UDC 579.864.1:576.524
ISSN 1330-9862

preliminary communication

(FTB-1186)

Adhesion of Two *Lactobacillus gasseri* Probiotic Strains on Caco-2 Cells

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Received: September 10, 2002
Accepted: January 24, 2003

Summary

Previous *in vitro* and *in vivo* studies showed that two human isolates of *Lactobacillus gasseri*, LF221 and K7 are able to survive the passage through the gastrointestinal tract and to colonise intestines of pigs at least temporarily. The aim of this study was to examine the adhesion ability of LF221 and K7 strains to Caco-2 cells. Adhesion of lactobacilli from early stationary growth phase was examined at two pH values of DMEM buffer (4.5 and 7). *Lactobacillus rhamnosus* GG, a widely used strain with clinical evidences of its efficiency, served as a positive control. The number of lactobacilli added to each well was found to be crucial in the adhesion assay. When added, lactobacilli were in range of $2.5 \cdot 10^6$ to $2.5 \cdot 10^8$ cfu/well, the linear correlation between the number of adhered cells (log cfu) and the number of added cells (log cfu) was found for all three strains ($R^2 > 0.99$) at both pH values (4.5 and 7). At the highest concentration of added K7 and GG cells tested (app. 10^9 cfu/well), the efficiency of adhesion was reduced. pH value of the medium strongly affected the adhesion, which was promoted in acidic conditions (pH=4.5). The adhesion of K7 strain was slightly weaker compared to GG strain at both pH values, while at pH=4.5 the adhesion of LF221 strain was even better than GG adhesion, at least at lower concentration of lactobacilli. The direct comparison of these strains was possible by regression analysis. At lower concentration of lactobacilli ($2.5 \cdot 10^6$), the best efficiency of adhesion (% of adhered bacteria) was observed for the strain LF221, reaching the values of 7.8 and 1.9 % at pH=4.5 and 7, respectively, while at higher lactobacilli concentration the ration of adhesion was higher for GG strain (3.3 % at pH=4.5). In conclusion, strains LF221 and K7 were demonstrated to be adhesive, especially in acidic conditions. The level of adhesion of K7 and GG strains positively correlates with the number of added lactobacilli only up to the certain point when the saturation of potential binding sites on Caco-2 cells probably occurs. As the adhesion to Caco-2 cell cultures alone does not guarantee the adhesion of examined strains *in vivo*, additional studies on experimental animals are in progress and human clinical studies are planned as well.

Key words: adhesion, *Lactobacillus gasseri*, Caco-2, probiotic

Introduction

Probiotics are organisms which are introduced orally in the gastrointestinal (GI) tract, where they are expected to contribute positively to the activity of the intestinal microbiota, and thus, to the health of the host (*1*). In order to exert such an activity, they have to compete with the autochthonous microflora. When selecting new probiotic strains or testing functional properties of the existing ones, the screening of adhesion properties is con-

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sidered an important step (*2*). The importance of adhesion to intestinal epithelial cells for the establishement of particular probiotic bacteria in the GI ecosystem has been shown in clinical studies (*3–5*). Adhesion to the intestinal mucosa is considered important for immune modulation (the intestine is the largest immune organ of the body), pathogen exclusion, enhanced healing of damaged mucosa and prolonged transient colonisation which is important for probiotic activity (*6–8*). The inhibition of adhesion of food-borne pathogens by different lactic acid bacteria has been demonstrated *in vitro* and *in vivo* in a number of studies (*9–11*).

Two human isolates of *Lactobacillus gasseri*, LF221 and K7, were tested previously for their probiotic properties, such as production of antimicrobial metabolites, survival at low pH and in the presence of bile (*12,13*). Besides those promising results obtained in *in vitro* examinations, both strains demonstrated good survival ability and at least temporary colonisation *in vivo* in piglets (*14, 15*). As both strains are believed to have a potential in future human application, additional studies in appropriate models and human studies are needed.

