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Research Note

Exopolysaccharides Produced by Probiotic Strains Modify the Adhesion of Probiotics and Enteropathogens to Human Intestinal Mucus

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ABSTRACT

Exopolysaccharides (EPSs) are exocellular polymers present in the surface of many bacteria, including *Lactobacillus* and *Bifidobacterium.* The genome sequence of several strains revealed the presence of EPS-encoding genes. However, the physiological role that EPSs play in the bacterial ecology still remains uncertain. In this study, we have assessed the effect of EPSs produced by *Lactobacillus rhamnosus* GG, *Bifidobacterium longum* NB667, and *Bifidobacterium animalis* IPLA-R1 on the adhesion of probiotic and enteropathogen strains to human intestinal mucus. The EPS fraction GG had no significant effect on the adhesion of *L. rhamnosus* GG and *B. animalis* IPLA-R1. However, the EPS fractions NB667 and IPLA-R1 significantly reduced the adherence of both probiotic strains. In contrast, the three EPS fractions increased the adhesion of *Enterobacter sakazakii* ATCC 29544 and *Escherichia coli* NCTC 8603. Higher adherence of *Salmonella enterica* serovar Typhimurium ATCC 29631 and *Clostridium difficile* ATCC 9689 was detected in the presence of the EPS fractions GG and NB667. In general, these effects were obtained at EPS concentrations of up to 5 mg/ml, and they were EPS dose dependent. The competitive exclusion of probiotics in the presence of EPS could suggest the involvement of these biopolymers in the adhesion to mucus. The increase in the adherence of enteropathogens could be explained if components of the pathogen surface are able to bind to specific EPSs and the bound EPSs are able to adhere to mucus. To the best of our knowledge, this is the first work reporting the effect of EPSs from probiotics on bacterial adhesion properties.

Probiotic has been defined as ''a viable microbial food supplement which beneficially influences the health of the host'' *(29).* Many of the health effects are related to the capability of probiotic strains to adhere to the intestinal mucosa. Therefore, this is one of the main criteria for the selection of probiotic strains *(5),* although the mechanisms and molecules involved in this process are not well known. Carbohydrate moieties on the mucosal surface might be responsible for the adhesion of lactic acid bacteria, including bifidobacteria *(15, 17).* Furthermore, strains of *Lactobacillus johnsonii, Lactobacillus reuteri,* and *Bifidobacterium longum* share mucosa carbohydrate-binding specificities with enteropathogenic bacteria, such as *Escherichia coli* and *Helicobacter pylori (7, 14, 16).* On the other hand, the identities of the bacterial molecules involved in adhesion of the probiotic bacteria are still not completely characterized. Some proteins from *Lactobacillus* species, which are able to adhere to mucus components *(8, 14, 24, 25),* and a mannose adhesion encoding gene from *Lactobacillus plantarum (20)* have been identified. In addition, lipoteichoic acids associated with the surface of *L. johnsonii* La1 also participate in its adhesion to intestinal-like Caco-2 cells *(9).* There is considerably less information about the adhesion-promoting molecules of *Bifidobacterium.* It has been suggested that the adhesion determinants of bifidobacteria could be glycoproteins or carbohydrate chains attached to the cell wall *(3). Bifidobacterium bifidum* EB102 expresses multiple proteinaceous components (still unidentified) on the cell surface, which are able to bind Caco-2 glycolipids *(15).* Some authors point to the hydrophobicity as an important factor for the adhesion properties of bifidobacteria *(4, 19).* From these studies, it seems that multiple adherence factors from the bacterial surface are responsible for the adhesion of probiotic strains to the intestinal mucosa.

Exopolysaccharides (EPSs) are exocellular polymers present in the surface of many lactic acid bacteria. A number of strains of *Lactobacillus* and *Bifidobacterium,* the most common probiotics included in commercial products, are able to produce these biopolymers *(2, 11, 22, 23).* Some of the EPS-producing strains are currently used in the manufacture of fermented dairy products as a source of natural thickeners and stabilizing ingredients. Positive health effects have been attributed to EPSs *(28).* However, the physiological role that these polymers play in the bacterial ecology of probiotic lactic acid bacteria still remains uncertain. It has been suggested that EPSs from other bacteria can act

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as protective agents against desiccation, antimicrobial compounds, bacteriophage attack *(6),* and phagocytosis *(12).* They can also be involved in adhesion to surfaces and biofilm formation, such as oral biofilm *(26).*

