

Human milk glycosaminoglycans inhibit *in vitro* the adhesion of *Escherichia coli* and *Salmonella ftyris* to human intestinal cells

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BACKGROUND: Breast-fed infants have a lower incidence of acute gastroenteritis due to the presence of several anti-infective factors in human milk. The aim of this work is to study the capacity of human milk glycosaminoglycans (GAGs) to inhibit the adhesion of some common pathogenic bacteria.

METHODS: GAGs were isolated from a pool of milk samples collected from different mothers during the first month of lactation. Experiments were carried out to study the ability of GAGs to inhibit the adhesion of two intestinal micro-organisms (enteropathogenic *Escherichia coli* serotype 0119 and *Salmonella ftyris*) to Caco-2 and Int-407 cell lines.

RESULTS: The study showed that the GAGs had an anti-adhesive effect on the two pathogenic strains studied with different degrees of inhibition. In particular, in the presence of human milk GAGs, the adhesion of *S. ftyris* to Caco-2 cells and to Int-407 cells of both tested strains was significantly reduced.

CONCLUSION: Our results demonstrated that GAGs in human milk can be one of the important defensive factors against acute diarrheal infections in breast-fed infants.

In infancy, a wide variety of bacteria, viruses, and parasites are responsible for gastrointestinal infections. There is strong evidence to support the correlation between breastfeeding and a lower incidence of diarrhea. In fact, several anti-infective substances (secretory antibodies, lactoferrin, oligosaccharides, etc.) are present in human milk (1,2). Oligosaccharides, in particular, play several important protective, physiological, and biological roles including growth stimulation for beneficial gut microbiota and inhibition of pathogen adhesion and immunoregulation (3,4).

Glycosaminoglycans (GAGs) are highly sulfated, complex, linear natural polysaccharides constituted by repeating disaccharidic units. They are generally grouped into four classes: hyaluronan, keratan sulfate, sulfated galactosaminoglycans represented by chondroitin sulfate and dermatan sulfate bearing D-galactosamine, and sulfated glucosaminoglycans with

heparan sulfate and heparin having D-glucosamine. Each specific disaccharidic unit is formed of a hexosamine (galactosamine or glucosamine) residue alternated with a hexuronic acid (glucuronic or iduronic acid) with the exception of keratan sulfate containing galactose. Sulfate groups are esterified on various positions of carbohydrate backbones producing structures possessing high heterogeneous sequences and charge density (5).

Contrary to oligosaccharides, GAGs are able to specifically (and aspecifically) interact with other biological components due to the presence of a great number of sulfate and carboxyl groups capable to generate highly specific sequences producing strong anionic interactions (5–7). As a consequence, it emerges that human milk GAGs could play a role as soluble receptors and would therefore have the power to inhibit the binding of different pathogens to the intestinal mucosa, thus protecting the infant from infections. Furthermore, human milk hyaluronan was recently demonstrated to stimulate protective antimicrobial defense in the newborn (8).

After the pioneering studies of Newburg *et al.* (9), a complete quantitative and qualitative evaluation of GAGs has recently been reported both in term and preterm human milk (10,11). From the quantitative point of view, the highest concentration of GAGs was found in colostrum (9.3 and 3.8 g/l in preterm and term milk, respectively), followed by a progressive decrease to 4.3 and 0.4 g/l at the end of the first month of lactation. From these data, it follows that breast-fed newborns daily ingest consistent amounts of GAGs. From the qualitative point of view, chondroitin sulfate and heparan sulfate/heparin represent 55 and 42% of total GAGs, respectively, whereas dermatan sulfate and hyaluronan make up the remaining 3% (12).

Milk GAGs are synthesized in the mammary gland by the sequential action of specific glycosyltransferases, linked to a protein “core” and excreted as proteoglycans. At the small intestine level, the proteolytic enzymes secreted in the pancreatic juice digest the “core” of proteoglycans, liberating the GAG chains. As the intestinal wall and microvilli lack specific glycosidases and sulfatases, the free GAGs persist undegraded

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in the upper part of the digestive system (13) where, behaving as soluble receptors, they could play an anti-infective role, as already demonstrated for human milk oligosaccharides (14).

