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Good adhesion properties of probiotics: a potential risk for bacteremia?

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Abstract

The ability to adhere to human intestinal mucus was tested for lactic acid bacteria of clinical blood culture, human fecal and dairy origin. The blood culture isolates were found to adhere better than the dairy strains. Of the *Lactobacillus rhamnosus* strains (nine clinical, 10 fecal and three dairy), blood culture isolates adhered better than the fecal strains. Although these results indicate a trend for blood culture isolates to bind to intestinal mucus in higher numbers than strains of dairy and human fecal origin, other factors are also likely to be involved in the etiology of lactobacillemia since some of the clinical *Lactobacillus* isolates exhibited a relatively low level of adhesion.

Keywords: Mucus; Adhesion; Lactic acid bacterium; Safety; Probiotic; *Lactobacillus* bacteremia

1. Introduction

Lactobacillus strains are generally regarded as safe due to their long history of safe use in fermented foods and, in many cases, their presence in the normal intestinal microflora of humans. However, *Lactobacillus* strains have been associated with some isolated cases of bacteremic infection in patients with immune suppression or underlying disease, such as endocarditis, meningitis, pneumonia and local suppurative conditions [1]. There has also been some speculation about a possible increase in *Lactobacillus* infections [2–4]. It is generally believed that lactobacilli involved in bacteremia originate from the patient's own gastrointestinal microflora [5]. When new potential probiotic strains are used, safety aspects must always be considered, as it is important to determine if the strain has any factors indicating virulence. The mechanism in opportunistic *Lactobacillus* infections is thus of great interest for determining the potential risk factors in probiotic lactobacilli.

The ability to adhere to the intestinal mucosa is considered one of the main selection criteria for potential probiotics [6] as it prolongs their persistence in the intestine [7] and thus allows the probiotic to exert its healthful effects longer. However, adhesion is also considered a potential virulence factor for pathogenic bacteria [8]. The intestinal mucus is an important site for bacterial adhesion and colonization [9] and thus provides a good model for in vitro studies of the binding abilities of probiotics.

Our previous studies indicate that the ability to adhere well to human intestinal mucus tends to be common among lactobacilli from bacteremia in comparison to those of dairy origin [10,11]. Our objective in this study was to evaluate whether the ability to bind in high numbers to mucus is a property promoting lactobacillar translocation and is as such a factor to consider when planning probiotic therapy. This is of particular concern in patients with underlying diseases predisposing to bacteremia. For this assessment, the ability to adhere to human intestinal mucus was determined for *Lactobacillus*, *Bifidobacterium*, *Carnobacterium* and *Enterococcus* strains, to determine whether strains of blood culture origin were able to bind in larger numbers to the intestinal mucus than the respective species of intestinal or dairy origin. This would indi-

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cate whether mucus adhesion is a general phenomenon among fecal lactobacilli or whether it is a factor in the potential translocation of a *Lactobacillus* strain.

2. Materials and methods

In the current study, 55 bacterial strains were tested for adhesion to intestinal mucus. Lactobacilli accounted for 48 of the strains. Sixteen of the strains were clinical blood culture isolates (Table 1), most were obtained from patients with severe underlying diseases [12]. Twenty-five of the strains tested were fecal isolates from healthy adult volunteers (Table 2), and 14 strains were of dairy origin (Table 3). Of the remaining seven strains, five were bifidobacteria (one clinical, three fecal, one dairy), one was a clinical *Carnobacterium* strain (Table 1) and one a dairy *Enterococcus* strain (Table 3). All strains were stored at −70°C in 40% glycerol and subcultured minimally to avoid changes in the properties of the strains due to continuous passage in laboratory media.

