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Survival and *in vivo* adhesion of human isolates *Lactobacillus gasseri* LF221 and K7 in weaned piglets and their effects on coliforms, clostridia and lactobacilli viable counts in faeces and mucosa

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In *in vivo* study on 24 weaned piglets (8 per group), the survival rates of human isolates *Lactobacillus gasseri* K7 and LF221 were quantified by selective enumeration on MRS agar with rifampicin, and the presence of both strains in intestinal mucosa was examined. Faeces from individual animals were analysed for the number (cfu/g) of coliforms, lactobacilli, clostridia and both of the two probiotic strains during 2-weeks probiotic application period (5×10^{10} cfu of individual strain/day) and 1 week after the probiotic treatment was ceased. Samples of duodenum, jejunum and ileum of sacrificed animals (5^{th} or 20^{th} day) were also examined microbiologically. A great variability in the microflora of faeces and mucosa was observed even between equally treated animals. The survival of both *Lb. gasseri* strains was established by their detection in the faeces ($2{\cdot}5 \times 10^5$ to $3{\cdot}3 \times 10^5$ cfu of K7 strain/g faeces; $4{\cdot}5 \times 10^5$ to 5×10^5 cfu of LF221 strain/g). In two animals, the LF221 or K7 viable cells were found in the faeces 6 d after ceasing probiotic application. In both animals from the group fed with *Lb. gasseri* K7 that were sacrificed 5 d after weaning, the presence of K7 strain was found either in the mucosa of duodenum (140 cfu/10 cm^2) and jejunum (170 cfu/10 cm^2) or in the ileum (1600 cfu/10 cm^2). LF221 cells were detected in the ileal mucosa of one piglet (820 cfu/10 cm^2). The results demonstrated the capability of both tested strains of *in vivo* adhesion to intestinal mucosa and of temporary colonisation of the piglets' intestine.

Keywords: *Lactobacillus gasseri*, probiotic, weaned piglets, *in vivo* adhesion, intestinal mucosa.

Members of *Lactobacillus gasseri* species are common inhabitants of human and animal intestinal tract (Walter, 2005). Since several positive functional effects of *Lactobacillus* sp. including *Lb. gasseri* have been demonstrated *in vitro* and in animal models, such as protection against infections, stimulation of immune system, reduction of allergy and many others, they are often described as probiotics, i.e. »live microorganisms which when administered in adequate amounts confer a health benefit on the host«(FAO/WHO, 2002; Dunne et al. 1999; Ouwehand et al. 2002).

Beside functionality testing *in vitro*, good survival during the passage of GI tract remains one of the crucial criteria for selecting probiotic strains (Dunne et al. 1999). However, since the presence of probiotic test strains in the faeces is not a satisfactory indication of their colonisation ability, the demonstration of *in vivo* adhesion to intestinal mucosa provides the only reliable evidence (Alander et al. 1999).

Two *Lb. gasseri* strains tested in the present study originate from human faeces. Their probiotic properties were previously extensively studied *in vitro*, and they were also successfully incorporated into cheese (Bogovič Matijašić & Rogelj, 1999, 2000; Perko et al. 2002; Rogelj et al. 2002; Bogovič Matijašić et al. 2006). Before human studies, which are recommended before commercial application of new probiotic strains, and for certain applications even obligatory (for example in the case of the use of probiotics as therapeutic agents), the functionality is usually demonstrated on pigs (Miller & Ullrey, 1987) or other animal models.

In a previously reported *in vivo* trial, *Lb. gasseri* LF221 and K7 were found to survive the passage through piglets'

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intestines (Bogovič Matijašić et al. 2004). In that study, discrimination between the test strains and other faecal microflora was performed by a combination of classical microbiological methods (use of selective media, testing of antimicrobial activity) and RAPD.

In the present work, the presence of orally applied probiotic bacteria in the faeces as well as in the mucosa of the small intestines (duodenum, jejunum, ileum) of weaned piglets were quantified by using rifampicin resistant variants which were selectively enumerated on the agar medium where rifampicin was included. In addition, the effect of feeding two probiotic strains on the coliforms, clostridia and lactobacilli counts in faeces and mucosa was examined.