Our aim in this study was to assess the effect of EPSs produced by probiotic bacteria on bacterial adhesion to intestinal mucus. For this purpose, an immobilized human intestinal mucus model was employed. The EPS fraction produced by three probiotic strains was purified. The adherence to intestinal mucus of two representative probiotic strains, currently found in functional foods, and four enteric pathogens was analyzed in the presence and absence of these EPSs. To the best of our knowledge, this is the first report on the effect of EPSs from probiotics on bacterial adhesion properties.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Three EPS-producing bacteria were used in this study: *B. longum* NB667, *Bifidobacterium animalis* IPLA-R1 and *Lactobacillus rhamnosus* GG (ATCC 53103). Strains were grown overnight at 37°C in MRS broth (Oxoid, Basingstoke, UK) containing 0.05% (wt/vol) L-cysteine (Sigma-Aldrich Finland, YA-Kemia Oy, Helsinki, Finland) under aerobic conditions for *L. rhamnosus* GG and under anaerobic atmosphere (10% H_2, 10% CO_2, and 80% N_2) in a Concept 400 anaerobic chamber (Ruskinn Technology, Leeds, UK) for bifidobacteria. To obtain cellular biomass for EPS isolation, plates of agar–MRS–cysteine were spread with 200 μl of grown cultures using sterile glass beads and incubated for 5 days at 37°C. For the adhesion experiments, fresh MRS–cysteine broth containing 10 μl/ml tritiated thymidine ([5-^{3}H]thymidine, 117 Ci/mM; Amersham Biosciences, Buckinghamshire, UK) was inoculated at 1% (vol/vol) with the cultures grown overnight and incubated as formerly indicated until the late-exponential phase. The enteric pathogens tested in this study were *Clostridium difficile* ATCC 9689, *Enterobacter sakazakii* ATCC 29544, *Salmonella enterica* biovar Typhimurium ATCC 29631, and *E. coli* NCTC 8603. Pathogenic strains were grown overnight in Gifu anaerobic medium (GAM; Nissui Pharmaceuticals, Japan) incubated at 37°C in aerobic conditions, except for *C. difficile,* which was incubated at the same temperature anaerobically. These grown cultures were employed to inoculate (1%, vol/vol) GAM medium containing 10 μl/ml tritiated thymidine. After incubation, radiolabeled probiotic and pathogenic strains were harvested by centrifugation, washed twice with *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–Hanks' buffer (pH 7.4) (10 mM HEPES) (HH) and resuspended in HH buffer with and without different concentrations of the isolated EPS fractions (1, 5, and 10 mg/ml). The A_{600} of the bacterial suspensions was standardized to 0.25 ± 0.01.

EPS isolation. Cellular biomass was collected from agar–MRS–cysteine plates using 2 ml of ultrapure water and a plastic L-shaped spreader. To release the polymer from the cell surface, 1 vol of 2 M NaOH was added to the cellular suspension and stirred for 24 h at room temperature. Cells were removed by centrifugation and EPSs from the supernatant were precipitated for 48 h at 4°C using 2 volumes of absolute ethanol. The precipitated EPS fraction obtained after centrifugation was resuspended in ultrapure water and dialyzed (3 days at 4°C) against the same daily-changed water using dialysis tubes (Sigma-Aldrich) of 12- to 14-kDa molecular mass cutoff. Finally, the dialyzed EPS fractions were freeze-dried. The protein content of the EPS fractions was measured using the BCA protein assay kit (Pierce, Rockford, Ill.), using bovine serum albumin (Sigma-Aldrich) as standard.

***In vitro* adhesion to human intestinal mucus.** Mucus was collected from the healthy part of human colonic tissue samples obtained during surgery of patients with colon cancer as described by Ouwehand et al. *(18).* Briefly, resected tissue was washed in PBS containing 0.01% gelatine, and mucus was collected by gently scraping the mucosa with a rubber spatula. The mucus was centrifuged to remove cell debris and bacteria and stored at −70°C. Before use, the protein content was measured by the Lowry method *(13)* using bovine serum albumin as standard. Then, mucus was dissolved in HH buffer at a concentration of 0.5 mg/ml protein and stored at −20°C before use. The adhesion of radioactively labeled probiotics and enteropathogens to immobilized colonic mucus was assessed as described by Kirjavainen et al. *(10).* In short, 100 μl of intestinal mucus was immobilized on polystyrene microtiter plate wells by overnight incubation at 4°C. Then, wells were washed twice with 200 μl of HH buffer to remove the excess of mucus and filled with 100 μl of the A_{600}-standardized (in HH buffer and in buffer containing EPS) radiolabeled bacterial suspensions. After 1 h of incubation at 37°C, the wells were washed twice with HH buffer to remove the unattached bacteria and the adhering cells were released and lysed with 200 μl of lysis solution (1% [wt/vol] SDS in 0.1 M NaOH) at 60°C for 1 h. The content of wells was transferred to microfuge tubes containing scintillation liquid (OptiPhase ''HiSafe 3,'' Wallac Oy, Turku, Finland) and the radioactivity of lysed bacteria was determined by liquid scintillation with a 1450 Microbeta Liquid Scintillation Counter (Wallac). Adhesion results were expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension initially added to the immobilized mucus.