2.1. Isolation and identification of fecal strains

The three adult fecal *Bifidobacterium* isolates were kindly provided by Dr. F. He (Takanashi Milk Products Co., Ltd., Yokohama, Japan). Fecal samples were collected from healthy individuals ($n = 28$; age 41 ± 8.6 years), the subjects refrained from consuming fermented dairy products in order to avoid isolation of dairy lactobacilli. Serial dilutions were made in phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2) and 50-μl aliquots were plated onto de Man, Rogosa and Sharpe agar (MRS; Merck, Germany). The plates were incubated for 24–72 h at 37°C under anaerobic conditions. From each sample, 20 randomly picked colonies were subcultured on MRS agar. Further analysis involved Gram staining, catalase test and identification by API 50 CHL carbohydrate fermentation pattern (BioMérieux, Marcy-l'Etoile, France). Where identification was equivocal (doubtful or unacceptable identification) strains were identified by protein patterns as described by Henriksson and co-workers [13]. A similar number of human fecal isolates to clinical isolates were then chosen for further study. Where possible these strains were chosen from a range of subjects and were strains with unequivocal identification.

Table 1
Lactic acid bacteria isolated from clinical blood cultures, their classification by 16S rRNA sequence or carbohydrate fermentation patterns, and their adhesion to immobilized human intestinal mucus

Strain identification by 16S rRNA sequence clustering[a]	Adhesion % (S.D.)
L. rhamnosus T 21162	18.45 (1.84)
L. rhamnosus T 19557	5.28 (2.38)
L. rhamnosus T 17221	22.85 (1.84)
L. rhamnosus T 15756	4.19 (1.36)
L. rhamnosus 5080	20.98 (1.03)
L. rhamnosus 4846	21.32 (3.01)
L. rhamnosus 8320	9.24 (3.14)
L. rhamnosus 4813	3.85 (1.25)
L. rhamnosus Sjögren	2.54 (0.12)
L. acidophilus T 12094	11.52 (2.85)
L. acidophilus T 17699	12.01 (3.86)
Bifidobacterium sp. T 17877	8.84 (3.58)
L. casei 9393	2.53 (0.52)
L. casei 7709	18.46 (2.47)
L. curvatus 3300	21.80 (1.27)
Carnobacterium divergens 4456	2.50 (0.82)

[a]V1 and V2 region sequences; 99% similarity within a cluster.

2.2. Culture conditions

All lactobacilli, the *Carnobacterium* and *Enterococcus* strains were cultured under anaerobic conditions for 17–20 h at 37°C in MRS broth containing 10 μl ml^{-1} of tritiated thymidine (5′-^{3}H, 117 Ci $mmol^{-1}$; Amersham International, UK) as radiolabel. The bifidobacteria were cultured in GAM broth (Nissui Seiyaku Co., Tokyo, Japan) for 24–36 h with the same radiolabel. All bacteria were harvested by centrifugation ($2000 \times g \times 7$ min), washed twice and resuspended in HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-2-[ethanesulfonic acid])-buffered Hanks' balanced salt solution (HH; 10 mM HEPES; pH 7.4). The concentration of each bacterial suspension was adjusted to an absorbance of 0.5 ± 0.01 at 600 nm, corresponding to approximately 10^8 CFU ml^{-1}.

2.3. Human intestinal mucus preparation

Mucus was isolated from feces by extraction and dual ethanol precipitation according to the method of Miller and Hoskins [14], modified by Kirjavainen and co-workers [15] and Ouwehand and co-workers [11]. In short, fecal samples were collected from healthy adults ($n = 17$; age: 41 ± 8.6 years) and suspended in PBS containing protease inhibitors. The suspension was centrifuged to remove particulate matter. The mucus was then isolated from the clear extract by dual ethanol precipitation, lyophilized and finally dissolved in HH at a concentration of 10 mg ml^{-1}, using an equal amount from each of the 17 mucus preparations, and stored at −70°C until use.

