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Review

Lactobacillus gasseri LF221 and K7 – from isolation to application*

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Abstract: The article presents research findings on two human strains with probiotic activity. On the basis of API 50 CHL fermentation pattern, PCR by species-specific primers and sequencing of the V2–V3 region of 16S rRNA both strains designated as LF221 and K7 were identified as members of the *Lactobacillus gasseri* species. Two LF221 bacteriocins, acidocin LF221 A and B were purified and sequenced. They were classified as members of the two-component class II bacteriocins. Among basic probiotic properties, the survival under conditions in gastro-intestinal tract, ability to adhere to cultured intestinal enterocytes and pig's mucosa and stimulation of the immune response were demonstrated. In *in vivo* study of 24 weaned piglets, the survival rate of K7 Rif^r and LF221 Rif^r was quantified by selective enumeration on MRS agar with rifampicin. The survival of both strains was good (2.9×10^5 cfu of K7 Rif^r /g faeces; 4.8×10^5 cfu of LF221 Rif^r/g) and the LF221 Rif^r/K7 Rif^r viable cells were found either in the mucosa of duodenum, jejunum or in the ileum. The possible effect of K7 to inhibit adhesion of *E. coli* O8:K88 to enterocytes was studied on Caco-2 cultured cells, on tissue obtained from small intestines of pigs and *in vivo* on gnotobiotic piglets. Lactobacilli were found to be effective in reducing *E. coli* adhesion to enterocytes in Caco-2 model, but not on mucosa of pig's jejunum under *ex vivo* conditions. Competitive exclusion, production of organic acids and stimulation of immune response, were involved in inhibition of *E. coli* by K7 strain in gnotobiotic piglets. Any inflammatory change in intestines of piglets treated with K7 was observed, which confirmed its safe use. Among the technological parameters the survival and activity of the strains during cheese-making are presented.

Key words: *Lactobacillus gasseri*; bacteriocins; probiotic properties; safety; technological properties.

Abbreviations: GIT, gastrointestinal tract; LAB, lactic acid bacteria; RAPD, random amplified polymorphic DNA.

Introduction

Lactic acid bacteria (LAB) colonize parts of the human and animal body, and environments where spontaneous fermentations of carbohydrate-containing substrates occur (Teuber et al., 1999). To survive and persist in such microbially diverse ecological niches, LAB are forced to produce various antibacterial substances. The antimicrobial activity of LAB has long been attributed to the production of metabolites such as organic acids, hydrogen peroxide, ethanol and diacetyl. However, it has gradually become clear that additional antimicrobial compounds often contribute to the antimicrobial capacity of LAB. One such group of antimicrobial compounds is represented by bacteriocins. These compounds are ribosomally-synthesized antimicrobial peptides that have been found to be widespread in LAB (Nes & Johnsborg, 2004).

Since food safety and bioconservation have become increasingly important concerns all over the world, the application of bacteriocins or LAB that produce bacteriocins with a wide range of inhibitory activity, has received great attention (Stiles, 1996; Cleveland et al., 2001). Our group also started to search for bacteriocinogenic LAB which could be interesting as protective starter cultures for the dairy industry. Our first task was screening of a huge number of LAB isolates from faeces and different fermented dairy products for antibacterial activity and bacteriocin activity.

Among all examined isolates, two strains isolated from infant faeces were distinctive because of their widely antagonistic effect that inhibits not only lactobacilli but also strains of other genera, including *Lactococcus*, *Pediococcus*, *Staphylococcus*, *Enterococcus*, *Listeria*, *Clostridium* and *Bacillus*. They were *Lactobacillus* sp. LF221 selected from the collection of the Istituto di Microbiologia, Università Cattolica del Sacro Cuore, in Piacenza, Italy and *Lactobacillus* sp. K7 obtained from the Dairy Research Laboratory, Biotechnical Faculty, University of Ljubljana, Slovenia. The LAB strains had a broad spectrum of antimicrobial activity and produced bacteriocins named acidocins LF221 and

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Table 1. Some bacteriocins with a wide spectrum of antibacterial activity.

Bacteriocin	Producer strain	Reference
Nisin A	*L. lactis* NIZOR5, NCFB894, ATCC11454	Twomey et al. (2002), Guinane et al. (2005)
Nisin Z	*L. lactis* N8, NIZO22186	Twomey et al. (2002)
Lacticin 3147	*L. lactis* subsp. *lactis* DPC3147, *L. lactis* IFPL105	Guinane et al. (2005)
Enterocin EJ97	*Ent. faecalis* EJ97	García et al. (2004)
Enterocin AS-48	*Ent.faecalis* A-48–32, *Ent. faecium* S-32–81	Ananou et al. (2005)
Mesentericin ST99	*Leuc. mesenteroides* subsp. *dextranicum* ST99	Todorov & Dicks (2004)
Plantaricin TF711	*Lb. plantarum* TF711	Hernández et al. (2005)
Gassericin T	*Lb. gasseri* SBT 2055	Kawai et al. (2000)
Gassericin A	*Lb. gasseri* LA39	Kawai et al. (2004)
Acidocin LF221 A and B	*Lb. gasseri* LF221	Bogovič Matijašić & Rogelj (1999, 2000), Majhenič et al. (2004)
Gassericin KT7	*Lb. gasseri* KT7	Zhu et al. (2000)
Acidocin CH5	*Lb. acidophilus* CH5	Chumchalová et al. (2004)

K7 with very promising properties for food and feed preservation. In addition, being of human origin they could be potential probiotic strains (Bogovič Matijašić & Rogelj, 1999, 2000).

