Original Article

Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus

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In this study we evaluated the ability of commercial strains (*L. rhamnosus* GG, *L. rhamnosus* LC705, and *P. freudenreichii ssp. shermanii* JS) in combination with *B. breve* 99 or *B. lactis* Bb12 to inhibit, displace and compete with model pathogens in order to test their influence on the adhesion of selected pathogens to immobilized human intestinal mucus. Our results demonstrate that specific probiotic combinations are able to enhance the inhibition percentages of pathogens adhesion to intestinal mucus when compared to individual strains. This suggests that combinations of probiotic strains are useful and more effective in inhibition of pathogen adhesion than individual strains. Such combinations should be assessed in clinical studies in subjects where the intestinal microbiota aberrancies have been identified.

Keywords: Adhesion, pathogens, *Lactobacillus, Bifidobacterium, Propionibacterium,* combinations, synergy

Introduction

The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms are of interest when new targets for probiotics are identified. Mainly single probiotic strains have been used in human interactions but researching probiotic combinations with added benefits is actively assessed. The most extensive studies and clinical applications of probiotics have been related to the management of gastrointestinal infections caused by pathogenic microorganisms on inflammatory microbiota aberrancies. The development of adjuvant or alternative therapies based on bacterial replacement is considered important due to the rapid emergence of antibiotic-resistant pathogenic strains and the adverse consequences of antibiotic therapies on the protective microbiota. [1]

Research using single probiotic strains has been reported earlier but at present probiotic combinations with possibly additional health benefits are being assessed prior to use in clinical studies. At present, only a few scientific reports on the effects of probiotic combinations are available.[2-6] The best known probiotic combination consisting of a mixture of eight lactic acid bacterial species (VSL#3) analyzed has been reported to be effective in several human diseases.[7-9] However, the mechanisms of action have not been clarified.

The ability to adhere to the gastrointestinal mucosa and competitive exclusion of pathogens are most frequent mechanism tools for the search of new probiotics[10-12] as the most important criteria for selection of probiotics. For instance, adhesion could be influenced by both the normal microbiota and the specific probiotics included in each preparation. However, few studies are available on the adhesion interactions of probiotics combination in the intestinal mucus system.[13] Thus we hypothesized that combinations of adherent probiotic strains will influence the pathogens adhesion to the human intestinal mucus, either enhancing or decreasing the adhesion and that specific probiotics should be assessed and selected based on *in vitro* tests to interact together for particular targets. The aim of this study was to assess the adhesive properties and the abilities to inhibit the adhesion, to displace and to compete with pathogens of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* 99, *B. lactis* Bb12 and *P. freudenreichii ssp. shermanii* JS strains alone or in different combinations using the human intestinal mucus model. [14, 15]

Materials and Methods

Bacterial strains and culture conditions

The lactic acid bacteria (LAB) strains used in this study were *Lactobacillus rhamnosus* GG (ATCC 53103), *L. rhamnosus* LC705 (DSM 7061), *B. breve* 99 (DSM 13692), *B. lactis* Bb12 (DSM 10140), *Propionibacterium freudenreichii* spp. *shermanii* JS (DSM 7067). The pathogens

