Lait 82 (2002) 123-130

DOI : 10.1051 / lait : 2001011

Original article

In vitro adhesion of propionic acid bacteria to human intestinal mucus

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Abstract — Propionic acid bacteria (PAB) have widely been used as starter cultures. Their potential as probiotics has, however, received little attention. Adhesion to the intestinal mucosa is considered one of the main selection criteria for probiotic micro-organisms. Therefore, in the current study the adhesion of PAB to human intestinal mucus was investigated in an in vitro model. The tested PAB exhibited a low to moderate level of adhesion (0.4 to 4.5% of the applied bacteria). Because adhesion to mucus and bovine serum albumin were similar, it is likely that the adhesion to mucus is the result of non-specific interactions. The adhesion to mucus could be significantly enhanced by prior adhesion of existing probiotic strains. The PAB did not affect the adhesion of these probiotics. Adhesion of the moderately adhering strains was found to be close to maximum within 30 s showing that the adhesion happens almost instantaneously, while the adhesion of the low binding strains increased until 1 h of incubation. These results together with earlier observations on the properties of PAB indicate that selected PAB have good prerequisites for probiotic use.

***Propionibacterium* / adhesion / intestinal mucus / probiotic**

Résumé — **Adhésion in vitro de bactéries propioniques au mucus intestinal humain.** Les bactéries propioniques ont été largement utilisées comme levains. Leur potentiel comme probiotiques a cependant reçu peu d'attention. L'adhésion à la muqueuse intestinale est considérée comme l'un des principaux critères de sélection des microorganismes probiotiques. Ainsi, dans la présente étude, l'adhésion des bactéries propioniques au mucus intestinal humain a été examinée dans un modèle in vitro. Les bactéries propioniques testées ont montré un niveau d'adhésion bas à modéré (0,4 à 4,5 % des bactéries appliquées). Comme l'adhésion au mucus et à la sérum-albumine bovine était similaire, il est probable que l'adhésion au mucus est le résultat d'interactions non-spécifiques. L'adhésion au mucus pourrait être significativement accrue par l'adhésion préalable de souches probiotiques existantes. Les bactéries propioniques n'affectent pas l'adhésion de ces probiotiques. L'adhésion des

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Oral communication at the 3rd International Symposium on Propionibacteria, Zurich, Switzerland, July 8–11, 2001.
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souches adhérant modérément était proche du maximum en 30 s, montrant que l'adhésion a lieu presque instantanément, alors que l'adhésion des souches adhérant faiblement augmentait jusqu'à 1 h d'incubation. Ces résultats ajoutés à des observations antérieures sur les propriétés des bactéries propioniques indiquent que les bactéries propioniques sélectionnées présentent de bonnes aptitudes pour une utilisation probiotique.

bactérie propionique / adhésion / mucus intestinal / probiotique

1. INTRODUCTION

Propionic acid bacteria (PAB) have traditionally been of interest for their use as dairy starters, especially for the production of Swiss-type cheeses. However, the potential use of PAB as probiotics has been little investigated [14]. Probiotics have been defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host [22]. PAB are members of the normal intestinal microflora, though not as a major component, where they are thought to play a role in the digestion of protein [12], and contribute to the production of vitamin K [6]. Some health effects of PAB have been reported; stimulation of the immune system [8,11], stimulation of the growth of bifidobacteria [3, 10] and reduction of mutagen-producing faecal enzyme activity [14, 21]. The ability of PAB to suppress the growth of yeasts and fungi could also have considerable therapeutic use [23]. In addition, PAB have been observed to survive gastrointestinal transit [13]. Their intrinsic properties and use in dairy products would make them interesting probiotic candidates.

In addition to survival of gastrointestinal transit, the ability to adhere to the intestinal mucosa is one of the main selection criteria for probiotics [7, 15, 17]. This has, to date, received little attention as far as PAB are concerned. Though, for many of the proposed health effects of probiotics adhesion to intestinal mucosa is considered important; antagonism against pathogens [4], modulation of the immune system [16] and enhanced healing of damaged intestinal mucosa [5]. Although good adhesion of probiotics does not cause long-term colonisation of a host [2] it is associated with transient colonisation [1, 9].

