

# The Effect of Digestive Enzymes on the Adhesion of Probiotic Bacteria *In Vitro*

A.C. OUWEHAND, S. TÖLKKÖ AND S. SALMINEN

**ABSTRACT:** Specific probiotics have several clinically proven health effects. Adhesion to the intestinal mucosa is considered important for many of these effects. In the current study, the effect of digestive enzymes and bile on the adhesion to intestinal mucus of 5 selected probiotics was studied. All of the digestive enzymes affected the adhesion of at least some of the tested strains. Bile was observed to reduce the adhesion of all strains tested. When the strains were sequentially exposed to 3 enzyme preparations and bile, the level of adhesion was reduced for all strains. The results suggest that the selection criterion “adhesion” for probiotics should be further refined.

**Keywords:** intestinal mucus, adhesion, *Lactobacillus*, digestive enzymes, bile

## Introduction

PROBIOTICS ARE MICROBIAL FOOD SUPPLEMENTS THAT BENEFICIALLY affect the health of the host. Several beneficial health properties have been clinically documented for specific probiotics (Salminen and others 1998). For many of these properties, adhesion of the probiotic to the intestinal mucosa is considered important; for immune modulation (Schiffirin and others 1997; O'Halloran and others 1998), enhanced healing of damaged gastric mucosa (Elliot and others 1998), antagonism against enteric pathogens (Coconier and others 1993) and transient colonization (Alander and others 1999). Therefore, the ability of a strain to adhere to the intestinal mucosa is one of the main selection criteria for probiotic microorganisms (Havenaar and others 1992).

We have previously shown the adhesion of selected probiotic strains in intestinal mucosal models; Caco-2 tissue culture cells (Tuomola and others 1998), intestinal mucus (Kirjavainen and others 1998) and ileostomy glycoproteins (Tuomola and others 1999; Ouwehand and others 1999a). The effect of the normal microflora on the *in vitro* adhesion of selected probiotics to immobilized intestinal mucus has been investigated, and reported not to affect the adhesion of the tested probiotics (Ouwehand and others 1999b). The effect of, especially, low pH and bile on the viability of probiotic microorganisms has been widely investigated as resistance to these factors is considered an important characteristic for a potential probiotic strain (Havenaar and others 1992). However, the effect of these factors on the adhesive ability of probiotics has not received much attention. The effect of certain proteases on the adhesion of probiotics has been investigated, but primarily for the purpose of studying the nature of adhesions and not for their possible effect on adhesion (Tuomola 1999).

In the present study, we examined the effect of digestive enzymes, bile and low pH on the mucus adhesion of five probiotic strains. The objective was to evaluate whether these treatments would alter the adhesive properties of the strains studied. This would indicate whether passage of probiotics through the stomach and small intestine can be expected to change the adhesive capacity of the strains and would validate the way adhesion assays are performed to date.

## Materials and Methods

### Bacteria and culture conditions

Four probiotic *Lactobacillus* strains and one probiotic *Bifidobacterium* strain were used; *L. rhamnosus* GG (ATCC 53103), *L. casei* strain Shirota (isolated from Yakult®, Yakult Honsha Co. Ltd.); *L. johnsonii* La1 (isolated from a LC1® product, Nestlé), *L. rhamnosus* LC 705 and *B. lactis* Bb12 (Chr. Hansen A/S). All strains were kindly provided by Dr. M. Saxelin (Valio Ltd., Helsinki, Finland). The bacteria were grown in 1 ml MRS broth (Merck) from stocks stored at -75 °C in 40% glycerol (1% inoculum), *B. lactis* Bb12 was grown under anaerobic conditions (80% N<sub>2</sub>, 10% H<sub>2</sub>, 10% CO<sub>2</sub>). To the medium, 10 µl of tritiated thymidine ([methyl-1,2-<sup>3</sup>H]thymidine, 120 Ci mmol<sup>-1</sup>) was added to metabolically radiolabel the bacteria. After 16 h (± 1h) growth at 37 °C, the bacteria were harvested by centrifugation (2000 × g) and washed twice with phosphate buffered saline (PBS; pH 7.2; 10 mM phosphate) and resuspended in PBS. The absorbance (600 nm) was adjusted to 0.25 ± 0.02 in order to standardize the number of bacteria (10<sup>7</sup> to 10<sup>8</sup> CFU ml<sup>-1</sup>). *L. johnsonii* La1 was subsequently diluted 10× with PBS (6.4 × 10<sup>6</sup> CFU ml<sup>-1</sup>) in order to avoid saturation of the substratum in which case low mucus adhesion may be observed as an artifact (Tuomola et al 1999), this would also make it difficult to observe changes in the level of adhesion.

