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Lactobacillus plantarum 1K from "SLAVONSKI KULEN" as natural probiotic starter culture

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Summary

The influence of the oral treatment with probiotic strain *Lactobacillus plantarum* 1K isolated from „Slavonski kulen" on an oral infection of mice by *Salmonella* sp. was investigated. Mice were fed with milk-based diets supplemented with *L. plantarum* 1K for three days prior and following an oral challenge with *Salmonella* sp. Survival, competition, adhesion and colonization of *L. plantarum* 1K and influence on infection with *Salmonella* sp. were monitored in the gastrointestinal tract of mice. After the oral treatment of mice with *L. plantarum* 1K in combination with *Salmonella* sp., the total number of lactic acid bacteria in faeces and in intestinal homogenates was increased, on the contrary, the total number of enterobacteria and *Salmonella* sp. was reduced. These results demonstrate that *L. plantarum* 1K can reduce the severity of infection due to the pathogenic *Salmonella* sp., and suggest that this reduction is associated with competitive exclusion in the intestinal tract. Considering that *L. plantarum* 1K has demonstrated the basic functional criteria for the selection of probiotic strains, as such, it can be used as functional autochthonous starter culture for fermented meat products.

Keywords: probiotic, *Lactobacillus plantarum*, *Salmonella* sp., infection, Slavonski kulen

Introduction

Nowadays, increasing number of studies is focused on the isolation and identification of autochthonous functional starter cultures, with the aim of developing new functional meat products, which will be recognized and labelled as autochthonous due to the influence of climate and vegetation of the region in which they are produced (Frece, 2007). Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation processes and are considered technologically fundamental. They are able to decrease pH by lactic acid production, produce bacteriocins to prevent the growth of pathogenic and

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spoilage microorganisms, provide diversity by the modification of raw material to obtain new sensory properties, improve the safety, the stability and the shelf life of meat products (Fontana et al., 2005; Frece et al., 2005 a, b, c; Frece et al., 2009) and they also contribute to the development of flavour, colour and texture (Kovačević, 2001). One of the most important properties of LAB as probiotics is protection against pathogens in the intestinal tract of the host (Šušković et al., 2001; Kos et al., 2003, Frece et al., 2005c; Frece et al., 2009). The role of antimicrobial compounds produced by probiotic strains as prophylactic agents against enteric infections is crucial and well documented (Šušković et al., 2001, Kos et al., 2003; Golowczyc et al., 2007). The antimicrobial activity of starter cultures and probiotic bacteria has been attributed to the production of metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide ethanol, diacetyl, acetaldehyde, other low molecular mass compounds with antimicrobial activity and bacteriocins (Šušković et al., 2001).

Enteric bacterial pathogens represent a major cause of gastrointestinal disease worldwide. Current measures to control gastrointestinal infections rely heavily on the use of antimicrobial chemotherapeutic and chemoprophylactic agents. However, widespread use of antibiotics in public health is discouraged due to complications including the emergence of drug-resistant strains and the potential for chronic toxicity (Shu and Harsharnjit, 2002). Therefore, there is an effort to develop alternative, non-pharmaceutical strategies for controlling gastrointestinal bacterial infection (Vinderola et al., 2007, Spinler et al., 2008).

Probiotic bacteria, mainly lactic acid bacteria (LAB) and bifidobacteria, are previously shown to have beneficial effects on immunomodulation, alleviation and prevention of diverse intestinal disorders (Servin and Coconnier, 2003; Golowczyc et al., 2007). This could be due to their property to prevent the adherence, establishment and invasion of specific enteropathogens (Servin, 2004, Silva et al. 2004; Frece et al., 2005a; Matijašić et al., 2006). Several mechanisms have been proposed: contribution to mucosal barrier function, competitive exclusion, modulation of the immune response, coaggregation to pathogens, decreasing of the luminal pH via the production of lactic acid and secretion of specific compounds such as bacteriocins (Coconnier et al., 2000; Kos et al., 2003, Frece et al., 2005a; Golowczyc et al., 2007; Kos et al., 2008; Frece et al., 2009).

Considering that *L. plantarum* 1K has satisfied the basic criteria for selection of probiotic strains *in vitro* conditions, and showed strong antimicrobial activity against *Salmonella* sp. (results were not shown), *in vivo* studies in experimental mice were carried out.

Therefore, the aim of this study was to investigate survival, competition, adhesion and colonization of *L. plantarum* 1K in the gastrointestinal tract of mice, and if the oral treatment with probiotic strain *L. plantarum* 1K had the protective effect against *Salmonella* sp., by influencing the intestinal microflora of mice.