In the current study, the adhesive ability of four PAB was assessed in a mucus adhesion model and the effect of selected probiotic strains on this adhesion was determined. Although in vitro adhesion models cannot simulate the in vivo conditions, their results [19] appear to correlate well with in vivo observations [1, 9] and can thus be expected to have some predictive value.

2. MATERIALS AND METHODS

2.1. Bacteria and growth conditions

In the current study four PAB strains, two *Lactobacillus* strains and two *Bifidobacterium* strains were used. Media and growth conditions are mentioned in Table I. To the medium 10 $\mu L \cdot mL^{-1}$ of tritiated thymidine ([*methyl*-1,2-^{3}H]thymidine, 120 $Ci \cdot mmol^{-1}$) was added to metabolically radiolabel the bacteria. After overnight growth under anaerobic conditions, the bacteria were harvested by centrifugation (7 min, room temperature, 2000 *g*) and washed twice with phosphate buffered saline (PBS; pH 7.2; 10 $mmol \cdot L^{-1}$ phosphate) and resuspended in PBS. The absorbance was adjusted to 0.25 ± 0.02 in order to standardise the number of bacteria in suspension (10^7–10^8 $cfu \cdot mL^{-1}$) before use in the adhesion assay (see below).

Table I. Bacterial strains used in the in vitro adhesion assay and their culture conditions.

Bacterium	Culture conditions		
	Medium	Incubation time (h)	Temperature (°C)
P. freudenreichii ssp. *shermanii* JS	YEL[a] + MRS[b]	48	30
P. freudenreichii P2	YEL + MRS	48	30
P. freudenreichii P6	YEL + MRS	48	30
P. freudenreichii ssp. *freudenreichii* P131	YEL + MRS	48	30
L. rhamnosus GG (ATCC 53103)	MRS	18	37
L. rhamnosus LC 705	MRS	18	37
B. lactis Bb12	GAM[c]	18	37
B. infantis Bbi	GAM	24	37

[a] Yeast extract (5 g·L^{-1}), Na-lactate (10 g·L^{-1}), casein peptone (10 g·L^{-1}).
[b] de Man, Rogosa, Sharp Broth (Merck, Darmstadt, Germany).
[c] Gifu anaerobic medium (Nissui Seiyaku Co., Tokyo, Japan).

2.2. Mucus preparation

Colonic mucus for the in vitro adhesion assay was prepared as described earlier [19]. In short, mucus was prepared from the healthy part of resected human colonic tissue. The use of resected human intestinal material was approved by the joint ethical committee of the University of Turku and Turku University Central Hospital. The resected material was collected on ice within 20 min after resection and processed immediately. The intestine was cut open and contents were removed. The tissue was subsequently gently washed in (PBS) containing 0.01% gelatine until no contents were visible. Mucus was collected into a small amount of *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid (HEPES)-Hanks buffer (HH; 10 mmol·L^{-1} HEPES; pH 7.4) by gently scraping human colonic mucosa with a rubber spatula. The collected mucus was centrifuged for 10 min at 13 000 *g* to remove cell debris and bacteria. The mucus was stored at –70 °C until use.

2.3. Adhesion assay

The adhesion assay was performed as described earlier [19]. In short, mucus was passively immobilised on microtitre plate wells (Maxisorp, Nunc, Denmark) at a concentration of 0.5 mg·mL^{-1} protein. Radiolabelled bacteria were added to the wells and incubated for 1 h at 37 °C. Non-bound bacteria were removed by washing with HH. Bound bacteria were released and lysed with 1% sodium dodecyl sulphate in 0.1 mol·L^{-1} NaOH. Radioactivity was determined by liquid scintillation and the adhesion was expressed as the percentage of radioactivity recovered after adhesion, relative to the radioactivity in the bacterial suspensions added to the immobilised mucus.

To determine non-specific adhesion of the PAB, the adhesion assay was performed as described above using immobilised BSA at a concentration of 0.5 mg·mL^{-1}. Adhesion to polystyrene was assessed by performing the adhesion assay with uncoated microtitre plate wells (the plates are made of polystyrene).