### Pretreatment of the bacteria

To mimic exposure to digestive enzymes *in vitro*, the radiolabelled bacteria were pretreated with enzymes for 1 h at 37 °C prior to adhesion. The following enzymatic treatments were performed: lysozyme (egg white, 0.1 mg ml<sup>-1</sup> in PBS pH 6.24), pepsin (porcine stomach, 0.1 mg ml<sup>-1</sup> in 0.2 M citrate-HCl buffer pH 3.0), amylase (bovine pancreas, 0.1 mg ml<sup>-1</sup> in PBS pH 6.9), lipase (porcine pancreas, 0.1 mg ml<sup>-1</sup> in PBS pH 7.7), trypsin (porcine pancreas, 0.1 mg ml<sup>-1</sup> in PBS pH 7.6), α-chymotrypsin (bovine pancreas, 0.1 mg ml<sup>-1</sup> in PBS pH 7.8) and pancreatin (porcine pancreas, 0.1 mg ml<sup>-1</sup> in PBS pH 7.5). In addition, bacteria were incubated in PBS pH 1.5 to mimic gastric juice (Conway and others 1986), and with bile (bovine, 1% and 10% in PBS pH 7.2). Bacteria were incubated with the respective buffers as control. The effect

of the treatment was calculated by comparing the mucus adhesion of the treatment to the respective buffer control.

The bacteria were also sequentially pretreated with amylase, pepsin (pH 2.0), bile (10%) and pancreatin, to simulate the effect of saliva, gastric juice, bile and pancreatic juice respectively. This order was chosen as it is similar during digestion. After the pretreatments, the bacteria were washed twice with PBS and used in the *in vitro* mucus adhesion assay as outlined below. The washing of the bacteria after the pretreatment could represent an additional stress factor. However, we did not observe major differences in adhesion between the untreated bacteria and the control bacteria treated with the respective buffers of the pretreatments.

### Mucus preparation

Mucus was prepared from human faecal samples essentially as described earlier (Kirjavainen and others 1998; Ouwehand and others 1999a). In short, faeces from fifteen healthy adult volunteers were suspended in PBS (4 °C) containing protease inhibitors and sodium azide. Faecal extracts were prepared by centrifuging the suspension at  $15000 \times g$  at 4 °C. Mucus was isolated from the faecal extract by dual ethanol precipitation. Mucus from the different subjects was pooled in equal amounts and dissolved in HEPES (*N*-(2-hydroxyethyl)piperazine-*N*-(2)ethane sulfonic acid) buffered Hanks' balanced salt solution (HH; 10 mM HEPES; pH 7.4) at a concentration of 5 mg ml<sup>-1</sup>.

### In vitro adhesion assay

The adhesion of the radioactively labelled bacteria to immobilized intestinal mucus was determined as described previously (Kirjavainen and others 1998; Ouwehand and others 1999a). Briefly, the intestinal mucus was passively immobilized on polystyrene microtiter plate wells by incubation at 4 °C (16 h ± 1h). The wells were washed twice with HH buffer and the radioactively labelled bacteria were added. After 1-h incubation at 37 °C, the wells were washed and the adhered bacteria were released and lysed with 1% SDS in 0.1 M NaOH at 60 °C for 1 h. The radioactivity of the lysed bacteria was assessed by liquid scintillation. The adhesion was expressed as the percentage of radioactivity recovered after adhesion, relative to the radioactivity in the bacterial suspension added to the immobilized mucus.

### Statistical analysis

The results from the adhesion experiments are expressed as the average of 4 independent experiments. Each experiment was performed with 4 parallels, in order to adjust for intra-experimental errors. A 2 tailed *t*-test for paired observations was used to evaluate the statistical difference ( $P < 0.05$ ) in adhesion of each strain after pretreatment in comparison to the control (corresponding buffer only).