Material and Methods

Bacteria and growth conditions

Lb. gasseri K7 and *Lb. gasseri* LF221 are two isolates from babies' faeces, previously identified as potential probiotic strains (Bogovič Matijašić & Rogelj, 2000; Rogelj et al. 2002) and deposited in a ZIM Collection of Industrial Microorganisms, Biotechnical Faculty, University of Ljubljana, Slovenia. LF221 strain was originally isolated at Microbiology Institute, UCSC, Piacenza, Italy (Prof. L Morelli). In a pig feeding trial, spontaneous rifampicin resistant cells (Rif^r) were used. These were derived by subculturing the strains in increasing concentration of rifampicin (Sigma-Aldrich Chemie, 89552 Steinheim, Germany), up to 250 μg/ml.

During two weeks application period, concentrated cell suspensions were prepared daily from 18 h MRS (Merck, 64271 Darmstadt, Germany) cultures. Each animal received once daily lactobacilli from 100 ml MRS culture ($5 \pm 0{\cdot}2 \times 10^{10}$ cfu), which were washed with and resuspended in 5 ml 1/4 Ringer solution (Merck). Aliquots of lactobacilli were stored on ice and, within 30 min dosed to piglets.

Animals and feeding trial

A total of 24 three week-old, commercial crossbreed piglets weighing $7{\cdot}24 \pm 0{\cdot}41$ kg were weaned at 21 d of age and the same day transferred and randomly distributed to 6 experimental cages and 3 experimental groups: control group, and two groups receiving either *Lb. gasseri* K7 or LF221 (K7 group and LF221 group, respectively). Prior to the weaning and throughout the experimental period, all the piglets were fed a non-medicated prestarter diet. Feed and water were provided *ad libitum*. The diet was formulated to contain 15 MJ metabolic energy per kg of feed, which was composed of 33·2% wheat, 30% barley, 8·5% fish meal, 20% skimmed milk powder, 3% sugar, 2% sunflower oil, 1% molases and 2·3% mixture of minerals and vitamins. The experimental period lasted for three weeks. The piglets in two experimental groups were given daily doses of 5×10^{10} cfu lactobacilli during the first two weeks in addition to feed and water. Animals were weighed at 5 d intervals. Fresh faecal samples were collected from individual animals each 5 d. Samples were immediately stored in bags where anaerobic atmosphere was generated using Genbag system (Bio-Merieux, France) and transported on ice to the laboratory within 30 min. Viable plate counts of microorganisms were performed immediately upon receipt of the samples.

On days five and twenty, two animals from each experimental group were sacrificed in order to obtain mucosal samples from jejunum, duodenum and ileum for microbiological examination.

The experiment was approved by the Veterinary Administration of the Republic of Slovenia, Ljubljana, Slovenia, number 323-02-84/01.

Analysis of faeces and mucosa

Approximately 10 g wet weight faeces were homogenised in final volume of 100 ml 1/4 Ringer solution. The segments of duodenum, jejunum and ileum were taken immediately after slaughter and transferred on ice to laboratory within 30 min. The content of small intestines was collected when possible, and the mucosa was washed with phosphate buffered saline (PBS) with 10 ml Tween 80/l (Biolife Italiana, 20128 Milano, Italy) until the residual intestinal fluid was removed, while the microflora attached to the mucosa remained entire. The pieces of 10 cm^2 were homogenised in appropriate volume of 1/4 Ringer solution to obtain 100 ml of suspension.

The appropriate dilutions of faecal and mucosal samples were plated on VRB, SPS and Rogosa agar media (Merck) for determination of coliform bacteria, sulphite reducing clostridia and lactobacilli, respectively. Selective enumeration of probiotic *Lb. gasseri* strains was performed on MRS media with added rifampicin (125 μg/ml).

RAPD analysis of selected colonies from MRS+rifampicin agar was performed as described previously (Bogovič Matijašić et al. 2004) by PCR, applying primers designed by Tynkkynen et al. (1999).

Statistical analysis

The data on the number of coliform and lactic acid bacteria were analysed by the General Linear Models (GLM) procedures in SAS® software (Release 8e, 2000). Data of bacterial counts were transformed by $\log_{10}$ before statistical analysis. The model used to analyse the microbial counts data included group (probiotic feeding), day of sampling and interaction between group and day of sampling.