2.4. Adhesion with lactobacilli and bifidobacteria

To investigate the effect of lactobacilli or bifidobacteria on the adhesion of PAB and vice versa, non-labelled lactobacilli or bifidobacteria were allowed to bind to the immobilised mucus (1 h, 37 ºC) followed by radioactively labelled PAB. For the effect of PAB on the adhesion of lactobacilli and bifidobacteria, non-labelled PAB were allowed to bind to the immobilised mucus first (1 h, 37 ºC), followed by radioactively labelled lactobacilli or bifidobacteria. The rest of the experiment was performed as described above for the adhesion assay.

2.5. Incubation time

In order to investigate the influence of incubation time on the adhesion of the PAB, labelled bacteria were incubated for 30 s, 5 min, 10 min, 15 min, 30 min, 60 min and 90 min. The rest of the experiment was performed as described above for the adhesion assay.

2.6. Statistical analysis

The results from the adhesion experiments are expressed as the average (± standard deviation) of at least three independent experiments. Each experiment was performed with four parallels, to adjust for intra-experimental errors. A paired, two-sided, t-test was used to determine the statistically significant ($p < 0.05$) difference in adhesion between the strains, to the substrata and with incubation time.

3. RESULTS

3.1. Adhesion to different substrata

The tested PAB were observed to exhibit a low to moderate level of adhesion to all tested substrata; 0.2 to 6.5% (Tab. II). There was no significant difference in adhesion to intestinal mucus or BSA. Because of poor growth on MRS and a trend to better adhesion upon growth in YEL for strains *P. freudenreichii* P6 and P 131 ($p = 0.09$), all further assays were performed with YEL-grown bacteria.

3.2. Influence of other bacteria on the adhesion

The effect of lactobacilli and bifidobacteria already adhered to the intestinal mucus was investigated. It was found that prior adhesion of *L. rhamnosus* GG, *B. lactis* Bb12 and *B. infantis* Bbi significantly

Table II. The adhesion of propionic acid bacteria to immobilised human intestinal mucus, bovine serum albumin (BSA) and polystyrene (PS).

Strain	Adhesion % (average ± standard deviation)			
	Mucus (MRS)[a]	Mucus (YEL)[b]	BSA (YEL)[b]	PS (YEL)[b]
P. freudenreichii ssp. *shermanii* JS	0.6 ± 0.3	0.4 ± 0.2	0.2 ± 0.1	2.1 ± 0.7
P. freudenreichii ssp. P2	1.6 ± 0.5	1.6 ± 0.3	1.1 ± 0.2	6.5 ± 2.9
P. freudenreichii ssp. P6	0.8 ± 0.2	2.0 ± 1.4	1.1 ± 0.9	5.2 ± 2.7
P. freudenreichii ssp. *freudenreichii* P131	0.4 ± 0.2	4.5 ± 2.0	2.7 ± 2.4	6.5 ± 4.1

[a] Grown in de Man, Rogosa, Sharp Broth.
[b] Grown in yeast extract, casein peptone, Na-lactate medium.

Table III. The influence of mucus adhered lactobacilli and bifidobacteria on the adhesion of propionibacteria.

Secondary[a]	Primary[a]				
	Control	LGG	LC 705	Bb12	Bbi
P. freudenreichii ssp. *shermanii* JS	0.6 ± 0.3[b]	0.5 ± 0.1	0.7 ± 0.3	0.7 ± 0.2	0.6 ± 0.2
P. freudenreichii ssp. P2	2.6 ± 1.4	2.8 ± 2.4	3.6 ± 3.4	3.8 ± 2.6*	2.7 ± 1.8
P. freudenreichii ssp. P6	1.9 ± 0.8	6.0 ± 0.6*	2.5 ± 1.4	3.6 ± 1.9*	2.8 ± 0.8
P. freudenreichii ssp. *freudenreichii* P131	4.4 ± 1.9	7.00 ± 1.6*	4.4 ± 1.3	6.7 ± 3.7	5.4 ± 1.9*

[a] Non-labelled *L. rhamnosus* GG (LGG), *L. rhamnosus* LC 705 (LC 705), *B. lactis* Bb12 (Bb12) or *B. infantis* Bbi (Bbi) were allowed to bind to immobilised mucus (primary), prior to radioactively labelled propionibacteria (secondary), the adhesion of which was measured.
[b] Adhesion (%) ± standard deviation.
* Significantly different from control $p < 0.05$ (paired, two-sided, t-test).