Materials and Methods

Bacterial strains and growth conditions

Probiotic strain *L. plantarum* 1K and *Salmonella* sp. are from the culture collection of the Department of Biochemical Engineering, Laboratory for general microbiology and food microbiology, University of Zagreb. *L. plantarum* K1 was stored at –70 °C in the Man Ragosa Sharpe (MRS) broth (Difco, Detroit, MI, USA) with 30 % (v/v) glycerol. *Salmonella* sp. was stored at –70 °C in the Brilliant green broth (Biolife, Milano, Italy) with 30 % (v/v) glycerol.

Mice

4 months old female Swiss albino mice weighing from 22 to 24 g were used after a month quarantine period. Each experimental group consisted of 4 mice, housed in cage, kept in a controlled atmosphere (temperature 22 ± 2 °C; humidity 55 ± 2 %) with a 12 h light/dark cycle. Mice had continual access to water and were fed *ad libitum* on skim milk powder (SMP)-based diet contained SMP (53 %), corn oil (8 %), vitamin (5 %), minerals (5 %), corn flour (28 %), and cellulose (1 %). All experimental procedures were carried out according to the standards set in the "Guide for the Care and Use of Laboratory Animal's of the National Research Council" (1996).

Feeding procedures and challenge with Salmonella sp.

L. plantarum 1K cells were cultured in MRS-broth and *Salmonella* sp. was cultured in the Brilliant green broth, both aerobically at 37 °C for 18 hours. Cells were removed by centrifugation at 10 000 *g* for 2 min, washed three times and resuspended in sterile 0.5 % NaCl solution to final concentration of 1 x 10^{11} viable bacterial cells per ml for *L. planatrum* 1K, and final concentration of 1 x 10^3 viable bacterial cells per ml for *Salmonella* sp. Mice were orally treated with 200 µl of prepared suspension of bacterial cells *L. plantarum* 1K during 7 consecutive days. On a 3rd day, single oral infection with *Salmonella* sp. followed. Another group (4 mice) was infected with 200 µl of prepared suspension of *Salmonella* sp. cells. Control group (4 mice) was fed only with standard rodent feed. The group of mice (4 mice) treated with 200 µl of prepared suspension of *L. plantarum* 1K cells during 7 consecutive days, were negative control.

Faecal sampling

After feeding period of 7 consecutive days, the survival of potential probiotic strain *L. plantarum* 1K and the infection of *Salmonella* sp., during transit through gastrointestinal tract of mice was determined in faecal samples.

Additionally, the influence of a challenge by *Salmonella* sp., alone and in combination with potential probiotic strain, on *Enterobacteriaceae* and *Salmonella* sp. count in faeces was also monitored after feeding period. 1g wet weight samples were homogenized in 1 ml sterile 0.5 % NaCl solution and serially diluted before plating on non-selective medium Peptone yeast extract glucose agar (PYEG, Biolife, Milano, Italy) and selective media: MRS-agar for LAB count, Violet red bile glucose agar (VRBG, Biolife, Milano, Italy) for *Enterobacteriaceae* count and finally Brilliant Green Phenol Red agar (BGPR agar, Merck, Darmstadt, Germany) for the *Salmonella* sp. counts. The plates were incubated aerobically for 48 h at 37 °C. LAB, *Enterobacteriaceae* and *Salmonella* sp. were identified on the basis of colony morphology, Gram staining, cell morphology, the catalase reaction. The identity of bacteria was also confirmed using API 50CH and API 20E identification kits (BioMérieux, France).

In vitro adhesion test

In vitro adhesion test was done according Frece et al., 2005 a, b, c.

In vivo adhesion test

Adhesion ability of examined probiotic strain was determined in homogenates of small and large intestine of Swiss albino mice 7 days after feeding with *L. plantarum* 1K with and without infection of mice with *Salmonella* sp. The influence of a challenge by *Salmonella* sp., alone or in combination with potential probiotic strain, on *Enterobacteriaceae* and *Salmonella* sp. count in the intestine of mice, was also monitored 7 days after expermimental period. The samples of small and large intestine, 5 cm long, were gently rinsed with sterile 0.5 % NaCl solution and homogenized using a Teflon homogeniser (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 *Feeding procedures and challenge with Salmonella* sp.