2.4. Adhesion assay

The in vitro adhesion assay was performed as described by Cohen and Laux [16]. Briefly, 100 μl of mucus was passively immobilized in microtiter plate wells (MaxiSorp™; Nunc, Denmark) by overnight incubation at 4°C. Any unbound mucus was removed by washing the wells twice with 250 μl of HH, then 100 μl of radioactively labeled bacteria was added to each well. After incubation at 37°C for 1 h, the wells were washed twice with HH and 200 μl of 1% SDS in 0.1 M NaOH added to lyse and

release the bacteria. The plate was further incubated at 60°C for 1 h and the contents of the wells quantitatively transferred to microfuge tubes containing scintillation liquid (OptiPhase 'HiSafe 3'; Wallac, UK). Radioactivity was assessed by liquid scintillation and expressed as the percentage of radioactivity recovered from the wells compared to 100 μl of the radiolabelled bacterial suspension.

2.5. Statistical analysis

The results of the adhesion experiments are presented as average with S.D. of three independent experiments, which were performed with four parallels. The Kruskal–Wallis test was used to evaluate the statistical significance ($P < 0.05$) of variation in the adherence abilities between bacteria of different origin (i.e. blood, fecal or dairy). Differences in adhesion between the origin groups and species were analyzed with the Mann–Whitney U test.

3. Results

3.1. The normal human fecal Lactobacillus flora

Lactobacilli were not cultured from seven of the 28 fecal samples (i.e. $< 2 \times 10^2$ lactobacilli g^{-1} feces). From the remaining samples, the most common *Lactobacillus* species isolated were *L. rhamnosus* (10/28 samples), *L. paracasei* (7/28 samples) and *L. acidophilus* (3/28). *L. curvatus* and *L. plantarum* were identified from two individuals and *L. coprophilus*, *L. crispatus*, *L. delbrueckii* subsp. *lactis* and *Lc. lactis* were identified from one sample only (but not the same sample). This indicates that *L. rhamnosus* is one of the most common *Lactobacillus* species in the intestine of the Finnish population.

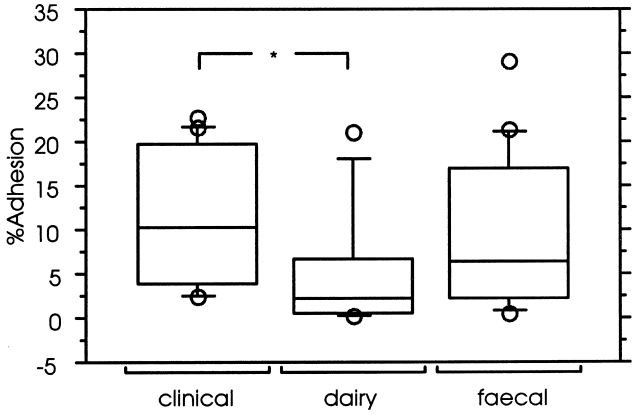

Fig. 1. Percentage adhesion of clinical, dairy, and fecal strains. *Significant difference ($P < 0.05$) in percentage adhesion. The boxes and whiskers indicate the 10, 25, 50, 75 and 90 percentiles, open circles indicate values outside the 10–90 percentile range.

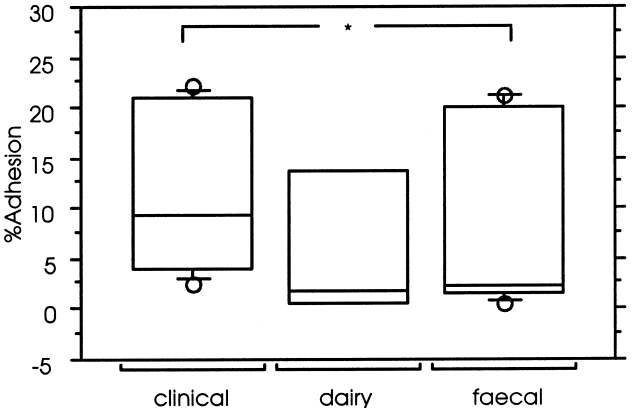

Fig. 2. Percentage adhesion of *L. rhamnosus* strains from clinical, dairy, and fecal origin. *Significant difference ($P < 0.05$) in percentage adhesion. The boxes and whiskers indicate the 10, 25, 50, 75 and 90 percentiles, open circles indicate values outside the 10–90 percentile range.