Table IV. The influence of mucus adhered propionibacteria on the adhesion of bifidobacteria and lactobacilli.

Secondary[a]	Primary[a]				
	Control	PJS	P2	P6	P131
L. rhamnosus GG	23.0 ± 2.0[b]	21.8 ± 3.1	22.7 ± 1.7	21.5 ± 2.0	23.4 ± 2.6
L. rhamnosus LC 705	1.3 ± 0.5	2.0 ± 0.3	1.7 ± 0.5	1.6 ± 0.6	1.7 ± 1.1
B. lactis Bb12	24.3 ± 2.1	23.1 ± 1.4*	21.8 ± 2.7*	22.1 ± 3.7	22.0 ± 1.5*
B. infantis Bbi	1.9 ± 1.6	2.4 ± 0.7	1.4 ± 0.7	1.8 ± 1.6	1.8 ± 1.5

[a] Non-labelled *P. freudenreichii* ssp. *shermanii* JS (PJS), *P. freudenreichii* ssp. P2 (P2), *P. freudenreichii* ssp. P6 (P6) or *P. freudenreichii* ssp. *freudenreichii* P131 (P131) were allowed to bind to immobilised mucus (primary), prior to radioactively labelled lactobacilli and bifidobacteria (secondary), the adhesion of which was measured.
[b] Adhesion (%) ± standard deviation.
* Significantly different from control $p < 0.05$ (paired, two-sided, t-test).

enhanced the subsequent adhesion of *P. freudenreichii* P6 and P131, *P. freudenreichii* P2 and P6 and *P. freudenreichii* P131 respectively (Tab. III). When the PAB were allowed to adhere first to the immobilised intestinal mucus, only the subsequent adhesion of *B. lactis* Bb12 was affected (Tab. IV).

3.3. Incubation time

The level of binding of the tested PAB was found to be only marginally affected by the incubation time (Fig. 1). For the strains exhibiting a moderate level of binding, *P. freudenreichii* P6 and P 131, most binding took place within the first 30 s of incubation, $p < 0.01$. Of these two strains, only a non-significant increase in binding was observed over time. The PAB strains exhibiting low adhesion, *P. freudenreichii* PJS and P2, showed a significant increase in adhesion for up to 60 min of incubation, $p < 0.01$ and $p < 0.05$ respectively. Longer incubation of these two strains did not further improve their adhesion.

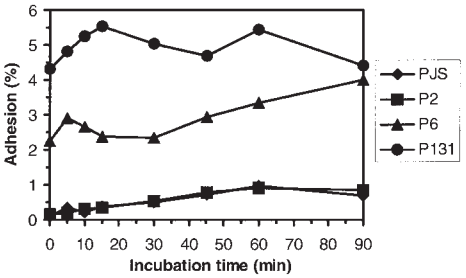

Figure 1. Relation between incubation time and adhesion to immobilised human intestinal mucus by *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS), *P. freudenreichii* P2 (P2), *P. freudenreichii* P6 (P6) and *P. freudenreichii* ssp. *freudenreichii* P131 (P131). Adhesion is expressed as the percentage of the added bacteria that bound to the mucus.

4. DISCUSSION

PAB have long been used as starter cultures. Their potential as probiotics has, unfortunately, received little attention. The aim of the current study was to investigate the ability of four PAB to adhere to immobilised human intestinal mucus, which is one of the main selection criteria for probiotic micro-organisms. This would indicate which of the tested PAB could be considered for further probiotic studies. The observed adhesion of the tested PAB to immobilised intestinal mucus was low to moderate; 0.2–6.5% of the applied bacteria depending on the growth medium (Tab. I). The tested lactobacilli and bifidobacteria were found to adhere between 1.3 and 24.3% (Tab. IV).