### Results and Discussion

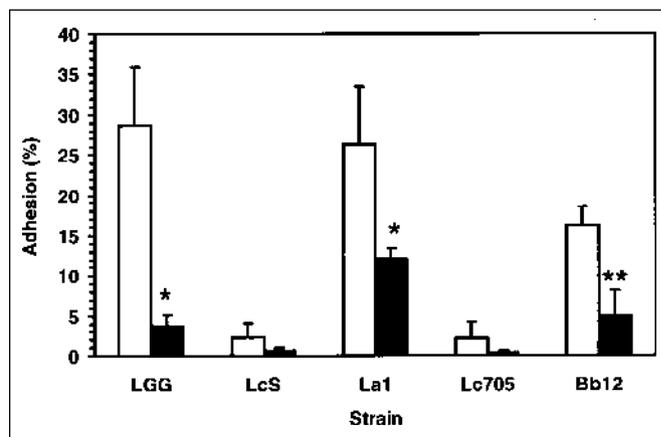
IN THE UNTREATED CONTROL SAMPLES, AT LEAST 3 OF THE tested strains were found to adhere well (= 10% of the added bacteria) to immobilized intestinal mucus; *L. rhamnosus* GG (28.8%), *L. johnsonii* La1 (27, 3%) and *B. lactis* Bb12 (10.0%). *L. casei* strain Shirota and *L. rhamnosus* Lc705 showed a low percentage (< 5% of the added bacteria) of binding to intestinal mucus; 4.0% and 1.3% respectively (Table 1). The adhesion of the untreated probiotic bacteria was similar to previous reports (Kirjavainen and others 1998, Tuomola and others 1999).

The adhesion of *L. rhamnosus* GG was reduced slightly,

by lysozyme, trypsin, chymotrypsin and 10% bile. The adhesion of *L. casei* strain Shirota was significantly reduced by all tested pretreatments except low pH and amylase. The adhesion of *L. johnsonii* La1 was significantly reduced by pretreatment with 10% bile and pepsin. The adhesion of *L. rhamnosus* Lc705 was significantly affected by pretreatment with lysozyme, bile, pepsin, lipase and low pH. Only pretreatment with bile significantly decreased the adhesion of *B. lactis* Bb12, none of the other treatments had a significant effect on the adhesion of this strain (Table 1). Proteases have earlier been reported to affect the adhesive ability of *L. johnsonii* La1 (Tuomola 1999; Bernet and others 1994) and *L. rhamnosus* GG (Tuomola 1999). Proteases are likely to degrade proteinaceous adhesions, while lysozyme treatment may release adhesions bound to the peptidoglycan. This can be expected to cause a reduced adhesion of the bacteria. Pretreatment with bile was found to significantly affect the binding of all the tested probiotics to immobilized intestinal mucus. It is possible that bile affects hydrophobic components on the bacterial cell envelope; for example, lipoteichoic acids. Lipoteichoic acids have been observed to be important for the binding of *L. johnsonii* La1 (Granato and others 1999) and bifidobacteria (op den Camp and others 1985).

A significant increase in adhesion of *L. johnsonii* La1 was observed after exposure of this strain to amylase and low pH; from 27% in the control to 35% and 42% respectively. These treatments may expose adhesins or enhance the adhesive properties of the cell envelope components. Amylase may hydrolyse bacterial capsules with  $\alpha$ 1,4-glycosidic bonds while acid treatment may alter the cell surface properties. This effect has been shown for the aflatoxin binding capacity of *Lactobacillus* sp. upon acid treatment (El-Nezami and others 1998). Similar effects may be possible in terms of adhesion properties.

To further simulate digestion, the test strains were treated sequentially with amylase, pepsin, bile and pancreatin, the adhesion of all strains was reduced, although it only reached significance for *L. rhamnosus* GG ( $P < 0.05$ ), *L. johnsonii* La1



**Figure 1**—The adhesion of probiotic bacteria; *Lactobacillus rhamnosus* GG (LGG), *L. casei* strain Shirota (LcS), *L. johnsonii* La1 (La1), *L. rhamnosus* Lc705 (Lc705) and *Bifidobacterium lactis* Bb12 (Bb12), to immobilised intestinal mucus after subsequent pre-treatment with amylase, pepsin, bile, and pancreatin (black bars). Significant differences as compared to the untreated control (white bars) are indicated; \*  $P < 0.05$ , \*\*  $P < 0.01$

**Table 1—Adhesion of probiotic bacteria to immobilised intestinal mucus isolated from human faecal samples, after pre-treatment with different digestive factors.**