Results

During the entire experiment, all piglets remained in good health and no cases of diarrhoea were observed. The

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Table 1. The average viable plate counts (log cfu/g) of coliform bacteria and lactobacilli in the faeces of piglets. The data presented are the means from 8 animals (d 3) or 6 animals (d 9, 14 and 20). Probiotic strains *Lb. gasseri* LF221 and K7 were given to the animals in groups designated LF221 and K7. D 14 represents the last day of probiotic administration which started at d 1, a day after weaning

		log cfu/g faeces		
Bacterial group/strain	Time (days)	Control group	LF221 group	K7 group
Coliforms	3	7·62 ± 0·77*	7·82 ± 0·30*	7·48 ± 0·60*
	9	5·81 ± 0·94	5·88 ± 0·34	6·30 ± 0·82
	14	6·03 ± 1·1	5·63 ± 0·45	6·76 ± 0·57
	20	5·93 ± 1·39	5·12 ± 0·81	6·19 ± 0·73
Lactobacilli	3	7·32 ± 0·99	7·10 ± 0·45	7·40 ± 0·30
	9	7·59 ± 0·54[a]	8·57 ± 0·41*[b]	8·33 ± 0·17*[b]
	14	7·33 ± 0·41	7·51 ± 0·56	7·64 ± 0·55
	20	7·42 ± 0·69	7·73 ± 0·52	7·63 ± 0·50
Lb. gasseri LF221	3	–	5·20 ± 0·32	–
	9	–	5·44 ± 0·55	–
	14	–	5·48 ± 0·48	–
	20	–	–[c]	–
Lb. gasseri K7	3	–	–	4·60 ± 0·60
	9	–	–	4·86 ± 0·96
	14	–	–	4·70 ± 0·90
	20	–	–	–[d]

– Not detected on MRS agar with rifampicin

* The effect of time: the coliform counts on d three were in all groups significantly higher ($P<0·05$) from those obtained at the following three samplings; the lactobacilli counts in groups K7 and LF221 were significantly higher on d 9

[a,b] The effect of treatment: the differences in lactobacilli counts between three groups were significant ($P<0·05$) on d 9 only; means with different letters differ significantly

[c] *Lb. gasseri* LF221 was found in the faeces of one animal only ($1·2 \times 10^4$ cfu/g)

[d] *Lb. gasseri* K7 was found in the faeces of one animal only ($3·6 \times 10^4$ cfu/g)

probiotic application did not have any significant influence on body weight gain as average weight gain of pigs did not significantly differ between the groups.

The viable counts of faecal coliform bacteria decreased significantly ($P<0·05$) in all groups of piglets for about 1·5 log from d 3 to d 9 (Table 1). During the following days, the coliform counts remained on the same level in control and K7 group and decreased slightly in LF221 group, however the decrease was not significant. No significant differences in the number of coliforms between the groups were observed.

The average concentrations of sulphite reducing clostridia ranged from 4·12 log to 5·55 log g^{-1} faeces, but the values did not differ significantly among the groups or at different time (data not shown).

Comparing the average total lactobacilli counts in the faeces of individual groups on different days (Table 1), in the two groups fed probiotic bacteria were found higher on d 9 than on any other sampling day. In control group, the lactobacilli concentration did not vary throughout three weeks. Except on d 9, significant differences between control group and K7 or LF221 groups, respectively were not observed.

The number of rifampicin resistant lactobacilli in K7 and LF221 groups during the feeding period reached on average 4·72 and 5·37 log cfu/g faeces, respectively and no significant changes were observed during the two weeks of probiotic application. In the post-feeding period the concentration of test lactobacilli in the faeces decreased, so that after 6 d K7 and LF221 strains were found in the faeces of one animal from each probiotic group only, in a concentration $3·6 \times 10^4$ cfu/g and $1·2 \times 10^4$ cfu/g, respectively. Obviously K7 and LF221 strains did not significantly contribute to the total lactobacilli count since their concentrations in the faeces were about 3 log lower in comparison with the total lactobacilli count on Rogosa agar. The increase in the lactobacilli counts on d 9 was probably a result of increased concentration of indigenous lactobacilli other than K7 or LF221 strains (Table 1).