Organisms are not likely to have evolved specific affinity for BSA; the adhesion to this substratum is therefore considered non-specific. Since there was no significant difference in adhesion of the tested PAB to mucus or BSA, it suggests that their adhesion to mucus is mainly due to non-specific interactions. Adhesion to polystyrene was low compared to earlier observations with lactic acid bacteria, suggesting low surface hydrophobicity. Surface hydrophobicity has been suggested to be positively correlated with adhesion [24]. The observed low surface hydrophobicity may thus be an explanation for the observed relatively low adhesive ability.

The growth medium was found to influence the adhesion, though not significantly. The influence of the growth medium on the adhesion is in agreement with earlier observations for lactic acid bacteria [20]. This may thus be a general phenomenon suggesting that appropriate growth media should be selected carefully.

Together with other intestinal bacteria, *P. freudenreichii* has been observed to contain a bifidogenic factor in its cell-free extract [10]. This may have a positive influence on the composition of the intestinal microflora. Because of this effect on growth of other bacteria, the effect of PAB on the adhesion of other bacteria was also investigated. We have earlier shown that *L. rhamnosus* GG and *L. delbrueckii* subsp. *bulgaricus* enhanced the binding of *B. lactis* Bb 12 to intestinal mucus [18]. It was hypothesised that a similar effect may be observed with combinations of PAB and lactic acid bacteria. Indeed, three of the four tested PAB exhibited a significant increase in adhesion in the presence of *L. rhamnosus* GG, *B. lactis* Bb12 or *B. infantis* Bbi. The adhesion of *P. freudenreichii* P6, especially, was more than tripled by the presence of *L. rhamnosus* GG and almost doubled by the presence of *B. lactis* Bb12 (Tab. III). Although the adhesion of *B. lactis* Bb12 was significantly reduced by three of the tested PAB, this represented only a small decrease. The adhesion of the other tested lactobacilli and *Bifidobacterium* strain was not affected by the PAB. These observations indicate that the adhesion of PAB can be significantly enhanced in combination with selected probiotic bacteria. A possible mechanism for this increase in adhesion could be co-aggregation between the PAB and the lactobacilli or bifidobacteria.

However, no significant co-aggregation was observed (results not shown).

The adhesion of the PAB was only slightly affected by the incubation time; only the low adhering strains exhibited an increased adhesion upon prolonged incubation time. Interestingly, the moderately adhering strains exhibited almost their maximal adhesion within 30 s. This indicates that only very short contact times are needed to obtain sufficient binding of the bacteria and is likely to enhance the chances for in vivo adhesion.

5. CONCLUSION

The observed in vitro adhesion of the tested PAB was low to moderate, but this can be increased significantly in combination with selected probiotics. The moderately adhering PAB reached almost maximum adhesion levels in a very short time, improving the likelihood of in vivo adhesion. These results, together with earlier results on the survival of gastrointestinal transit by PAB [13], indicate that selected PAB have good prerequisites for use as potential probiotics; especially in combination with established probiotic cultures. Their potential health effects deserve therefore to be further investigated.

ACKNOWLEDGEMENTS

Financial support was obtained from the Academy of Finland.

REFERENCES

[1] Alander M., Satokari R., Korpela R., Saxelin M., Vilpponen-Salmela T., Mattila-Sandholm T., von Wright A., Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus* GG, after oral consumption, Appl. Environ. Microbiol. 65 (1999) 351–354.

[2] Berg R., Probiotics, prebiotics or "conbiotics"? Trends Microbiol. 6 (1998) 89–92.

[3] Bougle D., Roland N., Lebeurrier F., Arhan P., Effect of propionibacteria supplementation on fecal bifidobacteria and segmental colonic transit time in healthy human subjects, Scand. J. Gastroenterol. 34 (1999) 144–148.

[4] Coconnier M.-H., Bernet M.-F., Kernéis S., Chauvière G., Fourniat J., Servin A.L., Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion, FEMS Microbiol. Lett. 110 (1993) 299–306.

[5] Elliott S.N., Buret A., McKnight W., Miller M.J.S., Wallace J.L., Bacteria rapidly colonize and modulate healing of gastric ulcers in rats, Am. J. Physiol. 275 (1998) G425–G432.