Statistical analysis. At least two replicated experiments, each done in triplicate, were carried out in all adhesion assays. Data obtained for each microorganism tested were statistically analysed by means of one-way analysis of variance (ANOVA) using the SPSS 11.0 software for Windows (SPSS, Chicago, Ill.). The ''EPS concentration'' was used as factor and the differences between the EPS concentrations were assessed by the least-significant difference (LSD) comparison of means test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

The percentage of protein content of the isolated EPS fractions was 6.08 ± 0.65, 4.49 ± 0.63, and 6.93 ± 2.26 for fractions GG, NB667, and IPLA-R1, respectively. Thereby, the major content of our fractions was EPS polymer with a molecular mass higher than 1.2×10^5 Da which was the cutoff size of the dialysis membrane used. Our EPS polymers had a molecular mass included in the range described previously for EPSs of different origins (from 2.5×10^4 to 2.2×10^7) *(27).* The preliminary results obtained by gas chromatography–mass spectrometry about the qualitative composition of the EPS fractions from the two bifidobacteria strains used in our study indicated that the main constituent monosaccharides are glucose, galactose, and rhamnose present in different ratios (data not shown). The structure of the repeating unit that built the EPS-GG has been reported previously *(11)* and is a hexasaccharide composed of four molecules of galactose, one rhamnose, and one *N*-acetyl-glucosamine.

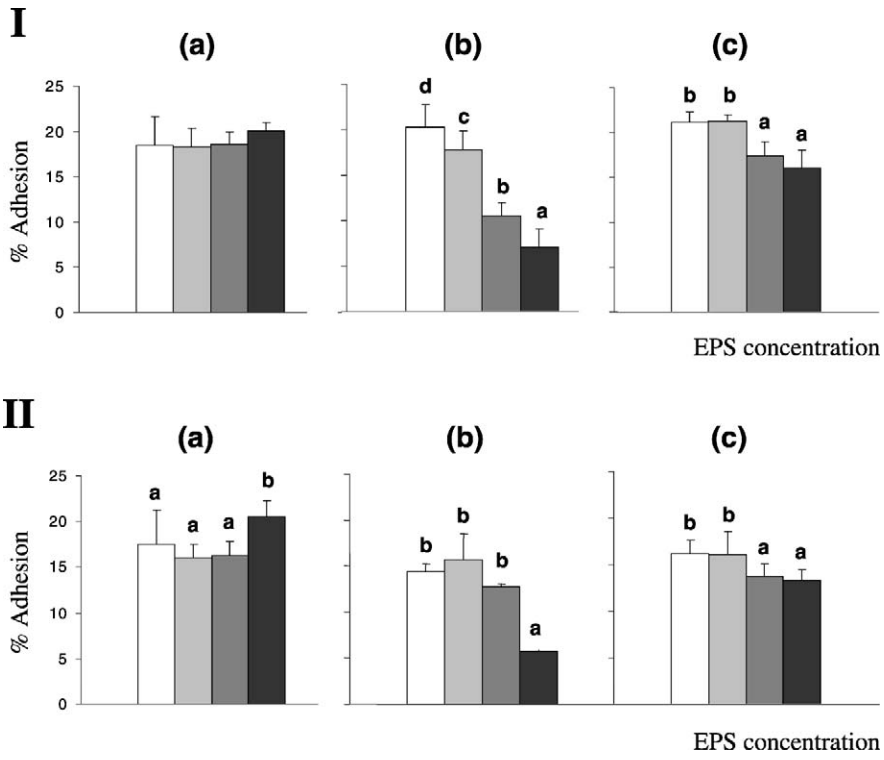

FIGURE 1. *Adhesion of* L. rhamnosus *GG (I) and* B. animalis *IPLA-R1 (II) to human intestinal mucus in the presence of increasing concentrations of the EPS fractions GG (a), NB667 (b), and IPLA-R1 (c). EPS concentrations:* □, *0;* ⬚, *1;* ▨, *5; and* ■, *10 mg/ml. Bars within the same plot that do not share equal letters are significantly different (*$P < 0.05$*).*

