approach using transmission electron microscopy

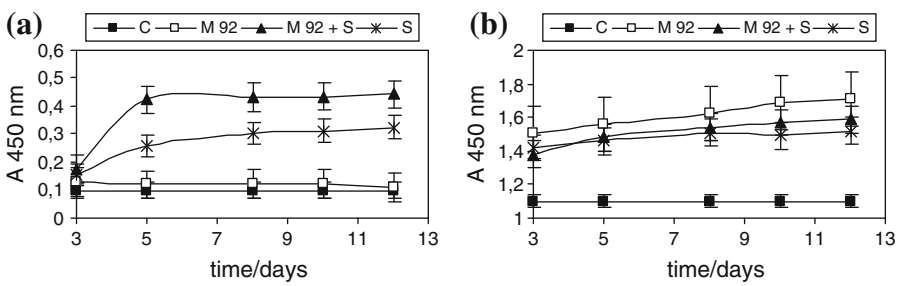

Fig. 5 Determination of **a** secretory-IgA (S-IgA) specific against *S.* Typhimurium FP1 and **b** total secretory IgA (S-IgA) by ELISA method in the intestinal fluid after the oral challenge of mice with viable cells of *S.* Typhimurium FP1 alone (S) or in combination with *L. helveticus* M92 (M92+S), or with *L. helveticus* M92 alone (M92), (C–control). *Error bars* represent standard deviations of the mean values

could be useful for the confirmation of both the presence of the paracrystalline SlpA and the functional role of SlpAs or any other cell surface structures important for probiotic activity, such as adhesion to intestinal epithelial cells (Johnson-Henry et al. 2007).

The possible role of *L. helveticus* M92 in the competitive exclusion of *S.* Typhimurium FP1 was investigated because *Salmonella* infections are one of the primary causes of gastroenteritis in humans. The use of antibiotics in the treatment of *Salmonella* often becomes less efficient due to the spread of antibiotic resistance (Casey et al. 2004). Therefore, the application of probiotic LAB bearing activity against *Salmonella* sp. could be effective as an alternative strategy (Coconnier et al. 2000; Casey et al. 2004; Golowczyc et al. 2007). Previously, Kos et al. (2008) found, by in vitro competition tests, that the growth of *S.* Typhimurium was completely inhibited after 10 h of incubation with *L. helveticus* M92 (Kos et al. 2008). The SlpAs are involved in coaggregation, but do not possess antimicrobial activity (data not shown). The intact probiotic cells, capable of producing antimicrobials, were necessary for the competitive exclusion of *Salmonella*. Therefore, in this study, involvement of the *L. helveticus* M92 in the reduction of gastrointestinal *Salmonella* infection in vivo was studied. The increased viable cell counts of LAB in small and large intestine of mice were detected 7 days after *L. helveticus* M92 administration. The viable cell counts of enterobacteria and *Salmonella* sp., in small and large intestine of mice, were lower compared with those obtained in the group of mice infected only with *S.* Typhimurium FP1. According to Golowczyc et al. (2007) SlpAs could interact with specific sites on *Salmonella* surface involved in the first step of mucosal infection or could modify or mask *Salmonella* structures necessary for the invasion of intestinal epithelial cells. Surface layer extracts from *L. helveticus* R0052 were recently shown to inhibit the adhesion of *E. coli* O157:H7 to epithelial cells (Johnson-Henry et al. 2007). Recently Liu et al. (2010) demonstrated that *Lactobacillus plantarum* surface layer adhesive protein decreased *E. coli* adhesion to Caco-2 cells and rescued *E. coli*-induced alterations in tight junction structures and permeability of Caco-2 cell monolayers. This process seems to be partly mediated by high hydrophobicity of the S-layers, and it is not yet known whether it involves interactions with specific receptors. Similar results were obtained for the S-layers of *L. crispatus* ZJ001, which were shown to play a role in the competitive exclusion against enterohemorrhagic *E. coli* and *S. enterica* serovar Typhimurium (Chen et al. 2007). Furthermore, Horie et al. (2002) reported that SlpAs of *L. crispatus* JCM 5810 inhibited the adhesion of *E. coli* to Matrigel, and this effect was ascribed to the competition with *E. coli* for the same binding sites in the extracellular matrix.

The possible competitive exclusion mechanisms of probiotic include ability of their cells to produce antibacterial substances and to compete for nutrients and receptors on the gut enterocytes, but also immune stimulation of the specific and non-specific immune system. Hence, the possible functional role of the orally administered, purified SlpA in the imunomodulation conferred by *L. helveticus* M92 in mice was studied. Here it must be emphasised that *L. helveticus* M92 SlpA evoked higher total serum IgA, IgG, and IgM than *L. helveticus* M92 cells without SlpA, but the S-layer did not evoke a specific humoral immune response after oral application and as such is suitable for probiotic application as an immunomodulator. In addition, the concentrations of the serum IgA, IgG, and IgM antibodies were lower when mice were orally immunised by *L. helveticus* M92 cells without S-protein compared to the levels of antibodies determined in the samples from the group of mice orally immunised with whole *L. helveticus* M92 cells, but were still higher compared to the control. SlpA, as the outer shell of proteins in lactobacilli (Delcour et al. 1999), may have the highest probability of the intimate interaction with the immune cells associated with the gut. Previously, between different probiotic strains assessed, *L. helveticus* M92, showed the highest capacity of activation of the immune system (Frece et al. 2005b). The immunomodulation capacity of the S-layer could be one of its functions, as was reported for the S-layer of the *Bacteroides* (Yoneda et al. 2003) and *Campylobacter* species (Grogono-Thomas et al. 2003). It seems that the S-layer, besides its involvement in the adhesive capacity and certain cell surface traits such as hydrophobicity and autoaggregation of *L. helveticus* M92, contributes to the immunostimulatory activity of this probiotic bacterium. Whereas bacterial interactions are the most accepted mechanism for the reduction of *Salmonella* count observed by *L. helveticus* M92 application, stimulation of an effective innate immune response

by the probiotic strain is more likely due to the rapidity of this response. Therefore, the effect of probiotic strain *L. helveticus* M92 on the total and specific mucosal antibody response levels in mice after challenge with *S.* Typhimurium FP1 was investigated. The significant increase in intestinal secretory IgA (S-IgA) antibody after *L. helveticus* M92 application is an important result. IgA is the predominant mucosal antibody and plays a key role in protection against dietary antigens and intestinal pathogens. This could be assigned to the surface SlpA of *L. helveticus* M92. Recently, Konstatinov et al. (2008) found that 45 kDa SlpA from the surface of *L. acidophilus* NCFM was involved in the regulation of immature dendritic cells (DC) as well as cytokine production. The cellular contacts of DCs and *L. acidophilus* NCFM involve interactions between dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), a DC-specific receptor DC-SIGN, and SlpA, the dominant protein expressed by *L. acidophilus* NCFM.

The enhanced immunity and reduced disease severity conferred by *L. helveticus* M92 in this study against *S.* Typhimurium FP1, with the evidences from the previous studies of immunity-enhancing and antimicrobial effect of *L. helveticus* M92 against pathogens (Frece et al. 2005a, b; Kos et al. 2008) suggest that dietary supplementation with this defined probiotic strain may represent an effective biotherapeutic means of countering gastrointestinal infections in humans.

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Conflict of interest None.

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