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In Vitro Evaluation of Prebiotics on Adherence of Lactobacilli

Krausova G*, Hyrslova I, Jakubec M and Hynstova I

Dairy Research Institute, Ke Dvoru 12a, 160 00 Prague, Czech Republic

Abstract

The ability to adhere to intestinal epithelia is a crucial probiotic property since the retention of bacteria in the large intestine is important for probiotic function. Previously, we have shown that the presence of prebiotics decreases the adherence in several prebiotic- probiotic combinations. The objective of this study was to test the effect of three commercially available prebiotics: Orafti GR, Orafti P95, and Orafti Synergy on the adherence of five Lactobacillus strains (*Lactobacillus delbrueckii* subsp. *bulgaricus* CCDM 66, *Lactobacillus casei* subsp. *paracasei* PE1TB-P, *Lactobacillus fermentum* RL25, *Lactobacillus animalis* CCDM 382, and *Lactobacillus gasseri* PHM-7E1). Adherence was tested using microtiter plates and was evaluated as the percentage of fluorescently labelled bacteria present in the wells after three washes. Adherence was evaluated in both uncoated and mucin-coated polystyrene plates. In accordance with our previous results, the majority of probiotic and prebiotic interactions resulted in at least a 10-fold decrease in adherence. Only one strain, *L. gasseri* PHM-7E1, exhibited increased adherence to mucin (from 5% to 8% and from 5% to 9%) after the addition of the prebiotics Orafti P95 and GR, respectively. These two combinations appear suitable for further synbiotic testing.

Keywords: Adhesion; Adherence; Lactobacilli; Prebiotics

Introduction

The ability to adhere to the intestinal epithelium is one of the most important properties of potential probiotic microorganisms [1]. The capacity of prebiotics to enhance probiotic properties is well described; prebiotics are known to serve as an energy source for bacteria that enhance their growth. Prebiotics are defined as non-digestible compounds, providing beneficial physiological effects for the host by selectively stimulating the growth or activity of a limited number of bacterial species present in the colon [2]. These compounds include oligosaccharides (usually fructo- and galacto-oligosaccharides) that can be fermented to organic acids by colonic microbiota [3,4] and are therefore considered selective substrates for probiotics [5]. The therapeutic potential of prebiotic oligosaccharides as well as their beneficial effect on bacterial growth is well established. However, little is known regarding the impact of prebiotics (or saccharides in general) on the adherence of beneficial bacteria, such as bifidobacteria or lactobacilli. Previous studies have mainly focused on the influence of prebiotics on the adherence of pathogens, such as *Helicobacter pylori, Campylobacter jejuni, Clostridium difficile* and others. As demonstrated, prebiotics can inhibit the adhesion of pathogenic bacteria [4,6,7]; infectious bacteria bind to prebiotics instead of epithelial receptor sites [4], thus blocking the attachment of microorganisms and their toxins to host cell surfaces. This anti-adhesive therapy is already in use as an alternative method to antibiotic therapy.

However, the effect of prebiotics on the adhesion of probiotics remains unclear. Previously, we have shown that the presence of prebiotics results in a general decrease in adherence in several pre- and probiotic combinations [8]. For this reason, the objective of this study was to examine the influence of prebiotics on the adherence of lactobacilli and thus furthering our knowledge regarding their interactions. This was conducted by testing the effect of commonly used commercial fructan-type prebiotics on the adherence of five lactobacilli strains.

Method

Bacterial strains and prebiotics

The *Lactobacillus delbrueckii* subsp. *bulgaricus* CCDM 66 and *Lactobacillus animalis* CCDM 382 strains used in this study were obtained from the Culture Collection of Dairy Microorganisms (CCDM, Laktoflora®, Czech Republic).Three new gastrointestinal tract isolates were tested as well; *Lactobacillus casei* subsp. *paracasei* PE1TB-P, *Lactobacillus gasseri* PHM-7E1 (both from biopsy samples), and *Lactobacillus fermentum* RL 25 (from infant faeces).

The inulin-type fructooligosaccharide prebiotics Orafti GR, Orafti P95, and Orafti Synergy (Beneo, Belgium) were used for testing. Orafti GR mainly contains chicory inulin, and Orafti P95 mainly contains oligofructose obtained by the enzymatic hydrolysis of chicory inulin. Orafti Synergy represents a combination of inulin with selected chain lengths and oligofructose. Glucose (Sigma-Aldrich, Czech Republic) was used as a monosaccharide source.