Treatment	Probiotic strain adhesion (%) <sup>a</sup> (mean ± S.D.)				
	<i>L. rhamnosus</i> GG	<i>L. casei</i> Shirota	<i>L. johnsonii</i> La1	<i>L. rhamnosus</i> Lc705	<i>B. lactis</i> Bb12
No treatment	28.8 ± 4.21	4.0 ± 0.79	27.3 ± 6.33	1.3 ± 0.45	10.0 ± 3.12
Lysozyme	24.3 ± 1.91*	2.1 ± 0.80*	29.3 ± 5.40	0.3 ± 0.08*	8.4 ± 0.73
1% bile	25.2 ± 2.61	1.2 ± 0.38***	23.2 ± 5.19	0.4 ± 0.16*	2.2 ± 0.84*
10% bile	17.5 ± 7.99*	1.3 ± 0.32***	14.7 ± 4.21*	0.4 ± 0.17**	1.8 ± 3.80*
Pepsin	24.9 ± 8.87	1.9 ± 0.38**	19.7 ± 3.19*	0.4 ± 0.21**	4.6 ± 1.18
Amylase	33.2 ± 3.96	4.5 ± 1.21	35.4 ± 3.52**	2.4 ± 1.43	14.7 ± 2.19
Lipase	24.6 ± 3.09	1.2 ± 0.63**	18.8 ± 4.77	0.3 ± 0.16*	7.5 ± 0.71
Chymotrypsin	28.3 ± 5.49*	1.1 ± 0.21**	24.1 ± 1.82	0.8 ± 0.75	6.9 ± 2.13
Trypsin	25.8 ± 4.69*	1.2 ± 0.58**	24.6 ± 2.25	0.9 ± 0.85	6.2 ± 0.36
Pancreatin	32.2 ± 4.75	2.9 ± 0.93*	27.2 ± 2.86	1.4 ± 0.86	9.7 ± 1.24
PBS pH 1.5	29.2 ± 3.79	3.0 ± 0.90	42.4 ± 6.37*	0.7 ± 0.23*	13.7 ± 4.34

<sup>a</sup> Adhesion expressed as the percentage of bacteria that adhered to the immobilised intestinal mucus relative to the bacteria added to the mucus.

\*Differences were calculated by comparing the results from treated samples with the results from control samples treated with the respective buffers.  $P < 0.05$ ,

\*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

( $P < 0.05$ ) and *B. lactis* Bb12 ( $P < 0.01$ ); adhesion after treatment 3.9%, 12.0% and 4.9% respectively (Figure 1). Thus, sequential exposure to the digestive factors appears to reduce the adhesive ability in a synergistic manner. This indicates that upon ingestion the adhesive capacity of the probiotic strains may be reduced considerably. We have recently shown that also pretreatment of probiotic bacteria with canine jejunal chyme reduces the adhesive capacity of probiotic bacteria (Rinkinen and others 2000). Further supporting the observation that combined digestive factors strongly reduce the adhesive ability of probiotics. This may be of significant importance for the selection and use of probiotics.

The ability to adhere to the intestinal mucosa is currently one of the main selection criteria for probiotic microorganisms (Havenaar and others 1992). Many different intestinal mucosal models have been used to assess the adhesive ability of probiotics. Here we have focussed on the adhesion to intestinal mucus which is overlying the enterocytes. Intestinal mucus provides a microbe the 1st contact with host tissue, and serves a dual role; a barrier for some organisms (Carlstedt-Duke 1989) and a habitat for others (van der Waaij and others 1996). Lactic acid bacteria have been shown to interact well with the intestinal mucosal layer (Roos and others 2000). We have recently shown that the normal human faecal microflora does not appear to affect the mucus adhesion of the probiotic microorganisms *in vitro* (Ouweland and others 1999b). The effect of digestive enzymes, and especially low pH and bile on the survival of probiotics has been well documented (Havenaar and others 1992). However, their effect on adhesion has not been thoroughly investigated and was therefore the subject of the current study.

The results from the present study indicate that passage through the intestinal tract may not only modulate the viability of probiotics (Marteau and others 1997; Miettinen and others 1998), but is also likely to alter their adhesive ability. The biological significance of the observations remains to be assessed. However, they may help explain why, even highly adhesive, probiotic strains colonize the gastrointestinal tract only transiently.

Probiotics are usually ingested in foods. The food matrix may protect the bacteria from some digestive factors, but may also interfere with the adhesion. The results provide a basis for further investigations with probiotics that have passed through the intestine of experimental animals or

through a validated dynamic gastrointestinal tract model (Marteau and others 1997). Such models would expose the bacteria to more physiological concentrations of digestive factors under physiologically relevant conditions, like incubation time and oxygen pressure. The results also indicate that the way adhesion assays for probiotics are performed to date may have to be reevaluated.

## Conclusion

THE RESULTS PRESENTED HERE SHOW THAT THE SELECTION criterion "adhesion" for probiotics should be further developed to include adhesion following sequential exposure to digestive enzymes and bile in relation to the pH in the gastrointestinal tract.