During the past two decades the probiotic microorganisms have been increasingly included in various food products, food supplements and OTC drugs. While probiotics have proven benefits, the optimism associated with their use is counterbalanced by the fact that many so-called "probiotic" products are unreliable in content and unproven clinically (Reid, 2005). Many criteria have been suggested for the selection of probiotics including safety, functional and technological characteristics. Safety aspects include specifications such as origin, the physiology, genetics and lack of pathogenicity. Strain identity is important to link a strain to a specific health effect as well as to enable accurate surveillance and epidemiological studies. Functional aspects include tolerance to gastrointestinal conditions, ability to adhere to the gastrointestinal tract (GIT) mucosa, competitive exclusion of pathogenes, modulation of gut flora and immunomodulation. In addition, to satisfy all these criteria the probiotic strain must also be technologically acceptable without loosing viability and functionality during technological processes and product shelf life (Saarela et al., 2000; Wright, 2005). This article summarizes research findings on *Lb. gasseri* LF221 and K7.

Bacteriocins of LF221 and K7

Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (Klaenhammer & Kullen, 1999; Fooks & Gibson, 2002). It may contribute to the colonization resistance of the host and its protection against gastrointestinal pathogens (Reid et al., 2001). Beside typical bacteriocins with bactericidal activity against species that are closely related to the producer bacteria, more and more bacteriocins with a wider spectrum of antibacterial activity have also been described (Table 1) or are in the process of characterization (Arici et al., 2004; Elegado et al., 2004). At present, biochemical and genetic characteristics classify bacteriocins into four different classes: (I) lantibiotics and (II) nonlantibiotics which are small, membrane-active and heat-stable proteins; (III) large, heat-labile proteins; and (IV) complex bacteriocins carrying lipid or carbohydrate moieties (Garneau et al., 2002, Papagiani, 2003). However, presently no complex bacteriocins have been purified and according to some researchers there is good reason to believe that this type of bacteriocin is an artifact due to the cationic and hydrophobic properties of bacteriocins which result in complexing with other macromolecules in the crude extract (Cleveland et al., 2001). Recently, a new class (V) of the cyclic bacteriocins has been proposed. These peptides have covalently linked their N- and C-termini (Kemperman et al., 2003, Kawai et al., 2004).

The main reason for isolation of bacteriocin(s) from the supernatant of the LF221 culture was a very interesting spectrum of inhibitory activity of the strain, when the effects of pH and hydrogen peroxide were excluded. Two different bacteriocins were isolated from the MRS culture of LF221 by ammonium sulphate precipitation, cation-exchange chromatography, C_8 hydrophobic-interaction chromatography and reverse-phase FPLC chromatography (Bogovič-Matijašić et al., 1998).

Two peptide fractions obtained by reverse-phase FPLC chromatography were analysed for N-terminal amino acid sequences, resulting in 46 and 35 amino acid residues for peptide A and B, respectively. Unusual amino acids, such as lanthionin or methyllanthionin were not found in the two peptides. The biochemical properties of the two LF221 bacteriocins – small (<10 kDa), heat-stable and hydrophobic peptides – mostly resembled class II LAB bacteriocins. The N-teminal amino acid composition of bacteriocins A and B was found to be different from those of the other bacteriocins. They were named acidocin LF221 A and acidocin LF221 B (Bogovič-Matijašić et al., 1998).

The next step was to analyze the genes encoding both LF221 acidocins because gene alignments can reveal certain homologies or even identities between

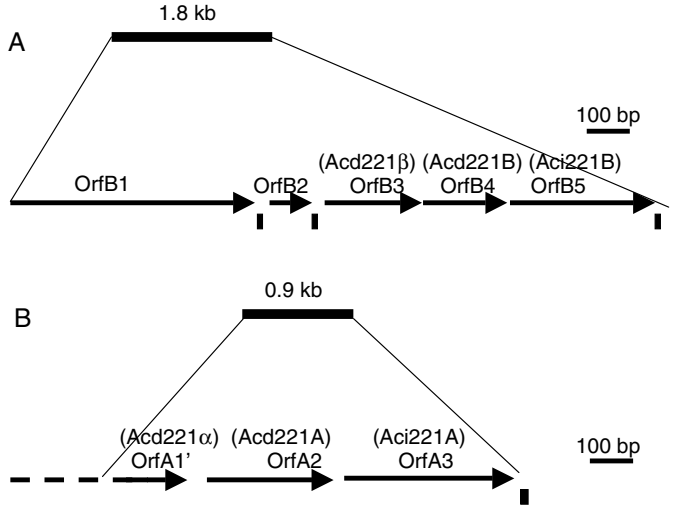

Fig. 1. Location and orientation of the ORFs on the 1.8 kb *Eco*RI/*Hind*III (A) and 0.9 kb *Bam*HI/*Hind*III DNA fragments (B) from LF221 carrying the genetic determinants for acidocin LF221 B and A, respectively.

newly identified and previously described bacteriocin(s) (Remiger et al., 1996; Van Belkum & Stiles, 2000). In addition, genetic information on bacteriocin(s) also enables tracking of the producer strain in complex environments.