The effect of the three EPSs tested in the adhesion of two representative probiotic strains (*L. rhamnosus* GG and *B. animalis* IPLA-R1) is presented in Figure 1. Results showed that the adhesion levels varied in the presence of EPS, the effect being EPS type dependent. The EPS fraction GG showed no effect in the adhesion of *L. rhamnosus* GG and a slight increase in the adherence of *B. animalis* IPLA-R1 at the highest dose. However, the EPS fractions NB667 and IPLA-R1 promoted a significant reduction in the adhesion of both probiotic strains. At a concentration of 10 mg/ml, this effect was more pronounced for the EPS fraction NB667 (adhesion decrease of 66 and 60% for *L. rhamnosus* GG and *B. animalis* IPLA-R1, respectively) than for the EPS fraction IPLA-R1 (24 and 18%, respectively). Moreover, a significant ($P < 0.001$) reduction in the adhesion of the *Lactobacillus* strain was obtained from the lowest dose of the EPS fraction NB667 tested (1 mg/ml), whereas the minimum effective dose of the EPS fraction IPLA-R1 for both probiotics was 5 mg/ml. These facts suggest that the different physicochemical and/or structural characteristics of the three biopolymers could account for their differential effect in probiotic adhesion. The reduction of adhesion in the presence of EPS fractions NB667 and IPLA-R1 was higher for *L. rhamnosus* GG than for *B. animalis* IPLA-R1. Therefore, the surface characteristics of the probiotic strains could also contribute to the differences in adhesion. The results obtained point to the involvement of the EPSs produced by probiotic strains in their adhesion to the intestinal mucus. The polymers could directly adhere to intestinal mucus and then competitively inhibit the adhesion of the probiotics (Fig. 2a), or they could stick to the probiotic surface and thereby mask the bacterial molecules involved in adhesion (Fig. 2b).

The three EPSs under study also modified the adhesion of the enteropathogens analyzed (Table 1). The EPS fractions GG and NB667 increased the adhesion of three out of the four pathogens studied. However, the EPS fraction IPLA-R1 had no effect on *C. difficile* and *S. enterica* serovar Typhimurium, but enhanced the adhesion of *E. sakazakii* and *E. coli.* In general, all these effects were detected at EPS doses higher than 5 mg/ml. With respect to *Clostridium* and *Salmonella,* which showed low adherence to mucus (around 3%), the adhesion was only influenced by one EPS fraction. The adherence of *Enterobacter* and *Escherichia,* with higher adhesion values, was modified by the three EPSs analyzed. The maximum increase of adherence was detected at 10 mg/ml for *E. sakazakii* in the presence of the EPS fraction NB667 (61%) and for *E. coli* with the EPS fraction GG (82%). Similar to that found with probiotic strains, the modification of the adhesion properties of pathogens by the EPSs was dependent on the EPS type and the pathogen tested. These results suggest that components of the pathogen surface might bind specific EPSs and the bound EPS would be able to adhere to mucus (Fig. 2c).

The results obtained in the present work indicate that the EPSs produced by probiotic strains could be able to adhere to intestinal mucus, the effect being dose and EPS type dependent. The genome sequence of probiotic *Lactobacillus* and *Bifidobacterium* strains predicts the occurrence of proteins needed for the production of glycoprotein-binding fimbriae and mucus- and fibronectin-binding proteins that could be involved in the bacterial adhesion to the gastrointestinal tract *(1, 21, 30).* This could reflect the adaptation of probiotics to their natural environment. Very often in the sequence of these genomes, genetic determinants for the synthesis of EPSs are also found *(1, 21, 30).* Thereby, both proteins and EPSs could act as adherence factors that