Although the most relevant approach to the study of colonisation properties of particular strains is to test the adhesion to intestinal mucosal tissue pieces of human origin, such an approach is not often used because of many disadvantages such as restricted availability and the need of immediate processing (*16,17*). Therefore, the intestinal tissue culture cells and mucus isolated from the intestines are most commonly used instead, providing convenient models for different parts of mucosa, enterocytes and mucus, respectively. Tissue culture cells most commonly used as models for enterocytes are Caco-2, HT-29 and mucus producing HT-20 MTX (*16,18*). The ability of adhesion to human intestinal cell lines tested *in vitro* is believed to be correlated with the actual persistence/colonisation in human gut *in vivo*, although good adhesion *in vitro* does not guarantee good adhesion *in vivo* (*19*). More studies on comparisons of *in vitro* and *in vivo* results are needed to make any conclusions (*2*).

As adhesion on the cultured epithelial cells is affected by many factors, such as pH, growth phase of bacteria, buffer used in the assay, the density of bacterial suspension and the assay protocol (volumes, time of adhesion, intensity of washing out the unbound cells *etc.*), the standardisation of assay protocol and the determination of optimal density of cell suspension are of crucial importance (*20–22*).

The main objectives of our study were to examine the adhesion ability of *Lactobacillus gasseri* K7 and LF221 to Caco-2 cell line in comparison with *L. rhamnosus* GG, a commercial probiotic strain with well documented probiotic properties, as well as to determine the effect of pH and the concentration of added lactobacilli cells on adhesion.

Material and Methods

Bacteria and growth conditions

Lactobacillus gasseri K7 and LF221 were previously isolated from babies' faeces and identified as potential probiotics (*12,13*). They are deposited in the Culture Collection at the Chair of Dairy Science, Zootechnical Department, University of Ljubljana. The *L. rhamnosus* GG strain with good adhesion properties was kindly provided by Dr. M. Saxelin (Valio Ltd., Finland). Lactobacilli were cultured in MRS broth (Merck, Germany) at 37 °C in microaerophilic atmosphere obtained by the use of GenBox system (BioMerieux, France).

Caco-2 cell culture

The Caco-2 human colon adenocarcinoma cell line (IZSBS BS TCL 87) was obtained from Istituto Zooprofilatico Sperimentale (Brescia, Italy). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) with L-glutamine, and 10 % (volume fraction) of fetal calf serum Fetalclone II (Hyclone, Germany) and 50 μg/mL of gentamycin sulphate (Sigma, USA). The incubation was carried out at 37 °C in 10 % CO_2 atmosphere.

Preparation of lactobacilli and Caco-2 for adhesion assay

For adhesion assay, the Caco-2 cells were seeded at a concentration of 10^4 cells/well in 24-well standard tissue culture plates.

The cells were maintained for 2 weeks after the confluence, when they were considered to be fully differentiated (*18*). At least 24 h before the tests, the DMEM medium with gentamycin was replaced with the same medium without antibiotic. The number of cells in a well was determined by trypsinisation of the monolayer (2 min) and counting by using a haemocytometer.

Lactobacilli cells from 18-hour MRS cultures were obtained by centrifugation (3500 g/10 min) and washed once with PBS buffer (pH=7.3) and once with the buffer used in the assay (DMEM without FBS and antibiotic, pH=4.5 or 7). The cell density was adjusted approximately to the desired levels by measuring the absorbance at 660 nm. The exact number of viable lactobacilli used in the assays was determined for each experiment by plate counting on MRS agar.

Adhesion assay

Preliminary assays for determination of appropriate concentration of Triton X-100 and minimal time needed to liberate lactobacilli from the surface of Caco-2 cells were done. Concentrations of 0.05, 0.2 and 0.5 % of Triton X-100 and 10, 20, 30 and 40 min of incubation time were tested on strains LF221, K7 and GG.

The assays were carried out in 24-well standard tissue culture plates. After washing the Caco-2 monolayer twice with PBS (pH=7.3), 0.5 mL of lactobacilli suspension was added to each well and incubated for 30 min in atmosphere with 10 % CO_2, at 37 °C. Afterwards, the unattached lactobacilli were removed by 3-fold washing with PBS. In order to enumerate the attached bacteria, each well was treated with 1 mL 0.05 % Triton X-100 for 10 min. Mixtures of lysed Caco-2 cells and lactobacilli were plated on MRS agar (Merck, Germany). The enumeration was done after 48-hour incubation at 37 °C in microaerophilic atmosphere.

Each of the trial conditions was tested in ten wells. Statistical analysis of the pH value effect was performed by paired t-test ($P<0.05$). The relation between the number of added and adhered cells in the assay was determined by the regression analysis according to the least square method.