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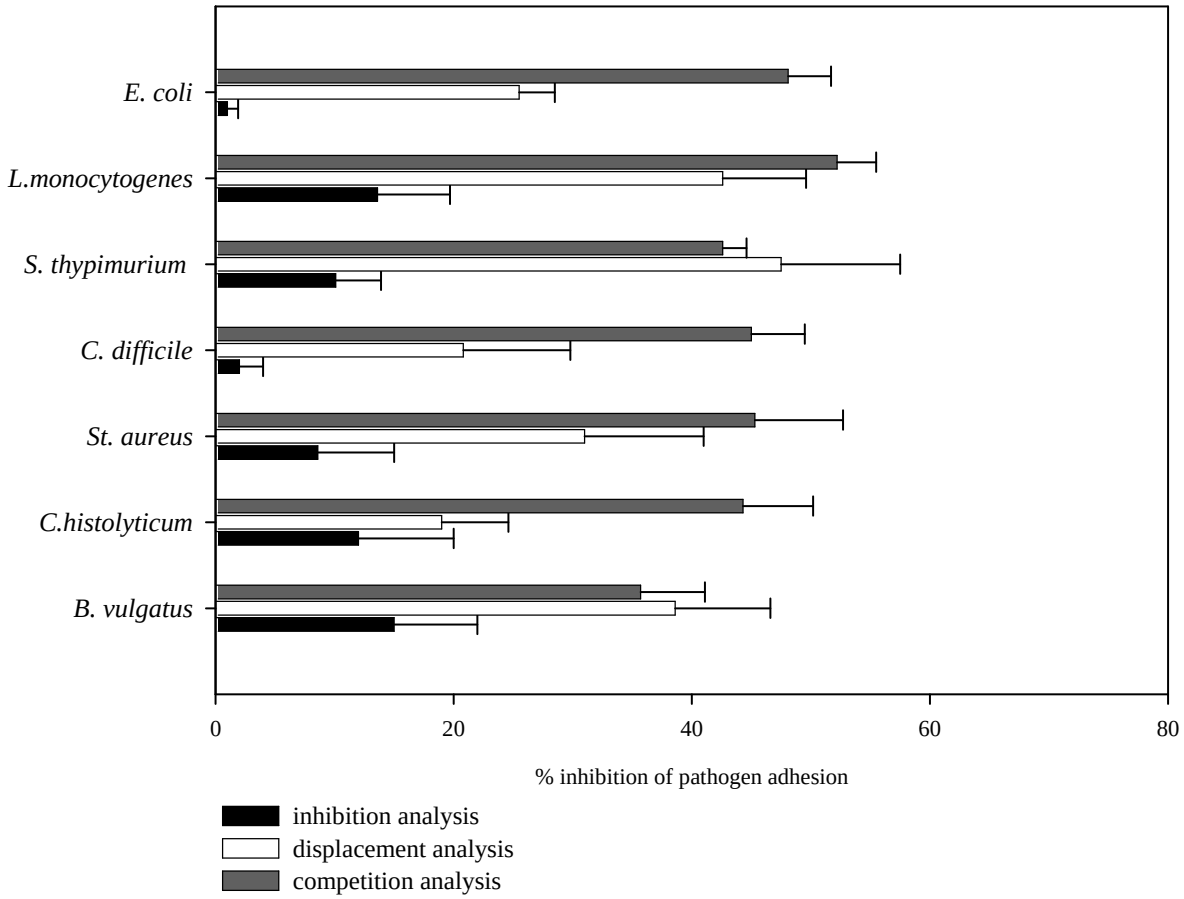

Figure 1. Inhibition of pathogen adhesion regarding to inhibition, displacement and competition with *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. lactis* Bb12 and *Propionibacterium* JS combination. Results are shown as media ± standard deviation. Controls (pathogen adhesion alone without probiotic combination presence) were taken as 0%.

pathogen strains used were *Bacteroides vulgatus* DSM 1447, *Clostridium histolyticum* DSM 627, *C. difficile* DSM 1296, *Escherichia coli* K2, *Listeria monocytogenes* ATCC 15313, *Salmonella enterica* serovar Typhimurium ATCC 12028, *Staphylococcus aureus* DSM 20231.

For assays, lactobacilli were cultured in MRS broth, bifidobacteria in MRS with 0.05% w/v cysteine-HCl, propionibacteria and pathogens were grown in Gifu anaerobic medium (GAM Nissui Pharmaceutical, Tokyo, Japan). All microorganisms were metabolically labeled by addition to the media of 10 µl/ml tritiated thymidine (5-^{3}H-thymidine 120 Ci/mM; Amersham Biosciences, UK) and they were incubated for overnight at 37°C under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, UK). Then, radiolabelled bacteria were harvested and washed twice with PBS buffer (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). Absorbance (A_{600nm}) was adjusted to 0.25±0.05 to standardize the bacterial concentration (10^8 CFU/ml approximately). Probiotic combinations were made by mixing equal amounts of each probiotic strains.

Adhesion assays to human mucus

Human intestinal mucus was collected from the healthy part of resected colonic tissue as previously described [11] and was dissolved (0.5 mg/ml protein) in HEPES-Hanks buffer (HH; 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanosulphonic acid, pH 7.4) and 100 µl of the mucus were immobilized on polystyrene microtitre plate wells (Maxisorp, Nunc, Denmark) by overnight incubation at 4 °C. The adhesion assessment was carried out as previously described.[12] Adhesion was calculated as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the mucus.

Inhibition of pathogen adhesion to intestinal mucus

To test the ability of the probiotic combinations to inhibit the adhesion of pathogens, the procedure described by Collado *et al*.,[12] was used. The inhibition was calculated as the difference between the adhesion of the pathogen in the absence and presence of probiotic combinations. Inhibition was determined in three independent experiments and each assay was performed in triplicate.