3.2. Adhesion ability of strains from different sources

With the exception of *L. rhamnosus* GG (LGG), *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus* LA5 the human fecal isolates and the clinical strains tended to adhere better to intestinal mucus than the dairy strains ($P = 0.098$; Tables 1–3). The difference in the ability of dairy and clinical strains to adhere to immobilized intestinal mucus was statistically significant ($P = 0.014$; Fig. 1). There is some overlap of the interquartile range of the

Table 2
Lactic acid bacteria isolated from feces of healthy human volunteers and their adhesion to immobilized human intestinal mucus

Strain classification[a]	Adhesion % (S.D.)
L. rhamnosus 14,4a	0.64 (0.23)
L. rhamnosus 7,1a	2.97 (1.33)
L. rhamnosus 5,5a	21.17 (0.55)
L. rhamnosus 5,3a	20.03 (3.17)
L. rhamnosus 5,1a	21.26 (2.00)
L. rhamnosus 3,8b	2.08 (1.42)
L. rhamnosus 3,7a	2.30 (1.43)
L. rhamnosus 3,3a	2.51 (1.17)
L. rhamnosus 3,1b	1.65 (0.66)
L. rhamnosus 4,17a	0.91 (0.85)
L. acidophilus 14,7b	6.27 (1.43)
L. acidophilus 16,13b	14.21 (2.41)
B. infantis H-1(1)	2.44 (1.18)
B. infantis H-1(2)	9.36 (2.22)
B. breve H-1(3)	0.94 (0.25)
L. paracasei 8,16b	17.01 (1.76)
L. paracasei 8,12a	16.90 (2.03)
L. paracasei 11,6b	3.32 (0.57)
L. paracasei 12,11a	18.07 (1.28)
L. paracasei 11,4a	29.07 (0.35)
L. curvatus 18,3b	0.66 (0.26)
L. coprophilus 14,5a	9.79 (0.78)
L. coprophilus 14,9a	4.53 (1.82)
L. plantarum 28,2b	6.74 (1.71)
L. plantarum 28,6b	6.96 (1.86)

[a]Identified by API 50 CHL carbohydrate fermentation patterns (BioMérieux, Marcy-l'Etoile, France) and where necessary, protein patterns.

Table 3
Lactic acid bacteria of dairy origin and their adhesion to immobilized human intestinal mucus

Bacterial strain	Origin	Adhesion % (S.D.)
L. rhamnosus GG	ATCC 53103	17.73 (2.03)
L. casei var. *rhamnosus*	Lactophilus® Laboratoires Lyocentre	0.30 (0.06)
L. rhamnosus 744	Fyos®, Nutricia	1.84 (0.32)
L. casei 01	Chr. Hansen	0.82 (0.99)
L. casei	BIO®, Danone	0.63 (0.53)
L. casei Imunitass	Actimel®, Danone	0.34 (0.07)
L. casei Shirota	Yakult®, Yakult Honsha	0.58 (0.08)
L. reuteri	Rela®, Ingman Foods	2.55 (1.33)
L. johnsonii LA1	LC1®, Nestlé	6.74 (1.42)
L. acidophilus LA5	Chr. Hansen	14.95 (4.40)
Bifidobacterium lactis Bb12	Chr. Hansen	6.70 (1.38)
L. plantarum 299v	Pro Viva	5.17 (0.73)
L. delbrueckii subsp. *bulgaricus*	Yogurt ATCC 11842	21.10 (3.53)
Enterococcus faecium	Gaio	1.35 (0.51)

clinical and dairy *Lactobacillus* strains. However, the median adhesion of the dairy strains is below the 10 percentile of the clinical strains.