Results

In order to liberate lactobacilli from the surface of Caco-2 cells, the cells were treated with Triton X-100. In preliminary trials, the concentration of Triton X-100 and the time of the treatment that were sufficient to liberate the attached bacteria without damaging them were determined. While after 40 min at 0.05 % Triton X-100 the number of viable *Lactobacillus* GG remained unchanged, the number of cfu/mL of K7 and LF221 strains was reduced for 47 and 20 %, respectively, the incubation for up to 30 min did not affect the number of viable cells.

Fig. 1 represents the average numbers of viable lactobacilli (log cfu/well) adhered to Caco-2 cells at four levels of added cells concentration (log cfu/well) and at two pH values, 4.5 and 7. The number of Caco-2 cells was determined in four wells in each trial, and an average number of $4.8 \cdot 10^5$ cells/well was used for all calculations.

At pH=4.5, more cells remained attached than at pH=7, at all concentration levels of added bacteria (Fig. 1). The differences in adhesion at two different pH values were statistically significant for all strains and concentration levels except for the GG strain at the lowest concentration of added cells ($2.53 \cdot 10^6$ cfu/well). The results from this trial differed from the others in higher variance (0.18), while in the other trials the values of variance ranged between 0.01–0.13.

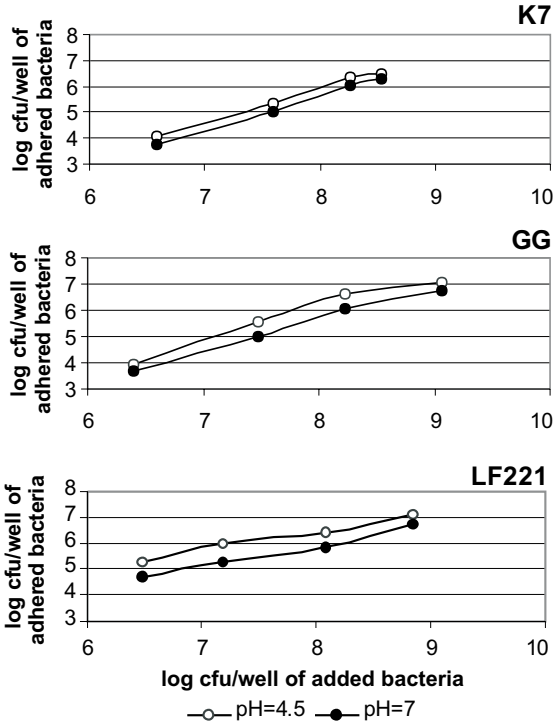

Fig. 1. Effect of pH value on the adhesion of *Lactobacillus rhamnosus* GG, *Lactobacillus gasseri* LF221 and K7 to Caco-2 cells at four concentrations of lactobacilli. All data are a mean of ten samples

Inside the range of added cells concentration from approx. $2.5 \cdot 10^6$ to $2.5 \cdot 10^8$ cfu/well, the linear correlation between the number (log cfu/mL) of added cells and the adhered cells was found in the case of K7 and GG strains, while at the highest concentration tested, the increase in the amount of added cells did not result in proportional increase of the added cells. The efficiency of LF221 strain adhesion was not reduced even when nearly 10^9 ($7 \cdot 10^8$) viable cells were added, which is the highest concentration tested in our experiments. Regresion analysis of the data for the three concentration levels except the highest one was performed and it showed high linear correlation, as regression coeficients were all high ($R^2 \geq 0.97$) (Fig. 2).