The aim of this study is to verify whether the purified complex of human milk GAGs is able to inhibit the adhesion of two pathogen bacteria of diarrhea in infancy, such as *Escherichia coli* and *Salmonella ftyris* (15) to Caco-2 and Int-407 cells.

RESULTS

Preliminary cell adhesion experiments demonstrated that both *E. coli* O119 and *S. ftyris* were able to adhere to Caco-2 and Int-407 monolayers (data not shown). The Caco-2 and Int-407 cells' viabilities after incubation with GAGs dissolved at a concentration of 1.5 mg/ml were evaluated using the Trypan blue assay. In this test, only living cells are able to exclude the dye. After 1-h incubation, the cell viability of GAGs-treated monolayer was identical to that of the corresponding controls. In particular, the percent of viability of Caco-2 in presence of GAGs was $99.68 \pm 0.56\%$ vs. control having $99.39 \pm 0.69\%$ and that of Int-407 was $97.19 \pm 1.08\%$ vs. control showing $96.32 \pm 0.95\%$.

In agglutination assays performed with GAGs at the same concentration used in cell adhesion inhibition experiments, no agglutination of both *E. coli* O119 and *S. ftyris* was detected. These results give us no possibility to measure any specific binding capacity between human milk GAGs and bacteria.

On the basis of these results, several adhesion experiments, performed in triplicate and repeated at least three times, were conducted by infecting Caco-2 and Int-407 cells with *E. coli* O119 and *S. ftyris*, both in the presence of GAGs and in their absence. A significant reduction in the percentage of adhesion of *S. ftyris* to Caco-2 cells and of *E. coli* O119 and *S. ftyris* to Int-407 cells was observed in the presence of purified human milk GAGs complex dissolved at a concentration of 1.5 mg/ml (Figure 1). In particular, the percentage of adhesion of *S. ftyris* to Caco-2 cells dropped from 7.7 ± 1.2 to 5.4 ± 0.6 ($P = 0.0412$) (Figure 1a) whereas that of *E. coli* O119 and *S. ftyris* to Int-407 cells dropped from 8.5 ± 2.6 to 2.1 ± 1.0 ($P = 0.0043$) and from 32.1 ± 5.5 to 15.2 ± 8.9 ($P = 0.0112$), respectively (Figure 1b). The reduction of *E. coli* O119 adhesion to Int-407 monolayers in the presence of GAGs was also appreciable by Giemsa staining (Figure 2a,b). On the contrary, no effect of purified GAGs on *E. coli* O119 adhesion to Caco-2 monolayers was observed (Figures 1a and 2c,d).

DISCUSSION

Microbial infection is a complicated process largely dependent on the host-microbe relationship. Usually, adhesion is the first step leading to colonization and subsequent infection (16). Various cellular components on eukaryotic cells and tissues have been identified as targets for adhesion by micro-organisms, and many of them are characterized as proteoglycans. Recent studies have suggested that carbohydrate-containing molecules rather than proteins mediate bacterial attachment to the surface of eukaryotic cells (17). A considerable number of carbohydrate-binding specificities have been reported and, in some cases, the bacterial attachment to host animal cells has

been linked directly to the interactions with GAGs (18,19). The overall process of binding involves the meeting of a solvated polyhydroxylated glycan placed on the surface of cells with a solvated protein-combining site (adhesin) present on the pathogenic agent. The forces involved in this link are represented by hydrogen binding, Van der Waals interactions, and charge and dipole attraction (20,21), suggesting that, if a surface on the glycan is complementary to the protein-combining site, water can be displaced and binding occurs.

Receptors present on the surface are made up of oligosaccharidic residues of glycoproteins and glycolipids of cell membranes. It has been proved that the adhesion to such receptors can be inhibited or reduced by the presence of free oligosaccharides with a structure analogous to that of cell receptors so that the pathogenic agent binds with them and not with the cell membrane, with a reduction of the pathogenic effect as a consequence (22).

Data available so far regarding the activity of human milk oligosaccharides toward *E. coli* and *S. ftyris* show the capacity of fucosyl-oligosaccharides containing fractions to inhibit the adhesion of enteropathogenic *E. coli* O119. Moreover, the anti-adhesive effect on *S. ftyris*, even though rather weak, was obtained by acid and neutral low-molecular-weight oligosaccharides (14).