Standard procedures for molecular techniques were used to locate, clone and sequence the fragments of LF221 chromosomal DNA carrying the acidocin LF221 A and B structural genes, respectively. Detailed procedures and results were presented by Majhenič et al. (2004). Degenerate primers for acidocin LF221 A and B were constructed on the basis of their N-terminal amino acid sequences. Sequencing analysis revealed the gene of acidocin LF221 A to be an open reading frame encoding a protein composed of 69 amino acids, including a 16-amino-acid N-terminal extension. The acidocin LF221 B gene was found to encode a 65-amino-acid bacteriocin precursor with a 17-amino-acid N-terminal leader peptide. DNA homology searches showed similarities of acidocin LF221 A to brochocin B (BrcB), lactococcin N (LcnN) and thermophilin B (ThmB) which complemented the BrcA, LcnM and ThmA, respectively, forming the two-component bacteriocins known as brochocin-C (McCormick et al., 1998), lactococcin MN (Van Belkum et al., 1991) and thermophilin 13 (Marciset et al., 1997). Acidocin LF221 B exhibited some homology to lactacin F (Allison et al., 1994) and was virtually identical to gassericin T (Kawai et al., 2000). As lactacin F and gassericin T belong to subgroup B of class II bacteriocins, it seems likely that acidocin LF221 B belongs to this group as well.

Figure 1 represents the location and orientation of the open reading frames on the DNA fragments from LF221 carrying the genetic determinants for acidocins A and B. On the 1.8 kb *Eco*RI/*Hind*III fragment carrying the acidocin LF221 B genetic determinants, 1 incomplete and 4 complete ORFs were revealed. Thirty-five amino acids of the purified acidocin LF221 B (Acd221B) corresponded to the sequence found in *orf*B4. The *orf*B3, positioned upstream of the *orf*B4 encodes a putative complementary component (Acd221β) of the two-peptide acidocin LF221 B. *orf*B5 might code for an immunity protein (Aci221B). ORFs B3, B4 and B5 form one operon. *orf*B1' encodes a truncated peptide which revealed some homologies to accessory factors for ABC-transporters found in some lactobacilli (Fig. 1A).

Similarly, from the analysis of the 0.9 kb *Bam*HI/*Hind*III fragment carrying the acidocin LF221 A genetic determinants, 1 incomplete and 2 complete ORFs were determined. *orf*A2 is structural gene for acidocin LF221 A (Acd221A), which possesses a typical Gly-Gly doublet *orf*A3, positioned immediately downstream of *orf*A2, and encodes a putative immunity protein (Aci221A), while truncated *orf*A1 most probably encodes the complementary component (Acd221α) of the acidocin LF221 A (Fig. 1B).

Based on the obtained results, acidocin LF221 A and acidocin LF221 B are predicted to be members of the two-component class II bacteriocins, where acidocin LF221 A appears to be a novel bacteriocin. The sequences were deposited in GenBank (Benson et al., 2004) under the accession numbers AY295874 and AY297947 for acidocin LF221 A and acidocin LF221 B, respectively.

The production of similar or even identical bacteriocins by different LAB is not a rare event. To take advantage of this finding, genetic determinants of the *Lactobacillus* K7 bacteriocins were tested for putative homologies with already described bacteriocins of the *Lactobacillus acidophilus* group. We used the PCR and specific primers for seven known bacteriocins produced by related species to shortcut the commonly used approach of bacteriocin characterisation and to avoid the needless work if the bacteriocins produced by K7 were identical to already described bacteriocins. Only when primers

specific for LF221 acidocins were used in PCR reaction on K7 genomic DNA as a template, the amplified products corresponded in size and nucleotide sequence to the amplicons generated with DNA from LF221 (Majhenič et al., 2003). To clarify the degree of similarity among K7 and LF221 bacteriocins, the complete nucleotide sequence of K7 bacteriocins had to be determined. It is important to stress that although K7 and LF221 strains are both of human origin and belong to the *Lb. gasseri* species, they differ in other characteristics such as the plasmid profile, random amplified polymorphic DNA (RAPD) profile, growth characteristics, the level of bacteriocins production, the optimal conditions for growth and bacteriocin production (Bogovič-Matijašić et al., 1998, 2001; Bogovič Matijašić & Rogelj, 1999, 2000).