Adherence assays

Black polystyrene 96-well microtiter plates (Nalge Nunc International, Penfield, NY, USA) were used for the adherence assays. The surface of the microtiter plates were coated with a 0.5 mg/ml porcine mucin (Sigma-Aldrich, Czech Republic) and plates without mucin were also used for testing. Plates with mucin were cultivated for 24 h at 4°C. Bacterial suspensions were diluted in phosphate buffered saline (PBS) subsequent to 24 h cultivation in de Man, Rogosa, and Sharpe (MRS) broth. The density of the suspension was adjusted to a cell concentration of McFarland No. 1. Bacteria were fluorescently stained using 5 µM of SYTO 24 solution (Life Technologies Corp., Carlsbad, CA, USA) and subsequently incubated for 30 min at 37°C. Stained bacterial suspensions (100 µl) were pipetted into the microtiter

***Corresponding author:** Krausova G, Dairy Research Institute, Ke Dvoru 12a, 160 00 Prague, Czech Republic, Tel: +420 296 792 111; E-mail: gabika.kunova@gmail.com

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plates (both with and without mucin), followed by the addition of 100 µl of prebiotic solution. After 60 min incubation at 37°C, free and weakly adherent cells were removed by three washes with 200 µl PBS buffer. Next, fluorescence was measured using a Synergy 2 reader (Bio Tek Instruments, Germany) at 485/20 nm (excitation) and 530/20 nm (emission). The data presented are the results of three independent measurements.

Percentage of fluorescence was calculated as follows:

$$X(\%) = \frac{X_{RFU-NC}}{PC - NC}$$

where X (%) =percentage conversion of the relative fluorescence units; X_{RFU}=well fluorescence in relative fluorescence units; NC=Negative Control (non-specific well fluorescence); PC=Positive Control (bacterial fluorescence without washing).

Statistical analysis

Statistically significant differences between the groups were determined with a Student's t-test at $P<0.05$ using Statistica 10 (StatSoft, Prague, Czech Republic) and Excel (Microsoft Corp., Redmond, WA). The following null hypothesis was selected: the adherence of bacteria with prebiotics/glucose is the same as bacteria in the control group (without prebiotics/glucose).

Results

The results shown in Tables 1 and 2 indicate the percentage of adherence to untreated microtiter plates and plates coated with mucin. An increase/decrease in adherence was determined by comparison to the control (blank) grown without any prebiotics or sugars. The presence of glucose and prebiotics resulted mainly in decreased adherence. Only one strain showed an increase in adherence without the addition of mucin and in the presence of prebiotics and glucose; *L. casei* subsp. *paracasei* PE1TB-P exhibited significantly enhanced adherence in comparison with the control (without the addition of prebiotics/glucose).

Adherence to mucin is important for applied uses. Overall, the adherence to mucin-coated plates was substantially lower, except for strains RL25 and CCDM 382. The robust adherence of *Lactobacillus animalis* CCDM 382 to mucin is of interest in terms of testing biofilm properties. The addition of prebiotics usually results in decreased biofilm production, however, when Orafti P95 was added, the adherence capacity remained at 32%. The only incidence of increased adherence to mucin-coated polystyrene in the presence of prebiotics was observed for strain *Lactobacillus gasseri* PHM-7E1 subsequent to the addition of the Orafti GR and Orafti P95. Interestingly, the prebiotic mixture Orafti Synergy caused a decrease in the adherence of this same strain to mucin. Similarly, the addition of glucose also resulted in decreased adherence. For all other tested strains, a significant decrease in adherence to mucin-coated plates was observed subsequent to the addition of prebiotics and glucose.

Discussion

In this study, we found that the presence of glucose and the tested prebiotics resulted mainly in decreased adherence of lactobacilli to the intestinal wall. In contrast, a significant increase in the adherence of *Lactobacillus gasseri* PHM-7E1 to mucin was observed subsequent to the addition of two fructan-type prebiotics (Orafti P95 and Orafti GR). This strain is an isolate from the colon of a child and is of interest for its probiotic properties and high hydrophobicity (unpublished), which may be related to its adherence ability.

Adhesion is a complex process in which both non-specific and specific mechanisms play a role [9]. Non-specific mechanisms are influenced by the hydrophobicity or hydrophilicity of bacteria as well as the surface charge of the substrate. Specific mechanisms are mediated by specific molecules such as adhesins and complementary receptors [10,11]. Surface adhesion is also affected by surface roughness, chemical composition, and texture [12]. The rate of adhesion also depends on pH and the presence of sugars, bile salts, calcium, and other compounds [13-15]. Bile salts significantly decrease adhesion, whereas the addition of calcium has a favourable effect [13,14]. Furthermore, it has been demonstrated that the degree of adhesion is also dependent on bacterial concentration; it is directly proportional to the number of colony forming units (CFU) [15]. Although many factors can influence adherence, there is a lack of information about how prebiotics (saccharides) can influence the adhesion process of beneficial bacteria. A limited number of studies have focused on the effect of prebiotics or single saccharides on the adhesion of beneficial bacteria. The sugars, mannose and fucose, for instance, increase the adherence of