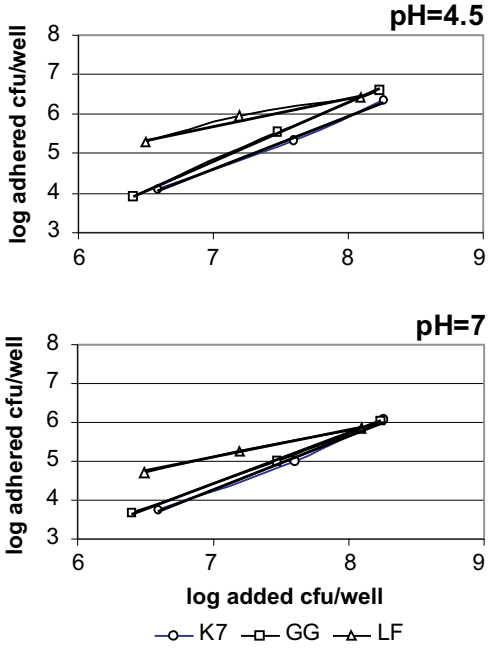

Fig. 2. Regression analysis of the relation between the concentration of lactobacilli cells used in adhesion assay and the observed number of adhered bacteria (log cfu/well). The lines indicate the linear fit according to the least-squares method. R^2 are the following: pH=4.5 (above): R^2 (K7) = 0.997, R^2 (GG) = 0.999, R^2 (LF221) = 0.970; pH=7 (below): R^2 (K7) = 0.995, R^2 (GG) = 0.999, R^2 (LF221) = 0.997

The direct comparison of the adhesion ability of three strains tested was possible only by assuming that the concentration of viable lactobacilli cells added to the wells was identical. Therefore, the number of adhered lactobacilli for three strains was calculated from the regression equations for two concentration levels of added cells, $2.5 \cdot 10^6$ and $2.5 \cdot 10^8$ cfu/well, both within the range of linear correlation between the amount of added cells and the adhered cells. The results are expressed as the number of adhered lactobacilli (cfu) per Caco-2 cell (Table 1) and the ratio of adhered cells per added cells (%) (Fig. 3). The differences in adhesion among the strains were more pronounced at lower concentration of added lactobacilli, where the adhesion of LF221 strain

Table 1. Adhesion of three lactobacilli strains, *L. rhamnosus* GG, *L. gasseri* K7 and *L. gasseri* LF221 to Caco-2 cell line; the values (cfu/Caco-2) for two concentration levels of lactobacilli were derived from the linear regression analysis

Test strain	pH	Adhered bacteria (cfu/Caco-2)	
		$2.5 \cdot 10^6$ cfu/well (=5.2 cfu/Caco-2) of added bacteria	$2.5 \cdot 10^8$ cfu/well (=520 cfu/Caco-2) of added bacteria
K7	7	0.006	3.25
	4.5	0.014	6.44
LF221	7	0.1	1.64
	4.5	0.41	10.44
GG	7	0.009	3.7
	4.5	0.018	16.93

was somewhat better than that of GG strain, and that of K7 strain only negligibly lower from the control strain. At higher concentrations of lactobacilli, the differences among three strains were small (Fig. 3) and can be better illustrated by the corresponding numbers of adhered lactobacilli per 1 Caco-2 cell (Table 1). The best adhesion was achieved by the strain GG at pH=4.5, at 16.93 lactobacilli/Caco-2. Under the same conditions, on average 10.44 and 6.44 of LF221 and K7 cells, respectively, would be adhered per 1 Caco-2 according to our calculations.

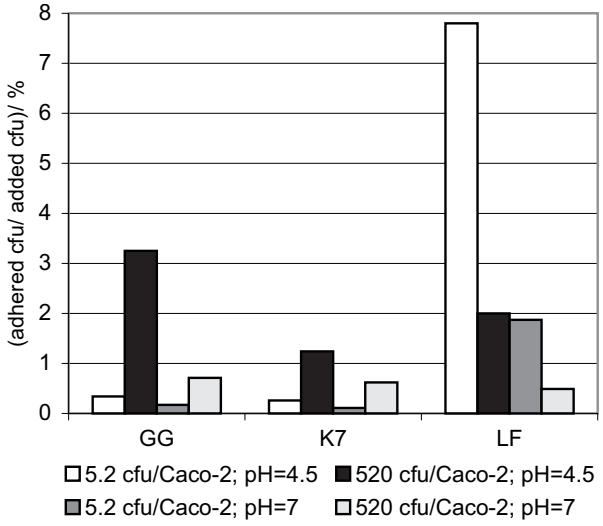

Fig. 3. Efficiency of adhesion expressed as the ratio (%) of lactobacilli viable cells that remained adhered to the Caco-2 enterocytes. The ratio was calculated from the values obtained by linear regression analysis

Another commonly used measure of adhesion is the ratio (%) of the added lactobacilli that remained adhered to the enterocytes. An interesting observation was that the ratio (%) was higher at lower concentration of added bacteria only in the assays with LF221 strain, while it was opposite for another two strains (Fig. 3). The same trend can also be seen from the slopes of regression curves, which is steeper for LF221 strain (Fig. 2). As expected from the observed number of adhered bacteria, their higher calculated ratio was found at pH=4.5.