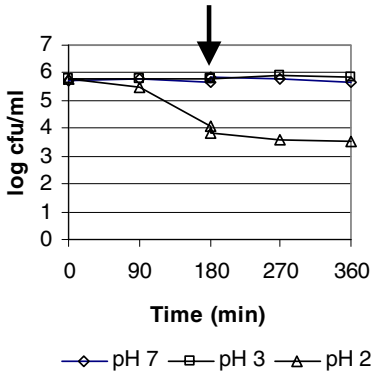

Fig. 2. Survival of *Lb. gasseri* K7 during exposure of cells to artificial gastric juice adjusted to different pH (pH = 2, 3 or 7) and simulated intestinal fluid (pH = 8). The transfer of K7 cells from gastric juice to intestinal fluid (after 180 min) is indicated with an arrow.

Identification of LF221 and K7

Identification on the species level was performed not only for safety reasons but also for diagnostic and epidemiological purposes. On the basis of physiological and biochemical properties including fermentation pattern determined by using API 50 CHL (BioMerieux, France) the two strains were identified as members of *Lb. acidophilus* group. With the development of modern taxonomy based on molecular techniques, six distinct species within the group of previously termed *Lactobacillus acidophilus* were identified: *Lb. acidophilus*, *Lb. amylovorus*, *Lb. crispatus*, *Lb. gallinarum*, *Lb. gasseri*, and *Lb. johnsonii*. The hyper variable 16S-23S intergenic spacer regions as well as 16S rRNA gene sequences were found to be sufficiently specific for differentiation between the closely related *Lactobacillus* species (Tannock et al., 1999; Kullen et al., 2000). PCR with species-specific primers and sequencing of the V2–V3 region of the 16S rRNA gene showed that LF221 as well as K7 strains belong to the *Lb. gasseri* species.

Probiotic properties of *Lb. gasseri* LF221 and K7

An important functional attribute for probiotic bacteria is survival in the GIT. In order to test *in vitro* the ability to survive at low pH and in the presence of bile, we found very useful an approach described by Fernandez et al. (2003) where artificial gastric juice (NaCl 125 mM + KCl 7 mM + $NaHCO_3$ 45 mM + pepsin 3 g/L; pH 2.3 and 7) and simulated intestinal fluid (0.1%, w/v) pancreatin + 0.3% bile salts (w/v, Bile salts No. 3, Biolife, Italy; pH 8) are used. After exposure of the bacterial cells to gastric juice for 180 min, the liquid part is removed, the cells are resuspended in intestinal fluid and incubated for additional 180 min. The survival of *Lb. gasseri* K7 in simulated intestinal conditions is presented in Figure 2. The transfer of K7 cells from gastric juice to intestinal fluid is indicated with an arrow. The cfu/mL of K7 strain was decreased for 2 log units in the simulated gastric juice at pH 2 only, while at higher pH values the viability was completely preserved. In addition, K7 cells were resistant to simulated intestinal fluid.

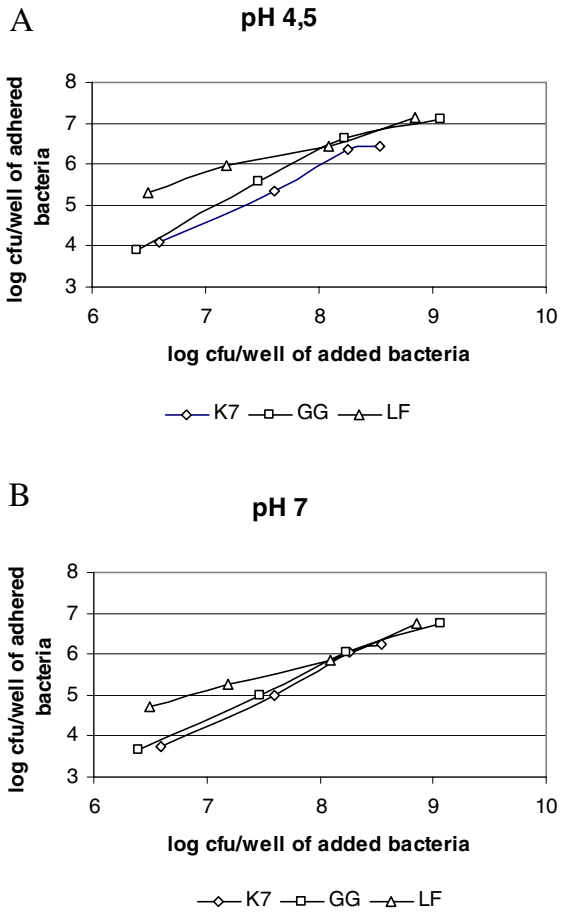

Fig. 3. Adhesion of *Lb. gasseri* K7, *Lb. gasseri* LF221 and *Lb. rhamnosus* GG on Caco-2 cells at pH 4.5 (A) and pH 7 (B). The number of adhered bacteria was obtained by plate counting (log cfu/well). Presented values are the means of ten wells. From Bogovič Matijašić et al. (2003).