The differences in the number of probiotic bacteria in the faeces among individual animals were very pronounced as is evident from Table 2 where bacterial counts at the end of probiotic feeding (i.e. on d 14) are presented. K7 an LF221 strains were detected in the faeces of all animals in test groups, and this was the case also on d 3 and 9 (data not shown). Great variability in lactobacilli and coliform counts among equally treated individual animals was observed throughout the experiment. The direct correlation between the concentration of lactobacilli and coliforms, or between the probiotic bacteria and coliforms in the faeces could not be established. While the applied probiotic strains represented less than 1 percent of the

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Table 2. Ratio of probiotic strains determined on MRS agar with rifampicin to total lactobacilli count, in the faeces of piglets at the end of probiotic feeding, i.e. on d 14

Group/ animal	Total coliforms (cfu/g)	Total lactobacilli (cfu/g)	Count on MRS+rif (cfu/g)	Ratio (%) of K7 or LF221
K7/1	$7{\cdot}1\times10^{6}$	$7{\cdot}3\times10^{7}$	$4{\cdot}0\times10^{4}$	0·05
K7/2	$5{\cdot}7\times10^{5}$	$6{\cdot}0\times10^{6}$	$1{\cdot}2\times10^{6}$	20
K7/3	$1{\cdot}5\times10^{7}$	$3{\cdot}1\times10^{8}$	$6{\cdot}0\times10^{4}$	0·02
K7/4	$7{\cdot}4\times10^{6}$	$3{\cdot}0\times10^{7}$	$1{\cdot}6\times10^{5}$	0·46
K7/5	$2{\cdot}4\times10^{7}$	$3{\cdot}8\times10^{7}$	$8{\cdot}0\times10^{3}$	0·02
K7/6	$3{\cdot}5\times10^{6}$	$3{\cdot}7\times10^{7}$	$4{\cdot}0\times10^{3}$	0·01
LF221/1	$3{\cdot}1\times10^{5}$	$1{\cdot}8\times10^{8}$	$5{\cdot}8\times10^{5}$	0·33
LF221/2	$1{\cdot}4\times10^{6}$	$7{\cdot}0\times10^{6}$	$7{\cdot}1\times10^{5}$	10·1
LF221/3	$1{\cdot}6\times10^{6}$	$8{\cdot}0\times10^{6}$	$1{\cdot}1\times10^{6}$	13·1
LF221/4	$2{\cdot}8\times10^{5}$	$3{\cdot}5\times10^{7}$	$1{\cdot}7\times10^{5}$	0·49
LF221/5	$1{\cdot}2\times10^{5}$	$9{\cdot}3\times10^{7}$	$5{\cdot}5\times10^{4}$	0·06
LF221/6	$2{\cdot}4\times10^{5}$	$3{\cdot}7\times10^{7}$	$2{\cdot}0\times10^{5}$	0·54

entire lactobacilli population in the faeces of most of the animals, levels as high as 13·1 and 20% were observed in individual animals from LF221 and K7 groups, respectively (Table 2).

Two piglets from each group were sacrificed on d 5 and another two on d 20, in order to carry out microbiological examination of small intestinal mucosa. The viable counts are presented in Table 3. Viable K7 cells were found in mucosal tissue derived from ileum of the first piglet sacrificed on d 5, and in samples of duodenum and jejunum from the second animal. The strain LF221 on the other hand, was found only in the ileal mucosa of one animal. Neither LF221 nor K7 cells were detected in mucosa from the animals sacrificed at the end of experiment, but one animal from K7 group and another from LF221 group contained viable probiotics in the content of ileum. The presence of K7 or LF221 strain in the mucosa was not correlated with the concentration of either in the faeces.

The concentration of viable coliform bacteria in all ileal mucosal samples was higher by about 2 log than those in duodenal and jejunal samples. In 5 samples of ileal and in 5 samples of jejunal mucosa, the viable counts were below the detection level (100 cfu/10 cm^2), irrespective of the treatment or day of execution.