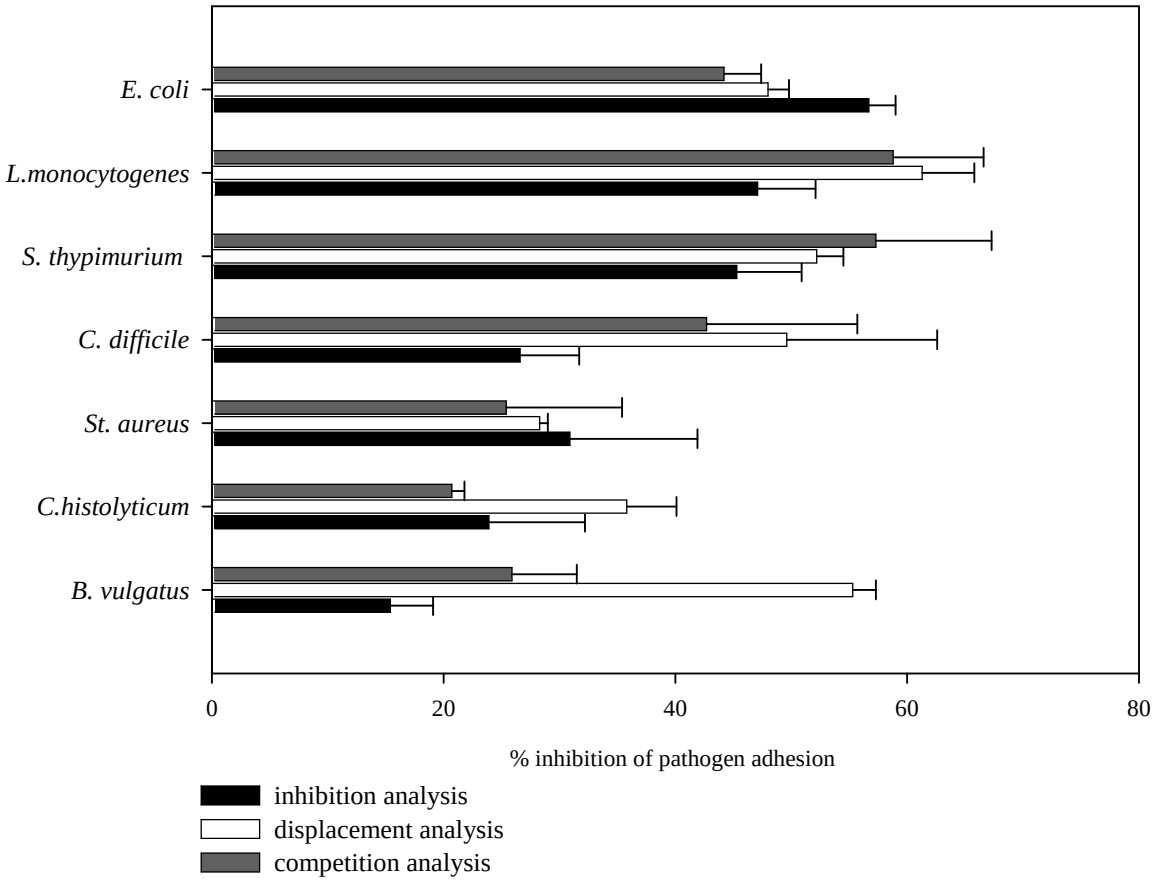

Figure 2. Inhibition of pathogen adhesion regarding to inhibition, displacement and competition with *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* 99 and *Propionibacterium* JS combination. Results are shown as media ± standard deviation. Controls (pathogen adhesion alone without probiotic combination presence) were taken as 0%.

Displacement of pathogens adhered to intestinal mucus

The ability of the studied probiotic strains to displace already adhered pathogens was assessed according to Collado *et al.*[12] Displacement of pathogens was calculated as the difference between the adhesion after the addition of the probiotic combinations and the corresponding control buffer. At least three independent experiments were carried out. Each assay was performed in triplicate to calculate intra-assay variation.

Competence between pathogens and probiotic strains to adhere to intestinal mucus

Competitive exclusion of the pathogens by tested probiotics was determined as described previously.[16] Competitive exclusion was calculated as the percentage of pathogens bound after the combination with probiotic combinations relative to pathogens bound in the absence of LAB (control).

Statistical analysis

Statistical analysis was done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Data were subjected to one-way ANOVA.