3.3. Adhesion ability of L. rhamnosus strains from different origin

Despite the considerable overlap of the interquartile range of the clinical and fecal *L. rhamnosus* strains, the median of the fecal stains is less than the 10 percentile of the clinical strains, 2.40 and 3.06 respectively (Fig. 2). The clinical *L. rhamnosus* strains were thus found to adhere significantly better than the human fecal strains ($P < 0.05$). The clinical *L. rhamnosus* strains did not adhere better than the other clinical *Lactobacillus* strains ($P > 0.05$). As there were only three dairy (specifically two dairy and one pharmaceutical) *L. rhamnosus* strains, statistical differences could not be analyzed.

4. Discussion

Regardless of the nature of action that ingested bacteria have on the host, bacteria have a greater impact when able to adhere to the intestinal mucosal surface and thus maintain their presence in the gut longer. Mucus has a dual role in the management of the microflora. It can promote bacterial colonization by serving as an initial binding site, nutrient source and matrix on which the bacteria can proliferate [9]. Conversely, it can inhibit bacterial adhesion to the epithelium. During many enteric infections mucus secretion is extensive, with the result that the offending microorganisms are dislodged from the mucosal surface and expelled from the intestinal tract. In this study, strains of clinical origin generally, especially *L. rhamnosus*, adhered in greater numbers to human intestinal mucus than the human fecal isolates and the dairy strains. This suggests that strong adhesion to mucus may be involved in mucosal translocation of lactobacilli in a host who is immune compromised or has a severe underlying disease. However, since also some low adhesive strains are present among the clinical isolates, it is likely that other factors than strong mucosal adhesion have been involved in the translocation of these lactobacilli from the intestine to the blood stream.

It has been speculated that *L. rhamnosus* is associated with infections more often than other lactobacilli [1] and that these strains are generally of intestinal origin [5]. In this study we showed that bacteremia isolates of *L. rhamnosus* strains adhered significantly better than fecal *L. rhamnosus* strains from healthy humans. This result correlates with our previous work where we tested the ability to adhere to intestinal mucus of eight of the clinical *Lactobacillus* isolates tested in this study [16]. We did not find, however, that the *L. rhamnosus* strains adhered better than other lactobacilli of clinical origin, so the ability to adhere strongly does not explain the greater number of infections caused by this species. However, *L. rhamnosus* was the most common *Lactobacillus* species isolated from the fecal samples. If it is one of the dominant *Lactobacillus* strains in the intestine in the studied population, then this may explain why it is more often involved in bacteremia cases than other lactobacilli. *L. rhamnosus* is also intrinsically resistant to vancomycin which is often used in antibiotic therapy of severe diseases.

In this study, the dairy strains LGG, *L. delbrueckii* subsp. *bulgaricus* (a normal yogurt strain) and *L. acidophilus* LA5 (a common strain in bio-yogurts) were among the most adhesive strains tested. It is likely that in a healthy gut, the ability to adhere may allow the probiotic to exert its healthful effects, albeit transiently. In a diseased gut, the ability to adhere may increase the possibility of translocation when the body's defense mechanisms are impaired.

The long history of safe use of yogurt attests to the safety of *L. delbrueckii* subsp. *bulgaricus*. Also *L. acidophilus* LA5 has been in common use around the world for more than 20 years and has not been connected to clinical

isolates although its implication has not been evaluated. *L. rhamnosus* GG has been used in dairy products in Finland for 10 years, and only one clinical case with a strain indistinguishable from LGG has been reported [17]. Although the results presented here indicate that high adhesion may play some role in the translocation of lactobacilli (and other genera) into the circulation, the results also indicate that other factors are likely to be involved in the development of the lactobacillemia since some of the clinical isolates tested exhibited a low level of adhesion. On the nature of these factors we can only speculate, though adhesion to extracellular matrix proteins, hemolytic activity, platelet aggregation and serum resistance are currently under investigation.

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