The identity of the colonies grown on MRS agar with rifampicin was confirmed by RAPD as described previously (Bogovič Matijašić et al. 2004). Some small colonies grown on rifampicin supplemented MRS agar plates and easily distinguishable from LF221 and K7, were also confirmed to be different by RAPD analysis. In general the uniform colonies were observed on the plates and by random checking of their DNA by RAPD, the identity with test strains was confirmed in all cases.

Discussion

In our previous *in vivo* studies, the capability of *Lb. gasseri* LF221 and K7 to survive passage through the pig gastrointestinal tract (GIT) was demonstrated, and the number of *Lb. gasseri* LF221 and K7 in the faeces was estimated to reach on average 0·9% and 0·2% of total lactobacilli population, respectively (Rogelj et al. 2002; Bogovič Matijašić et al. 2004). These estimations were quite good compared with the results in the present study (Table 2), where the direct quantification was made possible by the use of rifampicin resistant derivatives of test strains. Similarly, the recovery of K7 strain in the faeces seemed to be lower compared with LF221 strain, however, due to great variability among the animals the differences were not proved to be statistically significant.

The pig-feeding trial reported recently by Gardiner et al. (2004) differed from this study in the duration of lactobacilli feeding (3 weeks), and in the 10 d baseline period between the weaning and start of administration, while the daily dose was similar, i.e. 3×10^{10} cfu/d. Between 10^{5} and 10^{8} cfu of rifampicin resistant test strains were detected in the faeces during administration period, representing 1·1 to 1·3% of total lactobacilli in the case of *Lb. salivarius* and *Lb. pentosus* strains, and up to 23·7% for *Lb. murinus* strain which was reported to have the best survival ability. Compared with these results, LF221 and K7 strains could be described as strains with intermediate survival ability.

We included the analysis of total coliform and clostridial count in the experiment to examine possible effects of probiotic feeding on the total number of bacteria belonging to these groups of bacteria, as inhibition of particular strains of *Escherichia coli*, *Clostridium* sp. and other species by *Lb. gasseri* K7 and LF221 was observed previously *in vitro* on agar plates and on Caco-2 cells (Bogovič Matijašić & Rogelj, 1999, 2000; Bogovič Matijašić et al. 2006). The mean lactobacilli and coliform counts in the faeces of control group animals were comparable with those reported in other studies on pigs (Simpson et al. 2000; Gardiner et al. 2004; Bogovič Matijašić et al. 2004). Our observation that probiotic administration did not affect the total faecal coliform count was in accordance with reported results (Gardiner et al. 1999; Bogovič Matijašić et al. 2004). Because of the complexity of microflora, the ingested strains of lactobacilli usually do not influence the total lactobacilli count, however the composition of the group can be changed significantly (Shu et al. 2001).

Detection of ingested strains in the faeces during post-administration period has often been carried out in order to indirectly demonstrate their persistence in GI tract. Particular lactobacilli test strains in the study of Gardiner et al. (2004) were found in some of the test animals 5 d post-feeding, but not in all, except for *Lb. murinus* strains which were readily isolated from faeces. In another study, *Lb. reuteri* MM53, could be detected in the faeces 2 d after the end of administration, while *Enterococcus faecium* strain Fargo 688R persisted in weaned pigs for at least 8 d after 3-weeks application period, although not in all animals (Gardiner et al. 1999; Simpson et al. 2000).

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Table 3. Viable counts of coliform bacteria, lactobacilli and *Lb. gasseri* K7 or LF221 in the faeces and mucosa of different segments of small intestines of piglets sacrified on d 5 or 20. Probiotic feeding period lasted from d 1 to d 14