Results

In vitro adhesion assay to intestinal human mucus

All probiotic strains tested were able to adhere to intestinal mucus. The percentages expressed as mean ± SD were 20.0% ± 2.0 for *L. rhamnosus* GG, 1.2% ± 0.7 for *L. rhamnosus* LC705, 0.9% ± 0.5 for *P. freudenreichii* JS and 2.5% ± 0.3 for *B. breve* 99. The most adhesive strains was *L. rhamnosus* GG (20.0%) while the less adhesive strain was *P. freudenreichii* JS (0.9%). With regard to the pathogenic bacteria, *E. coli* K2 showed the highest adhesion value (13.8%), while the other pathogens tested showed adhesion values ranging from 4.6 to 12.6%. The less adhesive pathogens were *L. monocytogenes* ATCC 15313 and *Salmonella enterica* serovar Typhimurium ATCC 12028 that just showed a 0.5% and 0.6% of adhesion to human intestinal mucus, respectively.

Inhibition of pathogen adhesion to intestinal mucus

The inhibition of the adhesion of pathogenic microorganisms by the assessed probiotic combinations was dependent on the each probiotic strain and the pathogen assayed (Fig. 1 & 2). Probiotic combinations were able to significantly inhibit (P<0.05) the adhesion of all model

pathogens in this study. *Bacteroides vulgatus* were inhibited in 15.0-15.4%, *Clostridium histolyticum* in 12.0-23.9%, *Clostridium difficile* in 2.0-26.6%, *Staphylococcus aureus* (8.6-30.1%). *Salmonella enterica* serovar Typhimurium was inhibited by all combinations in 10.1-45.3% and *Listeria monocytogenes* in 13.6-47.1%. The *L. rhamnosus* LGG, *L. rhamnosus* LC705, *B. lactis* Bb12 and *P. freudenreichii* JS combination was able to significantly inhibit (P >0.05) the adhesion of other pathogens expect *E. coli* K2. The best combination to inhibit pathogen adhesion was *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* 99 and *P. freudenreichii* JS combination because it was able to inhibit all pathogens tested in higher percentages than the other combination with *B. lactis* Bb12.

Displacement of pathogens adhered to intestinal mucus

Results of pathogen displacement by commercial probiotic strains are showed in Figure 1 and 2. Both probiotic combinations were able to displace significantly (P <0.05) *Bacteroides vulgatus* (38.6-55.3%), *Clostridium histolyticum* (19.1-35.8%), *Clostridium difficile* (20.8-49.5%), *Staphylococcus aureus* (28.3-31.0%), *Escherichia coli K2* (43.0-48.0%), *Salmonella enterica* serovar Typhimurium (44.3-54.6%) and *Listeria monocytogenes* (25.5-48.0%). The best combination to displace the pre-adhered pathogens was *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* 99 and *P. freudenreichii* JS combination because it was able to displace all pathogens tested in the highest percentages.

Competition between pathogens and probiotic combinations to adhere to intestinal mucus

Results of competitive exclusion studies between pathogens and probiotic strains are presented in Figure 1 and 2. All probiotic combinations were able to compete significantly (P <0.05) for mucus sites with all pathogen strains tested. *Bacteroides vulgatus* was inhibited from 25.9% to 35.7% by probiotic combination. *Clostridium histolyticum* inhibition ranged from 20.7 to 44.4%, *Clostridium difficile* inhibition from 42.7 to 45.0%, *Staphylococcus aureus* from 25.4 to 45.3%, *Escherichia coli* from 44.3 to 48.2%, *Salmonella enterica* serovar *typhimurium* from 42.6 to 57.3% and *Listeria monocytogenes* between 52.2-58.8%.

Discussion

Our results are among the first to compare the *in vitro* properties and competitive exclusion abilities of different probiotic combination. Probiotic bacteria selected for commercial use in foods and in therapeutics must retain the characteristics for which they were originally selected.[10,11,17] Bacterial adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. In addition, adhesion to different mucosal surfaces, such as gastrointestinal, urogenital and respiratory tracts, is regarded a prerequisite for probiotic microorganisms, allowing the colonization, although transient, of the human intestinal tract[18] but also, it is an important step in pathogenic infection. Thus, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of probiotic bacterial strains and their combinations.[12,19]

It can be hypothesized that a combination of probiotic strains may complement each other's effects or improve benefits or properties.[3,4] We hypothesized that combinations of well-known probiotic strains as *B. lactis* Bb12 and *L. rhamnosus* GG, will influence pathogen adhesion in the human intestinal mucus, either enhancing or decreasing their adhesion. The objective of this study was therefore to determine if the chosen probiotics in the combinations tested may increase or enhance each other's beneficial properties and their potential applications in clinical studies. All probiotic strains included and tested in this study have documented health effects and also, the combination of the four strains.[3,4] All these strains tested were found to adhere well in the model system; this is in agreement with earlier observations.[12,20,21] The adhesion levels of the tested commercial probiotic strains showed a great variability depending on the strain, species and genera. All combinations of four probiotic strains tested were able to reduce the adhesion of all pathogen strains to intestinal mucus.