Immunization

The potential probiotic bacteria were orally administrated to each mice (4 mice/group) eight times over a period of eight successive days. A dose of 200 µl of sterile 0.5 % NaCl solution containing 1 x 10^{11} viable bacterial cells of *L. plantarum* 1K was daily directly given to each mice, 3 days before the challenge with *Salmonella* sp. (1 x 10^3 viable bacterial cells) and then throughout the remaining experimental period. The control group (4 mice) was given 200 µl of sterile 0.5 % NaCl solution. All the mice were fed *ad libitum*. On 1stand 14 th day after first immunization, treated and control animals were anesthetized and sacrificed by cervical dislocation. The small intestine from each mouse was recovered and its contents were flushed with 1.5 ml PBS.

Statistical methods

A randomized complete block design which incorporated the 4 treatments (control, *L. plantarum* 1K (1K), *L. plantarum* 1K in combination with *Salmonella* sp. (1K + S), and *Salmonella* sp. (S)) and three block trials was used for analysis of the response variables. Analysis of variance of the randomized complete block design was carried out using a general linear model of SAS (1995) where the effect of treatment and replicates were estimated for all response variables. Duncan's multiple comparison test was used as a guide for pair comparisons of the treatment means. Differences between treatments that are described subsequently as being significant were determined at least $P< 0.05$.

Results and Discussion

Consumption of some strains of LAB has been shown to protect animals and human against a wide range of gastrointestinal pathogens (Servin et al., 2003; Servin, 2004, Vinderola et al., 2007). Antagonistic activity of LAB against *Salmonella* sp. has been extensively studied (Coconnier et al., 2000; Tsai et al., 2005; Vinderola et al., 2007, Golowczyc et al., 2007), however there is a lack of information on the protective effects of LAB against *Salmonella* sp. infection *in vivo*.
The adhesion of *Salmonella* sp. to the surface of epithelial cells represents the first direct contact with host. This is a prerequisite for the subsequent steps in pathogenesis that lead to mucosal infection, systemic spread and disease (Darwin and Miller, 1999). Inhibition of the *Salmonella* sp. invasion into the epithelial cells is the first step forward in the disease prevention. There is an effort to develop alternative strategies for controlling *Salmonella* sp. infections, like possibility to use live, oral biomodulatory agents, such as probiotic LAB.
One of the selection criteria for probiotic strains is to adhere to intestinal tract of the host. Therefore we investigated *in vitro* adhesion of *L. plantarum* 1K on intestinal epithelium cells of mice. *L. plantarum* 1K has shown very good adhesion capability on intestinal epithelium cells of mice (Fig. 1).

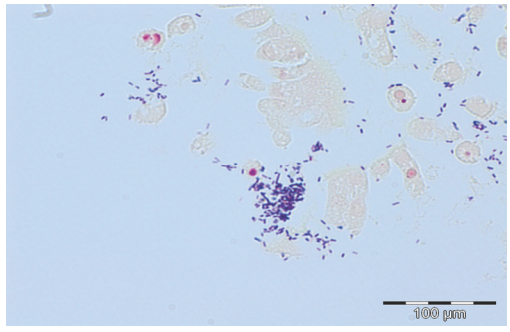

Fig.1. Adhesion of *L. plantarum* 1K to the intestinal epithelium cells of the mouse

Considering that *L. plantarum* 1K fulfilled *in vitro* selection criteria demanded for probiotic strains and exerted inhibitory activity against a wide range of bacteria including some pathogens (results have not shown), therefore, the influence on *Salmonella* sp. infection in Swiss albino mice by treatment with potential probiotic strain *L. plantarum* 1K was studied. The results have shown that the adhesion of *Salmonella* sp. to intestinal epithelial cells of mice was reduced (Tables 1-3). *L. plantarum* 1K survived transit trough gastrointestinal tract of mouse and interacted and competed with other microorganisms within the gut environment (Table 1). *L. plantarum* 1K showed better affinity to the large intestinal, than to the small intestinal epithelial cells of mice (Tables 2-3). Furthermore, the increased number of LAB in small and large intestine was detected 7 days after *L. plantarum* 1K administration, and the number of enterobacteria and *Salmonella* sp. in small and large intestine of mice was lower in comparison to the group of mice infected only with *Salmonella* sp (Tables 2-3). These results could be a consequence of lactic acid and bacteriocin production. Namely, the antibacterial activity of this strain was confirmed *in vitro* against some lactic acid bacteria and some enteropathogenic bacteria including *Salmonella* sp. (results are not shown).

Other authors have also reported that administration of certain strains of LAB can decrease the numbers of faecal *Escherichia coli*, anaerobic coci and sulphite-reducing clostridia (Lund et al., 2002; Marquina et al., 2002). The possible competitive exclusion mechanisms of probiotic action include ability of probiotic 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 (Marquina et al., 2002).