		log cfu/10 cm^2 of mucosa				
Group	Day of execution	Duodenum	Jejunum	Ileum	log cfu/g of ileum's content	log cfu/g of faeces
Control	5	3·40/5·35/–	3·49/4·14/–	5·65/5·84/–	n.d.	5·90/8·96/–
Control	5	3·41/2·96/–	–/–/–	4·20/4·92/–	n.d.	6·51/7·59/–
Control	20	2·78/4·87/–	3·49/4·14/–	5·65/5·84/–	7·29/7·65/–	7·89/7·26/–
Control	20	2·69/5·36/–	2·43/4·67/–	4·10/4·81/–	7·34/8·47/–	6·59/7·60/–
LF221	5	–/4·01/–	–/3·20/–	3·85/4·04/2·91	6·38/7·79/–	6·29/8·85/6·85
LF221	5	4·26/5·14/–	2·56/3·54/–	4·23/5·25/–	6·20/7·90/–	5·62/8·58/4·62
LF221	20	2·41/4·68/–	3·51/5·29/–	4·45/4·84/–	6·25/7·40/2·90	6·00/7·20/–
LF221	20	–/4·18/–	–/3·85/–	3·53/4·28/–	6·08/7·36/–	3·95/8·04/4·08
K7	5	–/4·73/–	–/3·64/–	4·77/6·13/3·20	6·90/8·71/5·18	6·21/8·49/4·73
K7	5	2·93/4·08/2·14	3·79/4·68/2·23	4·90/4·34/–	6·98/7·64/–	6·46/6·26/4·36
K7	20	–/2·78/–	–/–/–	4·38/4·98/–	6·55/6·88/3·59	5·64/7·04/–
K7	20	–/4·54/–	2·91/4·41/–	4·48/4·54/–	6·81/7·81/–	6·78/7·73/–

–: viable count bellow detection level, 100/10 cm^2

Lb. gasseri SBT2055SR in healthy human subjects was still detected in the faeces one month after digestion in five out of eight subjects, and at some lower counts even 90 d after the end of administration (Fujiwara, 2001). *Lb. rhamnosus* GG was reported to be detected in two from 8 subjects in the faeces 9 d after the last dose of lactobacilli (Alander et al. 1999). Usually, continuous consumption is necessary to maintain high concentrations of probiotic strains in the faeces (Sui et al. 2002).

Actually, concentration of introduced strains in the faeces may give an insight into their survival in the gut, but may not be an appropriate reflection of colonisation and persistence, which are important properties of probiotic strains since functionality of probiotics is largely dependent on their activity *in situ* (Alander, 1999; Simpson et al. 2000). Therefore the *in situ* demonstration of the adhesion capability of bacteria to the mucus is crucial. However, studies where analysis of mucosa were performed in addition of faecal analysis are scarce. Alander et al. (1999) readily observed *Lb. rhamnosus* GG in colonic biopsies after termination of administration, but more rarely in the faeces of the same subjects. The authors concluded that the colonisation by probiotic strains can be underestimated on the basis of their detection in the faeces.

Some authors have already pointed out that the influence of indigenous bacterial population, which vary very much between individual subjects or animals, is usually very strong, and therefore survival rates and effect of introduced strains are host-specific and the average values are not very informative (Simpson et al. 2000; Gardiner et al. 2004). As binding to mucosal surface is dependent on the composition of mucus and on the colonisation resistance of the existing microflora, the differences between individual subjects or animals are to be expected. This can be an explanation for our observation that LF221 or K7 strains were found in individual animals either in faeces or in mucosa, or only in some parts of the small intestines. It is also worth mentioning that we analysed quite large samples of tissue (10 cm^2) having in mind possible non-uniform distribution of adhered bacteria in the mucosa, however as the total intestinal surface is extremely large, it is difficult to make final conclusions on the basis of examination of a few samples of intestinal tissue.

In conclusion, in the present study *Lb. gasseri* K7 and LF221 were confirmed to have a survival ability in piglet GIT and were demonstrated to adhere *in vivo* to the small intestinal mucosa. The colonisation of mucosa was probably transitional, as 6 d after termination of probiotic feeding, the concentration of test strains in the mucosa dropped below the detection limit. However, both strains were still present in the faeces or ileum's content of individual animals six d post administration. The total numbers of clostridia and coliforms were not directly affected by probiotic feeding. The number of lactobacilli increased significantly in K7 and LF221 groups during the period from d 3 to d 9, but later decreased on the level observed on the third day of the trial and was again comparable with the control group. Great differences between the animals should be considered. As all animals were healthy, particular health effect could not be observed, however the demonstration of adhesion ability to intestinal mucosa *in vivo* justifies the performance of additional studies on animals challenged with pathogens, as well as human studies.

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