The ability to exclude and displace pathogens from mucus by specific probiotic strains has been reported in other studies[12,21,22] but few studies with probiotics combinations have been related. Interestingly, all the pathogens tested showed a high adherence to intestinal mucus, with the exception of *Listeria monocytogenes* that only adhered a 0.5% and *Salmonella enterica* serovar Typhimurium ATCC 12028 that show only 0.6% of adhesion to mucus. These results suggest that they have the capacity to bind the intestinal mucus, which could assist the pathogens in the invasion into the human intestinal mucosa. In this context, to find appropriate probiotic microorganisms with the ability to prevent the adhesion of these pathogenic bacteria is important, and also, to test new potential probiotic combinations with synergistic in vitro properties would be important.

The ability to inhibit the adhesion of pathogens appears to be dependent in both, the probiotic combination and the pathogen tested, indicating a very high specificity and requiring identification of the pathogens or related microbiota aberrancies involved in the probiotic target population. The displacement of pre-adhered pathogens was also found to be probiotic combination and pathogen dependent and as in the case of the inhibition of pathogen adhesion no direct correlation was found between adhesion of commercial probiotics strains and displacement of pathogen. Nevertheless, adhesion seems to be one of the factors implied. The displacement profiles were different from those observed for the inhibition of pathogens. These results, together with previous observations[12,22] appear to confirm that different mechanisms are implied in both phenomena. Also, no relation was found between the results obtained for the adhesion inhibition and displacement of pathogens, suggesting us that different mechanisms could be implied in both processes.

We were able to demonstrate that both combinations had improved synergistic properties against pathogens than the individual strains.[23] However, the combination with better properties against the model pathogens re-

garding to inhibition, displacement and competition behaviors was *L. rhamnosus* GG and LC705, *B. breve* 99 and *Propionibacterium* JS.

Probiotic combinations that inhibit and displace pathogens may be excellent candidates to use in fermented milk products. Our results demonstrate that all probiotic combinations tested in this study showed good probiotic characteristics but it is important to take into account the high specificity of these in order to select the best strain combinations to prevent or treat infection by a specific pathogen. This would allow the development of new probiotic combinations for specific diseases caused by specific pathogens and they could be useful in their prevention or treatment. Our results suggest that it is possible that these combinations could increase the beneficial effects in the health regarding to their pathogens adhesion inhibition properties and their influence in their colonization. It could be suggested that combinations of different probiotic strains may be more effective in *in vivo* than monostrain probiotics, and there are also other reports that demonstrate this hypothesis.[3,25-27] The results report a high specificity in the inhibition of the adhesion and displacement of enteropathogens by different probiotic strain combinations, belonging to different genus and species, indicating the need of a case-by-case characterization of these combinations. However, it must be taken into account that *in vivo* studies are necessary to confirm their potential effect prior to introducing such combinations to clinical intervention studies.

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益生菌結合體對抗人體病原菌在體外實驗中吸附於人類腸黏液的保護機制

本研究我們評估商業菌種(*L. rhamnosus* LGG, *L. rhamnosus* LC705, 及 *P. freudenreichii ssp. shermanii* PJS)與*B. breve* Bb99或*B. lactis* Bb12 結合 後對 於 抑制、取代和對抗模式病原體的能力，以测試這些益生菌結合體對於吸附在停止不動的人體腸黏液上的病原體之影響。我們的結果指出單一菌種相比，特定的益生菌結合體可以提高抑制病原體黏著於腸黏液的比例。這些結果指出益生菌菌種結合體比起單一菌種，對於抑制病原體的吸附較有用且功效更佳。此結合體應該對那些腸道微生物菌叢異常已經被確認的對象進行臨床研究加以評估。

關鍵字：附著、病原體、乳酸菌、雙歧桿菌、丙酸桿菌、結合體、協同作用。