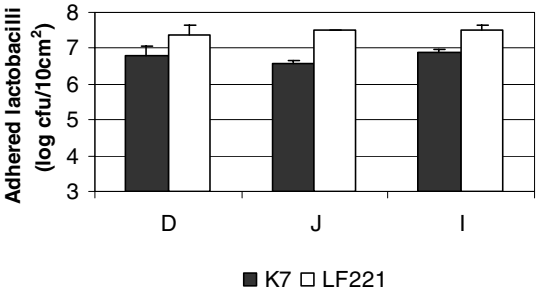

Fig. 4. *Ex vivo* adhesion of *Lb. gasseri* LF221 and *Lb. gasseri* K7 on different parts of pig's small intestines: D – duodenum; J – jejunum; I – ileum. Concentration of bacteria in suspension was 5×10^7 cfu/mL.

Among the probiotic properties which can be tested *in vitro* or *ex vivo* is the ability of the strain to adhere to epithelial cells. In our experiments, Caco-2 cells were used as the *in vitro* model and pig intestinal epithelium as the *ex vivo* model. Figure 3 represents the adhesion of three strains on Caco-2 monolayer. The adhesion of two *Lb. gasseri* test strains and of *Lb. rhamnosus* GG reference strain with previously established adhesion ability was better in acidic conditions and at lower concentrations (up to 2.5×10^8 of added cfu/well) linearly correlated to the concentration of added cells. It is obvious that the adhesion of our test strains was comparable to that of *Lb. rhamnosus* GG, at both pH tested. At lower concentrations, LF221 adhered even better than GG strain (Bogovič Matijašić et al., 2003).

Ex vivo adhesion studies comprised a three-step procedure. Immediately after slaughtering, the segments of pigs' small intestine were treated in a way to remove the microflora. The pieces of tissue (10 cm^2) were held in phosphate buffered saline (PBS) at 4 ℃ for 30 min in order to loosen the surface mucus and extensively washed three times with PBS (Mäyrä-Mäkinen et al., 1983). The adhesion was carried out in 100 mL suspensions (250 mL flasks) of single strains in PBS at 37 ℃ for 30 min by shaking (75 rpm). Unattached cells were removed by washing tissue samples in PBS by shaking (150 rpm), two times for 5 min. Adhered bacteria were quantified after being released from enterocytes, by plate counting. First, *ex vivo* adhesion of *Lb. gasseri* LF221 and K7 on different parts of small intestine, i.e. duodenum (D), jejunum (J) and ileum (I) was examined. Significant differences in adhesion to intestinal tissue derived from different segments (D, J, I) were not demonstrated for either of the test strains (Fig. 4). We therefore performed the additional experiments on jejunal tissue only.

The possible effect of K7 strain to inhibit adhesion of *Escherichia coli* O8:K88 to intestinal mucosa was studied by two models: (i) on cultured Caco-2 cells and (ii) on pigs' small intestinal tissue. Lactobacilli were added simultaneously with *E. coli* (for competition assay), or the addition of lactobacilli alone was followed by the washing of unbound cells and addition of *E. coli* cells (for exclusion assay). To test the possible displacement, the incubation of enterocytes with *E. coli* was carried out first and followed by incubation with K7. Although the *E. coli* cells adhered more effectively to Caco-2 than K7 strain, the latter was able to reduce adhesion of *E. coli* in exclusion and competition assays even when applied at the same concentration as *E. coli*. In addition, significant displacement was observed when the concentration of *E. coli* was about 2 log lower than that of K7 strain (Bogovič Matijašić et al., 2006a).

Functionality and safety of *Lb. gasseri* LF221 and K7

In vitro studies of basic probiotic properties confirmed both strains as potential probiotics, therefore we have proceeded with *in vivo* studies. To investigate the LF221 strain with respect to its survival and persistence in complex ecosystems, viable LF221 cells were orally administered to laboratory mice and piglets. It was revealed that in both animal models, LF221 administration did not significantly influence the total count of lactobacilli in faecal samples of mice and piglets and it had no negative health effects on animals (Rogelj et al., 2002). Another interesting observation was the persistence of LF221 in the GIT that was clearly demonstrated in piglets. At the final sampling carried out 10 days after the last application of LF221 strain, viable cells of the strain were still found in faeces. Colony hybridisation with bacteriocin-specific probe and PCR amplification of the part of acidocin LF221 A structural gene were used for confirmation of putative LF221 colonies.