[6] Falk P.G., Hooper L.V., Midtvedt T., Gordon J.I., Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology, Microbiol. Mol. Biol. Rev. 62 (1998) 1157–1170.

[7] Havenaar R., ten Brink B., Huis in't Veld J.H.J., Selection of strains for probiotic use, in: Fuller R., (Ed.) Probiotics, The scientific Basis, Chapman and Hall, London, UK, 1992, pp. 209–224.

[8] Isenberg J., Ko H., Pulverer G., Grundmann R., Stützer H., Pichlmaier H., Preoperative immunostimulation by *Propionibacterium granulosum* KP-45 in colorectal cancer, Anticancer Res. 14 (1994) 1399–1404.

[9] Johansson M.-L., Molin G., Jeppsson B., Nobaek S., Ahrné S., Bengmark S., Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa end effect on the indigenous flora, Appl. Environ. Microbiol. 59 (1993) 15–20.

[10] Kaneko T., Mori H., lwata M., Meguro S., Growth stimulator for bifidobacteria produced by *Propionibacterium freudenreichii* and several intestinal bacteria, J. Dairy Sci. 77 (1994) 393–404.

[11] Kirjavainen P.V., El-Nezami H.S., Salminen S.J., Ahokas J.T., Wright P.F., Effects of orally administered viable *Lactobacillus rhamnosus* GG and *Propionibacterium freudenreichii* subsp. *shermanii* JS on mouse lymphocyte proliferation, Clin. Diagn. Lab. Immunol. 6 (1999) 799–802.

[12] Macfarlane G.T., Cummings J.H., Allison C., Protein degradation by human intestinal bacteria, J. Gen. Microbiol. 132 (1986) 1647–1656.

[13] Mantere-Alhonen S., Propionibacteria of the dairy industry in the gastrointestinal tract, Meijeritieteellinen Aikauskirja. 40 (1982) 1–92.

[14] Mantere-Alhonen S., Propionibacteria used as probiotics – A review, Lait 75 (1995) 447–452.

[15] McNaught C.E., MacFie J., Probiotics in clinical practice: a critical review of evidence, Nutr. Res. 21 (2001) 343–353.

[16] O'Halloran S., Feeney M., Morrissey S.D., Murphy L., Thornton G., Shanahan F., O'Sullivan G.C., Collins J.K., Adhesion of potential probiotic bacteria to human epithelial cell lines, Int. Dairy J. 8 (1998) 596.

[17] Ouwehand A.C., Kirjavainen P.V., Shortt C., Salminen S., Probiotics: mechanisms and established effects, Int. Dairy J. 9 (1999) 43–52.

[18] Ouwehand A.C., Isolauri E., Kirjavainen P.V., Tölkkö S., Salminen S.J., The mucus binding of *Bifidobacterium lactis* Bb12 is enhanced in the presence of *Lactobacillus* GG and *Lactobacillus delbrueckii* ssp. *bulgaricus*, Lett. Appl. Microbiol. 30 (2000) 10–13.

[19] Ouwehand A.C., Tuomola E.M., Lee Y.K., Salminen S., Microbial interactions to intestinal mucosal models, Meth. Enzymol. 337 (2001) 200–212.

[20] Ouwehand A.C., Tuomola E., Tölkkö S., Salminen S., Assessment of adhesion properties of novel probiotic strains to human intestinal mucus, Int. J. Food Microbiol. 64 (2001) 119–126.

[21] Pérez Chaia A., Zarate G., Oliver G., The probiotic properties of propionibacteria, Lait 79 (1999) 175–185.

[22] Salminen S., Ouwehand A.C., Benno Y., Lee Y.K., Probiotics; how should they be defined, Trends Food Sci. Technol. 10 (1999) 107–110.

[23] Suomalainen T.H., Mäyrä-Mäkinen A.M., Propionic acid bacteria as protective cultures in fermented milks and breads, Lait 79 (1999) 165–174.

[24] Wadström T., Adherence traits and mechanisms of microbial adhesion in the gut, Baillièr's Clin. Trop. Med. Communicable Dis. 3 (1988) 417–433.