The results of this study demonstrated that dietary supplementation with *L. plantarum* 1K can reduce the severity of *Salmonella* sp. infection in mice (Tables 1-3). After oral infection with the pathogen, mice orally treated with probiotic *L. plantarum* 1K exhibited lower cumulative morbidity, but maintained significantly higher feed intake, compared to mice challenged with *Salmonella* sp. alone (results are not shown).

The reduced disease severity conferred by *L. plantarum* 1K in this study against *Salmonella* sp., suggest that dietary supplementation with this defined probiotic strain may represent an effective biotherapeutic means of countering gastrointestinal infection in humans.

Antimicrobial properties of *L. plantarum* 1K and its protection against pathogen infections are of great importance for the application of this strain in fermented dairy products as functional starter culture.

Table 1. Comparison of the total bacterial counts in faeces of mice, fed only with standard rodent feed (control), after the oral treatment of mice with probiotic strain *L. plantarum* 1K (1K), *L. plantarum* 1K in combination with *Salmonella* sp. (1K + S), or after the challenge of mice only with *Salmonella* sp. (S). Total number of bacteria (A) on Peptone yeast extract glucose agar; total lactic acid bacteria (B) on MRS-agar; *Enterobacteriaceae* (C) on Violet Red Bile Glucose agar; *Salmonella* sp. (D) on Brilliant green violet agar.

	log $_{10}$cfu/g faeces			
Growth media	**Control**	**1K**	**1K + S**	**S**
A	8.26 ± 0.12	8.71± 0.15	8.99 ± 0.12*	8.81 ± 0.19*
B	6.95 ± 0.29	10.12± 0.23	9.15 ± 0.16	4.11 ± 0.15
C	3.13 ± 0.26	1.78± 0.12	4.35 ± 0.12	7.22 ± 0.15
D	-	-	1.01 ± 0.11	3.78 ± 0.12

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 ($P \leq 0.01$)

Table 2. Bacterial counts in small intestine of mice 7 days after the oral treatment with *L. plantarum* 1K, *L. plantarum* 1K in combination with *Salmonella* sp., or after the challenge with *Salmonella* sp. Total number of bacteria (A) on Peptone yeast extract glucose agar; total LAB (B) on MRS-agar; *Enterobacteriaceae* (C) on Violet red bile glucose agar, *Salmonella* sp. (D) on Brilliant green violet agar.

	log cfu/g faeces			
Growth media	**Control**	**1K**	**1K + S**	**S**
A	8.11 ± 0.15	8.31± 0.15	8.03 ± 0.12*	8.29 ± 0.12*
B	6.45 ± 0.17	9.15± 0.13	8.45 ± 0.21	3.89 ± 0.17
C	1.15 ± 0.26	1.78± 0.12	3.55 ± 0.15	6.12 ± 0.21
D	-	-	1.15 ± 0.15	3.57 ± 0.21

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 ($P \leq 0.01$)

Table 3. Bacterial counts in large intestine of mice 7 days after the oral treatment with *L. plantarum* 1K, *L. plantarum* 1K in combination with *Salmonella* sp., or after the challenge with *Salmonella* sp. Total number of bacteria (A) on Peptone yeast extract glucose agar; total LAB (B) on MRS-agar; *Enterobacteriaceae* (C) on Violet red bile glucose agar, *Salmonella* sp. (D) on Brilliant green violet agar.

	log cfu/g faeces			
Growth media	**Control**	**1K**	**1K + S**	**S**
A	8.35 ± 0.12	8.55± 0.31	8.63 ± 0.11*	8.69 ± 0.32*
B	6.78 ± 0.11	9.55± 0.23	8.65 ± 0.17	3.76 ± 0.12
C	1.25 ± 0.14	1.56± 0.33	3.23 ± 0.11	6.35 ± 0.24
D	-	-	1.03 ± 0.15	3.73 ± 0.12

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 ($P \leq 0.01$)

Conclusions

L. plantarum 1K survived and adhered to intestinal tract of the mice. Furthermore, *L. plantarum* 1K reduced *salmonella* infection in mice. From these results, we can conclude that the bacterial strain *L. plantarum* 1K isolated from „Slavonski kulen" could be used as a probiotic strain to establish balance of intestinal microflora because it reduced the growth of undesirable pathogens. Also, research results show that *L. plantarum* 1K, since it is isolated from „Slavonski kulen", could be used as functional starter cultures for controlled fermentation of meat products, because it inhibits the growth of pathogenic microorganisms, and would thus extend shelf life of meat products.

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