In another trial, the effects of LF221 and K7 strains on the faecal coliform and lactobacilli counts and on the production parameters (weight gain, feed conversion) were studied in 18 weaned piglets. The experimental period lasted 25 days. For the discrimination among strains LF221, K7 and other faecal microflora, a combined approach that included culturing on selective media, testing of antimicrobial activity and RAPD analysis was used. The LF221 and K7 strains survived the passage through the intestines and were successfully detected in the faeces. The RAPD method enabled discrimination between LF221 and K7 strains, as well as discrimination between each probiotic strain and indigenous faecal microflora. An example of RAPD analysis of some antimicrobially active isolates from the faeces of piglets fed with K7 strains is presented in Figure 5. Of 6 isolates tested, the pattern typical for K7 strain was obtained with one of them (Fig. 5, lane 3). All the piglets remained healthy and no case of diarrhoea was observed. Administration of a single probiotic strain (5×10^{10} cfu per piglet/day) did not significantly influence the viable counts of the coliforms but influenced lactobacilli counts in K7 group. In addition,

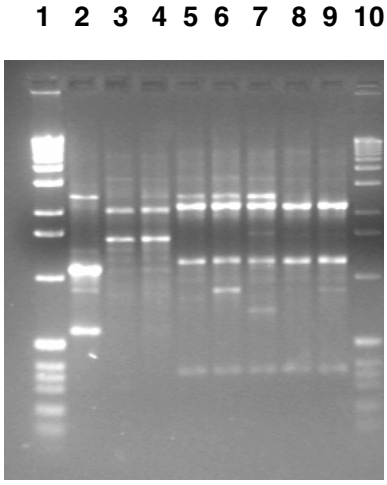

Fig. 5. RAPD profiles generated from DNA of faecal isolates from the group of piglets fed K7 strain which expressed antimicrobial activity against *Lb. sakei* NCDO 2714. Lanes 1 and 10: 1 kb DNA ladder; lane 2: *Lactobacillus* LF221 strain; lane 3: *Lactobacillus* K7 strain; lanes 4–9: isolates from faeces of animals from the group fed with K7 (primer 5'AGTCCAGCCAC3'; Tynkkynen et al., 1999). From Bogovič Matijašić et al. (2004).

the average feed conversion efficiency calculated for the whole experimental period was significantly improved in the group given the K7 strain (Bogovič Matijašić et al., 2004).

In *in vivo* study of 24 weaned piglets (8 per group), the rifampicin-resistant derivatives of parental K7 and LF221 strains were applied in order to enable their quantification in the faeces and mucosa by selective enumeration on MRS agar with rifampicin. Faeces from individual animals were microbiologically examined during 2-week probiotic application period (5×10^{10} cfu of individual strain/day) and 1 week after the probiotic treatment had ceased. In addition, the samples of duodenum, jejunum and ileum of 12 animals sacrificed on the 5^{th} or 20^{th} day were examined. The total lactobacilli count in all mucosal samples as well as in ileal content and faeces was higher from the coliform count. The recovery of both strains during the application period was good, while 6 days after ceasing of probiotic application, the LF221 or K7 viable cells were still found in the faeces of two animals. In both animals from the group fed with *Lb. gasseri* K7 that were sacrificed 5 days after weaning, the presence of K7 strain was found either in the mucosal samples of duodenum and jejunum or in the ileal samples. LF221 cells were associated with the ileal mucosa of one piglet. The presence of K7/LF221 in the mucosa was not correlated to the concentration of either in the faeces (Bogovič Matijašić et al., 2006b).

The antagonistic activity against *Escherichia coli* O8:K99:H8 ent$^-$ was studied *in vivo* in gnotobiotic piglets. In addition, we tried to confirm the safety of K7 strain. Twenty-five germ-free piglets were included in the experiment. They were obtained by open hysterectomy, divided into three groups (E: 9 piglets, L: 8 piglets and L-E: 8 piglets) and reared in three isolators. The animals were fed by sterilized milk replacement, Sanolac Ferkel (Sano, Germany), every four hours for 15 days. Piglets of L and L-E groups were inoculated daily with 2 mL of *Lactobacillus gasseri* K7 (1×10^9 cfu/mL) cultivated in MRS broth (Merck, Germany), while piglets from E group received 2 mL of MRS broth daily, throughout the whole period of experiment. On the 3^{rd} day of life, piglets from L group received 2 mL of PYG broth (Merck, Germany), while piglets from E and L-E groups were inoculated with a single dose (2 mL) of *Escherichia coli* O8:K99:H8 ent$^-$ (1×10^5 cfu/mL) cultivated in PYG broth. Piglets were sacrificed at the age of 3 (2 piglets from E group, 2 from L-E and 2 from L), 7 (4 piglets from E group, 1 from L-E and 1 from L) and 15 days (3 from L-E group and 5 from L). Immediately after slaughtering, tissue samples of duodenum, jejunum, ileum and colon (Dm, Jm, Im and Cm), and contents of particular intestinal parts (Dc, Jc, Ic and Cc) were obtained for determination of lactobacilli and *E. coli* counts. Tissue samples (5 cm^2) intended for determination of micro-organisms adhered to the intestinal mucosa were washed 3 times with 0.15 M PBS (pH 7.2), homogenised in 45 mL of the same buffer, diluted and plated on agars. For morphological analysis the samples of jejunum were prepared and visualised by electron microscope (TESLA BS 500, Czech Republic). The clinical observations of animals showed that more intensive infection symptoms, such as low condition, loss of appetite, cramps, diarrhoea, bleeding under the skin and in internal organs or even death, appeared in piglets which were inoculated with *E. coli* O8:K99:H8 ent$^-$ only (E). Preventive inoculation with K7 strain (L-E) decreased the severity of infection and protected piglets against perishing, although it did not totally protect them against infection. Only the piglets inoculated with K7 strain did not show any infection symptoms and were in very good condition, which indicates a safe use of K7 strain. Comparison of coliform bacteria (*E. coli*) numbers between E and L-E groups showed a time-independent statistically significant higher number on mucosa as well as in the contents of all intestinal parts in the group receiving *E. coli* only (E) (Table 2). Comparing the lactobacilli counts in both groups of piglets inoculated with K7 strain (L and L-E), we did not find any significant differences. These results confirmed the inhibition of *E. coli* in the group of piglets preventively inoculated with K7 strain (L-E group). The applied lactobacilli (K7 strain) probably prevented the adhesion of *E. coli* by competitive exclusion, which can include the occupation of specific *E. coli* binding sites or non-specific sterical hindrance of *E. coli* binding by lactobacilli. The infection of E group piglets with *E. coli* obviously caused intensive changes and damage to intestinal tissue observed by the presence of huge vacuoles and total destruction of micro-villi (Fig. 6A). The continuous inoculation of L and L-E group with K7 strain re-established the normal