Discussion

Besides two test strains, *L. gasseri* LF221 and K7, a strain with good adhesion properties, *L. rhamnosus* GG, was included in adhesion ability testing as a positive control strain. *L. rhamnosus* GG is a widely used commercial strain (Valio Ltd., Finland) with clinical evidence of its probiotic activities and its adhesion was tested in a number of experiments carried out by different groups (*23–27*).

Although time consuming, the number of adhered cells was determined by counting colony forming units (cfu), since it enables the enumeration of all attached bacteria per well, while a limited number of microscopic fields can be examined by microscopic enumeration (*16*). The main disadvantage of this method is that only viable organisms can be counted, but this did not have significant importance in our assays as the number of non-viable lactobacilli in the 18-hour culture of lactobacilli was negligible (results not shown). The other possible methods of bacterial quantification, such as radiolabelling of bacteria prior to the adhesion assay and measurement of radioactivity by liquid scintilation, detection with antibodies and ELISA or flow cytometry, are faster but need to be introduced carefully, with appropriate controls, including the determination of cfu and microscopic counting. Tumuola *et al.* (*26*) measured the adhesion of 12 tested *Lactobacillus* strains by flow cytometry and found out that the number of bacteria thus determined correlated well with plate counting.

Our observation that the direct relation exists between the number of bound bacteria and the number of added bacteria is in accordance with reported studies on lactobacilli (*26,27*). Lee *et al.* (*27*) tried to describe the adhesion ability of two commercial probiotic strains, *L. casei* Shirota and *L. rhamnosus* GG, and of two *E. coli* strains quantitatively, by mathematical equations. When a simple dissociation process was involved, it was possible to predict the maximal number of binding sites on Caco-2 cells and the adhesion affinity quite well; where other mechanisms were involved as well, the observed values deviated significantly from the predicted ones. In the above study, the predicted maximum number of adhered *L. rhamnosus* GG cells, namely the number that could not be exceeded by increasing the concentration of bacteria in the assay, was 16.13 per 100 Caco-2. In our study, on average 16.93 cfu of GG strain would adhere to 1 Caco-2 at pH=4.5 if 520.8 cfu/Caco-2 were added. Comparing the results of both studies, it could be speculated that the saturation of binding sites on Caco-2 cells by GG strain was nearly reached in our study. However, additional trials with higher bacterial concentrations should be performed to make conclusions about the number of receptor sites on Caco-2 cells for each particular strain. The number of adhered cells of different strains on Caco-2 cells is not dependent only on the number of binding sites but also on the adhesion affinity of a given strain, which shows how strongly the adhered strain is attached.

Considering the great influence of cell density, we should be critical when comparing absolute values such as the number of adhered bacteria per Caco-2 cell. Quite often, insufficient attention was given to the determina-

tion of the exact concentration of bacterial suspensions tested in adhesion assays. The adjustment of the cell density simply by measuring the optical density seems to be too inaccurate. In addition, the surface components of the cells can have influence on the turbidity of the cultures and therefore the cell suspensions with identical OD can contain very different numbers of viable cells (*26*). Commonly reported absolute values from the other studies are up to 3.5 cfu of *Lactobacillus* GG/Caco-2, while these values are most often a result of microscopic counting (*26–28*). Except for the significantly higher values at pH=4.5 and the highest bacterial concentration, in our study the average number of viable *Lactobacillus* GG cells adhered per one Caco-2 mainly fell into that range. The differences between our test strains were expected since it has been shown in many studies that adhesion is usually not species dependent, but strain specific (*9*).

The influence of pH was observed previously as well. The binding of *Lactobacillus johnsonii* La 1 occured at any pH between 3 and 7, but seemed to be better in acidic conditions, although the differences between pH values 4 and 7 were not statistically significant. In the case of La 1 strain, good adhesion was observed at pH=3, while at pH>4 the strain remained unadherent (*29*). *Lactobacillus* GG was found to be susceptible to the changes in pH as well (*25*). The mechanisms of the effect of pH value have not been reported so far. There are also different opinions about the pH values in the assays that would better resemble the microenvironment in the gut. While the pH of luminal small bowel is about 7, in the vicinity of the brush border the environment is acidic due to the presence of mucus overlay and metabolic activity of intestinal cells and adhered bacteria, therefore the adhesion tests should be performed at different pH values (*25,29*).