## References

- Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, von Wright A. 1999. Persistence of colonisation of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. *Appl Environ Microbiol.* 65:351-354.
- Bernet MF, Brassart D, Neeser JR, Servin AL. 1994. *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut.* 35:483-489.
- Carlstedt-Duke B. 1989. The normal microflora and mucin. In: Grub R, Midtvedt T, Norin E, editors. The regulatory and protective role of the normal microflora. Stockholm Press, New York. p 109-127.
- Cocconier M-H, Bernet M-F, Kernéis S, Chauvière G, Fourniat J, Servin AL. 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. *FEMS Microbiol. Lett.* 110:299-306.
- Conway PL, Gorbach SL, Goldin, BR. 1986. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci.* 70:1-12.
- Elliott SE, Buret A, McKnight W, Miller MJS, Wallace JL. 1998. Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. *Am J Physiol.* 275:G425-G432.
- El-Nezami H, Kankaanpää P, Salminen S, Ahokas J. 1998. Physico-chemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. *J Food Protect.* 61:466-468.
- Havenaar R, ten Brink B, Huis in 't Veld JHJ. 1992. Selection of strains for probiotic use. In: Fuller R, editor. *Probiotics, the scientific basis.* Chapman & Hall, London. p 209-224.
- Granato D, Perotti F, Masserey I, Rouvet M, Golliard M, Servin A, Brassart D. 1999. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Appl. Environ. Microbiol.* 65:1071-1077.
- Kirjavainen PV, Ouweland AC, Isolauri E, Salminen SJ. 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiol. Lett.* 167:185-189.
- Marteau P, Minekus M, Havenaar R, Huis in't Veld JHJ. 1997. Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J Dairy Sci.* 80:1031-1037.
- Miettinen M, Alander M, von Wright A, Vuopio-Varkila J, Marteau P, Huis in't Veld JHJ, Mattila-Sandholm T. 1998. The survival of and cytokine induction by lactic acid bacteria after passage through a gastrointestinal model. *Microb Ecol Health Dis.* 10:141-147.

- O'Halloran FM, Morrissey SD, Murphy L, Thornton G, Shanahan F, O'Sullivan GC, Collins JK. 1998. Adhesion of potential probiotic bacteria to human epithelial cell lines. *Int Dairy J.* 8:596.
- op den Camp HJM, Oosterhof A, Veerkamp JH. 1985. Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells. *Infect Immun.* 47:332-334.
- Ouwehand AC, Isolauri E, Kirjavainen PV, Tölkö S, Salminen SJ. 1999a. The mucus binding of *Bifidobacterium lactis* Bb12 is enhanced in the presence of *Lactobacillus* GG and *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Lett Appl Microbiol.* 30:10-13.
- Ouwehand AC, Niemi P, Salminen SJ. 1999b. The normal faecal microflora does not affect the adhesion of probiotic bacteria *in vitro* FEMS Microbiol. Lett. 177:35-38.
- Rinkinen M, Mättö J, Salminen S, Westermarck E, Ouwehand AC. 2000. *In vitro* adhesion of lactic acid bacteria to canine small intestinal mucus. *J Anim Physiol Anim Nutr.* 84:43-47
- Roos S, Karner F, Axelsson L, Jonsson H. 2000. *Lactobacillus mucosae* sp. nov., a new species with *in vitro* mucus-binding activity isolated from pig intestine. *Int J Syst Evol Microbiol.* 50:251-8.
- Salminen S, Bouley C, Boutron-Ruault M-C, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau M-C, Roberfroid M, Rowland I. 1998. Functional food science and gastrointestinal physiology and function. *Brit J Nutr.* 80:S147-S171.
- Schiffrin EJ, Brassart D, Servin AL, Rochat F, Donnet-Hughes A. 1997. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *Am J Clin Nutr.* 66:515S-520S.
- Tuomola EM. 1999. *In vitro* adhesion of probiotic lactic acid bacteria. Ph.D. dissertation, Dept. of Biochemistry and Food Chemistry, Univ. of Turku, Turku, Finland
- Tuomola, E.M. and Salminen, S.J. 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol.* 41:45-51.
- Tuomola EM, Ouwehand AC, Salminen SJ. 1999. Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. *Lett Appl Microbiol.* 28:159-163.
- van der Waaij LA, Limburg PC, Mesander G, van der Waaij D. 1996. *In vivo* IgA coating of anaerobic bacteria in human faeces. *Gut.* 38:348-354.

---

Financial support was obtained from The Academy of Finland.

---

*Authors are affiliated with the Dept. of Biochemistry and Food Chemistry, Univ. of Turku, Fin-20014 Turku, Finland. Direct inquiries to author Ouwehand (E-mail: arthur.ouwehand@utu.fi).*

---