Table 2. The number of *E. coli* and *Lb. gasseri* K7 in mucosa (m) and contents (c) of duodenum (D), jejunum (J), ileum (I) and colon (C) of sacrificed gnotobiotic piglets.[a]

Bacteria	Sample	Average number of bacteria[A] (log cfu/cm^2 of mucosa (m); log cfu/g of content (c))		
		E (n)	L (n)	L-E (n)
E. coli	Dm	6.50 ± 0.42[a] (6)	0	5.24 ± 0.19[b] (11)
L. gasseri K7		0	4.0 ± 0.07[a] (16)	4.49 ± 0.07[a] (12)
E. coli	Jm	5.85 ± 0.05[a] (2)	0	5.18 ± 0.53[b] (9)
L. gasseri K7		0	4.91 ± 0.10[a] (14)	5.05 ± 0.09[a] (11)
E. coli	Im	7.46 ± 0.11[a] (4)	0	6.48 ± 0.22[b] (11)
L. gasseri K7		0	5.02 ± 0.30[a] (14)	5.65 ± 0.56[b] (12)
E. coli	Cm	7.83 ± 0.01[a] (6)	0	7.31 ± 0.13[b] (11)
L. gasseri K7		0	5.67 ± 0.49[a] (16)	5.68 ± 0.33[a] (10)
E. coli	Dc	9.01 ± 0.55[a] (4)	0	6.15 ± 0.34[b] (6)
L. gasseri K7		0	7.42 ± 0.15[a] (10)	7.61 ± 0.15[a] (8)
E. coli	Jc	8.91 ± 0.49[a] (4)	0	7.27 ± 0.23[b] (10)
L. gasseri K7		0	7.87 ± 0.16[a] (16)	7.54 ± 0.07[b] (12)
E. coli	Ic	9.65 ± 0.02[a] (4)	0	9.02 ± 0.19[b] (10)
L. gasseri K7		0	7.79 ± 0.15[a] (15)	8.07 ± 0.17[b] (12)
E. coli	Cc	10.02 ± 0.05[a] (6)	0	9.74 ± 0.09[b] (11)
L. gasseri K7		0	8.25 ± 0.33[a] (14)	8.15 ± 0.15[a] (11)

[a] n = number of processed data; [A] = the average values were calculated from all samples of particular intestinal parts and groups, independently of sampling time; [a,b] = group effect, significant statistical diversity between means with different superscripts in the same row ($P < 0.05$).

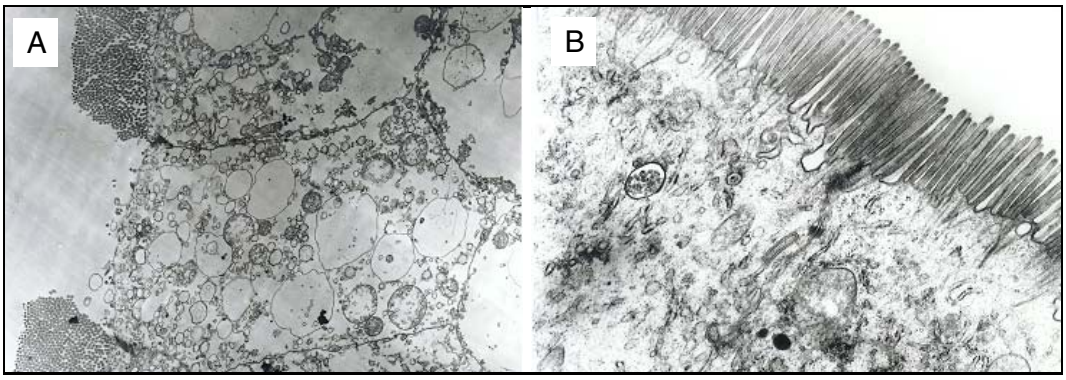

Fig. 6. Electron micrograms of the jejunal enterocytes of gnotobiotic piglets inoculated with *E. coli* (A – damaged intestinal tissue with huge vacuoles and destroyed micro-villi; magnification 4,800×) and *Lb. gasseri* K7 (B – well expressed micro-villi; magnification 8,300×). Experiments were performed at University of Veterinary Medicine, Research Institute of Veterinary Medicine, Laboratory of Gnotobiology and Diseases of Young, Košice, Slovakia.