	Orafti GR	Orafti P95	Orafti Synergy	Glucose	blank
Lactobacillus delbrueckii subsp. *bulgaricus*–**CCDM 66**	45.54 ± 1.66*↓	50.33 ± 2.83*↓	43.24 ± 3.98*↓	48.30 ± 2.58*↓	69.13 ± 4.69
Lactobacillus casei subsp. *paracasei*–**PE1TB-P**	99.12 ± 1.02*↑	95.71 ± 6.12*↑	97.20 ± 3.25*↑	80.59 ± 3.16*↑	55.35 ± 3.71
Lactobacillus fermentum–**RL 25**	12.88 ± 1.95*↓	11.88 ± 2.32*↓	10.83 ± 2,64*↓	10.53 ± 1.92*↓	16.33 ± 1.38
Lactobacillus animalis–**CCDM 382**	11.48 ± 2.57*↓	10.37 ± 2.74*↓	12.19 ± 3.32*↓	12.35 ± 2.10*↓	38.54 ± 2.74
Lactobacillus gasseri - **PHM-7E1**	27.95 ± 1.59*↓	27.16 ± 1.34*↓	29.12 ± 2.14*↓	25.57 ± 1.53*↓	47.93 ± 3.58

Prebiotics: Orafti P95, Orafti GR, Orafti Synergy, glucose; blank, a sample without the addition of prebiotics or sugar; ↓ lower adherence than that of the blank; ↑ higher adherence than that of the blank; * statistically different from the sample without prebiotics (blank). $P<0.05$.

Table 1: Percentage of adherence in untreated (without mucin) polystyrene microtiter plates.

	Orafti GR	Orafti P95	Orafti Synergy	Glucose	blank
Lactobacillus delbrueckii subsp. *bulgaricus*–**CCDM 66**	2.85 ± 0.34*↓	1.92 ± 0.29*↓	1.51 ± 0.17*↓	3.56 ± 0.56*↓	22.04 ± 1.05
Lactobacillus casei subsp. *paracasei*–**PE1TB-P**	2.00 ± 0.41*↓	2.50 ± 0.56*↓	2.35 ± 0.29*↓	0.98 ± 0.37*↓	12.90 ± 1.54
Lactobacillus fermentum–**RL 25**	5.10 ± 0.53*↓	8.39 ± 0.37*↓	5.76 ± 0.89*↓	11.13 ± 0.56*↓	32.29 ± 1.61
Lactobacillus animalis–**CCDM 382**	26.69 ± 0.89*↓	35.25 ± 0.87*↓	25.45 ± 0.30*↓	20.08 ± 0.55*↓	55.87 ± 1.34
Lactobacillus gasseri-**PHM-7E1**	8.50 ± 1.34*↑	9.22 ± 1.46*↑	5.22 ± 0.46 ↓	4.50 ± 0.87 ↓	5.50 ± 0.34

Prebiotics: Orafti P95, Orafti GR, Orafti Synergy, glucose; blank, a sample without the addition of prebiotics or sugar; ↓ lower adherence than that of the blank; ↑ higher adherence than that of the blank; * statistically different from the sample without prebiotics (blank). $P<0.05$.

Table 2: Percentage of adherence in polystyrene microtiter plates coated with mucin.

Bifidobacterium bifidum MIMBb75 to Caco-2 cells [13]. Recently, Koh et al. [16] demonstrated that the oligosaccharide tagatose has a positive effect on the adhesion of *Lactobacillus rhamnosus* GG. Another study [3] examined the effect of several prebiotic oligosaccharides and commercial prebiotics on the adhesion of probiotic strains (*Bifidobacterium longum* subsp. *infantis* ATCC 15697 and *L. rhamnosus* GG) to HT-29 and Caco-2 cell lines. The combination of 3'- and 6'-Sialyllactose (the two predominant human and bovine milk oligosaccharides) and 6'-Sialyllactose on its own caused an increase in the adhesion of *B. longum* subsp. *infantis* ATCC 15697. In contrast, the combination of 3'-sialyllactose and the commercial prebiotic product Orafti P95 did not enhance adhesion [3]. In a previous study [8], we demonstrated that the interactions between pro- and prebiotics are diverse and strongly strain-specific. In these *in vitro* experiments we have shown that the addition of the most commonly used fructan-based prebiotics resulted mainly in a decrease in the adherence of the tested strains. It seems that certain oligosaccharides are able to enhance the adhesion of probiotics; the effect on adherence is variable and strain specific, especially for commercially available prebiotic mixtures. In the next step, additional experiments are needed to simulate more precisely the gut conditions (e.g., using biological substrates). It is of importance that colon is a complex system where various bacterial species are present and their metabolic cooperation, fermentation products, or bacteria-substrate interactions and other factors can play a role. In conclusion, these results support our previous hypothesis and indicate the need for future research that will examine single combinations of pre- and probiotics individually. In addition, the strain *Lactobacillus gasseri* PHM-7E1 seems to be perspective from the point of adherence ability and should be used to determine new suitable symbiotic combinations.

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