It is difficult to comment the different behaviour of LF221 strain from the other two strains on the basis of the presented results, regarding the differences (%) of adhered bacteria at lower or higher concentration of added cells. As the adhesion process can include specific and non-specific binding, the influence of these two mechanisms may not be the same for all three strains. Additional studies on mechanisms could explain such behaviour; nevertheless, the high rate of LF221 cells adhesion at low concentrations can mean that in practice the desired probiotic effects could be obtained already at lower intake of bacteria.

In conclusion, strains LF221 and K7 were demonstrated to be adhesive, especially in acidic conditions. In the range between 5.2 and 520.8 cfu of added viable lactobacilli per Caco-2 cell, the level of adhesion positively correlated with the number of added lactobacilli. The saturation of binding sites on Caco-2 by GG and K7 cells was reached at the highest concentration of lactobacilli tested. Beside the *in vivo* studies on conventional and gnotobiotic piglets and *ex vivo* on the intestinal mucosa which are currently going on, human clinical studies are planned to demonstrate the adhesion of lactobacilli from the present study in the target organism.

Acknowledgements

This work was funded by the Slovenian Ministry of Education, Science and Sport (L4-3191-0402–01).

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Adhezija dvaju probiotičkih sojeva *Lactobacillus gasseri* na stanice Caco-2

Sažetak

Prethodna istraživanja *in vitro* i *in vivo* pokazala su da su dva humana izolata *Lactobacillus gasseri*, LF221 i K7, sposobna preživjeti u crijevima te da barem određeno vrijeme mogu kolonizirati gastrointestinalni trakt svinja. Svrha je rada bila ispitati sposobnost adhezije sojeva LF221 i K7 na stanice Caco-2. Adhezija laktobacila iz rane stacionarne faze rasta ispitana je pri dvjema pH-vrijednostima (4,5 i 7) u DMEM-puferu. *Lactobacillus rhamnosus* GG, zbog svoje učinkovitosti često upotrebljavan soj u kliničkim ispitivanjima, poslužio je kao pozitivna kontrola. Utvrđeno je da je broj dodanih laktobacila u svaku jažicu bitan za ispitivanje adhezije. Kada je dodano od $2{,}5 \cdot 10^6$ do $2{,}5 \cdot 10^8$ cfu laktobacila/jažica, nađena je linearna korelacija za sva tri soja ($R^2 > 0{,}99$) između broja međusobno povezanih stanica (log cfu) i broja dodanih stanica (log cfu) pri obje pH-vrijednosti (4,5 i 7). Pri najvišim koncentracijama dodanih K7 i GG stanica (približno 10^9 cfu/jažica) smanjen je učinak adhezije. Različite pH-vrijednosti (4,5 i 7) bitno utječu na adheziju koja je jača u kiselim uvjetima (pH=4,5). Adhezija soja K7 nešto je slabija u usporedbi s GG sojem pri obje pH-vrijednosti, dok je pri pH=4,5 adhezija soja LF221 bila čak bolja od adhezije GG barem pri manjoj koncentraciji laktobacila. Direktna usporedba sojeva bila je moguća regresijskom analizom. Pri manjim koncentracijama laktobacila ($2{,}5 \cdot 10^6$) najbolja adhezija (postotak povezanih bakterija) opažena je sa sojem LF221, pri čemu su postignute vrijednosti od 7,8 (pri pH=4,5) i 1,9 % (pri pH=7), dok je pri višim koncentracijama laktobacila postignuta veća adhezija sa sojem GG (3,3 % pri pH=4,5). Dakle, sojevi LF221 i K7 imaju sposobnost adhezije, osobito u kiselim uvjetima. Razina adhezije sojeva K7 i GG pozitivno korelira s brojem dodanih laktobacila, ali samo do određene granice gdje vjerojatno dolazi do zasićenja potencijalnih mjesta vezanja na stanicama Caco-2.