conditions in intestines of piglets and lowered the appearance of tissue damage. The micro-villi on the tissue samples obtained from the K7 group animals were well expressed (Fig. 6B). In addition, we did not observe any inflammatory changes in the intestines of piglets treated only with K7, which confirmed its safe use (Stojković, 2003).

Technological properties of *Lb. gasseri* LF221 and K7

Cheese was tested as a possible carrier of probiotic bacteria. A derivative of *Lb. gasseri* K7 and LF221 strains resistant to rifampicin (250 μg/mL) was prepared (Perko et al., 2002). Semi-hard cheese was produced in a pilot plant from 80 L of milk. After heat-treatment of raw milk at 65 °C for 30 min and rapid cooling to 32 °C, the milk was inoculated with *Lb. gasseri* K7 (Rif^r) or LF221 (Rif^r) cells (10^7 cfu/mL milk). Attempts to produce cheese with *Lb. gasseri* as a single starter culture were unsuccessful because of poor acidification activity of this strain (Perko et al., 2002). This could be expected, as it is well known that proteolytic activity of bacteria from *Lb. acidophilus* group is negligible, therefore they lack free amino acids which are essential for the growth. That is why *Streptococcus thermophilus* TH-4 (Christian-Hansen, Denmark) with good proteolytic activity was added simultaneously with *Lb. gasseri* strains not only to carry out the initial acidification, but also to create the conditions for growth and activity of both probiotic strains. Thirty min after rennet (CHY-MAX® Powder Extra,

Christian-Hansen, Denmark) addition, the curds were cut into particles of 0.5 cm diameter and heated at 42 ℃. The curds were transferred into moulds, pressed at room temperature until pH reached the value 5.3, and salted for 18 h in 20% brine. Cheeses were ripened at 15–17℃ for eight weeks. In order to obtain good organoleptic properties, the manufacturing process was optimized by varying the amount of *Str. thermophilus* culture, the heating temperature and the intensity of curd washing. The colonies grown on MRS agar with rifampicin were successfully confirmed by RAPD analysis to be identical to K7 or LF221 strain. After 5 and 8 weeks of ripening, total DNA was isolated directly from selected cheeses. *Lb. gasseri*-specific and acidocin-A-specific primers were used in PCR reaction for detection of K7 or LF221. Analyses of cheese demonstrated that both strains maintained a high viability during ripening and that it could be detected for at least 8 weeks at a level of at least 10^7 cfu per g of cheese. According to these results, semi-hard cheese represents a suitable carrier for introducing LF221 and K7 strains into a diet (Perko et al., 2002; Rogelj et al., 2002).

In some studies, the production of bacteriocins by LAB was demonstrated in cheese. The most extensively studied were nisin-producing *Lactococcus lactis* strains found to be efficient against non-desirable bacteria, such as *Listeria* and *Staphylococcus aureus*, as well as against *Clostridium tyrobutyricum* growth in a semi-hard cheese (Rilla et al., 2003; Rodríguez et al., 2005). Less-examined *in situ* in food are bacteriocins of lactobacilli, probably because most of them have a very narrow spectrum of activity. Bacteriocins produced by *Lb. gasseri* K7 were found to inhibit several *C. tyrobutyricum* vegetative cells and spores *in vitro* (Bogovič Matijašić et al., 2000). The possible use of *Lb. gasseri* K7 as a protective starter culture preventing growth and activity of clostridia was demonstrated recently (Bogovič Matijašić et al., (2007). A semi-hard cheese produced from the milk artificially inoculated with *C. tyrobutyricum* spores (2.5 x 10^3 spores/mL) was used as a model for studying the ability of bacteriocin producing *Lb. gasseri* K7 (Rif^r) to inhibit clostridia outgrowth. Late blowing occurred in all (ten) cheeses, however, the butyric acid fermentation was reduced in cheeses (five) with added *Lb. gasseri* (Rif^r) cells. The average amount of butyric acid was significantly higher after 6 weeks in cheeses without added lactobacilli (1.43 g/kg vs. 0.70 g/kg of cheese). Although bacteriocins were not detected, we suppose that they could contribute to total antagonistic activity against clostridia, as pH and concentration of organic acids did not differ significantly between cheeses with added bacteriocinogenic strain, and control ones.

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