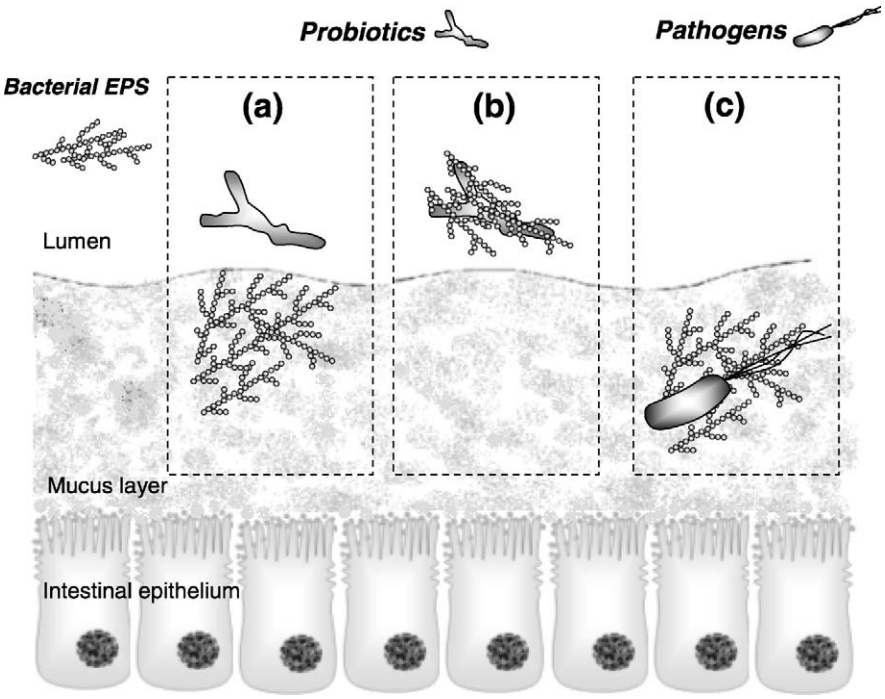

FIGURE 2. *Putative explanation of the interference of EPSs on the adhesion of probiotics and pathogens to intestinal mucus. Decrease of probiotic adhesion: (a) EPS could directly adhere to mucus and then competitively inhibit the adhesion of probiotics or (b) they could stick to the probiotic surface and thereby mask bacterial molecules involved in adhesion. Increase of pathogen adhesion: (c) components of the pathogen surface might bind specific EPSs and the bound EPS would be able to adhere to mucus.*

may play a role in the transitory colonization of the intestinal mucosa by probiotics. As far as we know, data are not currently available in the literature dealing with the production of EPSs by probiotics under the gastrointestinal tract conditions, nor data for EPS concentration from bacterial origin in this location. However, the ubiquity of EPS gene clusters on probiotic genomes allows us to suggest that a number of strains from the intestinal microbiota may produce extracellular polymers in this environment and that high EPS concentrations could be locally reached in the gastrointestinal tract. A future challenge would be the determination of EPS concentration in the gastrointestinal tract and the effect of these polymers in the in vivo adherence of bacteria. On the other hand, more studies about the chemical composition, the structure of the EPSs produced by probiotics, and their relationship to the bacterial adhesion are needed in order to propose a mechanism of action for these biopolymers.

TABLE 1. *Percentage of adhesion to human intestinal mucus of enteropathogenic strains in the presence of increasing concentrations of the EPS fractions GG, NB667, and IPLA-R1 isolated from the strains* L. rhamnosus *GG,* B. longum *NB667, and* B. animalis *IPLA-R1, respectively*

		% adhesion (mean ± SD)[a]			
EPS fraction	EPS concn (mg/ml)	*C. difficile* ATCC 9689	*E. sakazakii* ATCC 29544	*E. coli* NCTC 8603	*Salmonella* Typhimurium ATCC 29631
EPS-GG	0	2.73 ± 1.05	9.52 ± 1.52 A	6.79 ± 1.04 A	1.91 ± 0.82 A
	1	2.58 ± 0.36	10.68 ± 0.52 AB	7.56 ± 1.94 AB	2.05 ± 0.41 A
	5	3.17 ± 0.85	11.70 ± 1.91 B	8.82 ± 1.79 B	3.13 ± 0.87 B
	10	3.11 ± 0.31	13.65 ± 1.47 C	12.33 ± 2.53 C	3.09 ± 0.47 B
			***[b]	***	**
EPS-NB667	0	1.44 ± 0.34 A	4.63 ± 1.53 A	4.37 ± 0.55 A	1.65 ± 0.43
	1	1.75 ± 0.14 A	6.24 ± 2.40 A	4.56 ± 0.83 AB	1.43 ± 0.29
	5	1.91 ± 0.34 B	7.49 ± 1.81 B	5.59 ± 1.79 B	2.04 ± 0.48
	10	2.29 ± 0.23 C	7.46 ± 2.60 B	5.90 ± 2.25 B	2.01 ± 0.72
		***	*	**	
EPS-IPLA-R1	0	1.66 ± 0.68	5.27 ± 0.72 A	4.37 ± 0.57 A	1.72 ± 0.36
	1	2.66 ± 1.60	5.39 ± 1.46 A	4.51 ± 0.67 A	2.34 ± 0.77
	5	2.77 ± 0.80	6.38 ± 1.22 A	5.29 ± 1.02 C	1.79 ± 0.26
	10	2.67 ± 1.40	7.83 ± 0.46 B	4.53 ± 1.67 B	2.38 ± 0.49
			***	***	

[a] Columns with different letters are significantly different ($P < 0.05$) according to the test of means comparison LSD.
[b] One-way ANOVA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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