As already demonstrated for other human milk (oligo)saccharides, exogenous GAGs have shown the ability as competitive inhibitors of bacterial adhesion, among which heparin has been studied most (3,23). As concerns the possible physiological role of GAGs in the gastrointestinal tract of breast-fed newborns, it is important to underline that undigested GAGs reach the small intestine because there are no specific enzymes present on the intestinal wall able to degrade them. Therefore, these molecules could behave as soluble receptors having the power to interact with pathogens and to compete for their adhesion to the intestinal wall.

The present study confirms that GAGs are effective in inhibiting the adhesion of *S. ftyris* to Caco-2 and Int-407 cells and adhesion of *E. coli* O119 to just Int-407. On the other hand, the presence of GAGs does not significantly inhibit the adhesion of *E. coli* O119 to Caco-2 cells. The adhesion values for *S. ftyris* and *E. coli* O119 to Int-407 cells are concordant with those previously reported by comparing different configurations of cell cultures as an intestinal epithelium model (24). The adhesion percentages of these bacteria were reduced when the cell suspensions were co-incubated with GAGs, suggesting that GAGs may be involved in bacteria-epithelial cell interaction.

As concerns *E. coli* O119 and the lack of specific adhesion inhibition to Caco-2, such a phenomenon could be explained by the different molecular structures of the receptors belonging to the two cellular types. The human milk GAG pool might show a greater similarity to the Int-407 receptors than the GAGs located on Caco-2. Moreover, previous studies suggested that reduced adherence is due to lower levels of adherence proteins rather than to physical inhibition (25,26). Finally, the different degree of the adhesion inhibition to Int-407 cells compared with Caco-2 cells could also reflect the origin (embryonic and

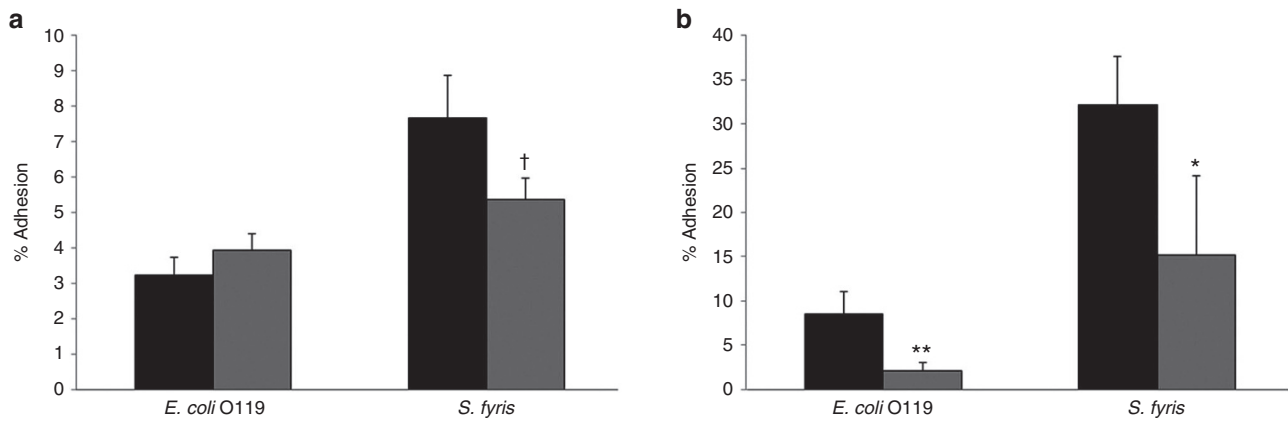


Figure 1. Inhibition of bacterial adhesion to intestinal cells by glycosaminoglycans (GAGs). Adhesion (percentage of the initial inoculum) of *E. coli* O119 and *S. ftyris* (a) to Caco-2 and (b) to Int-407 cells in presence (gray bars) and absence (black bars) of GAGs. Values are expressed as mean \pm SD. Symbols on the figure denote statistically significant values compared with the control. * $P = 0.0112$. ** $P = 0.0043$. † $P = 0.0412$.

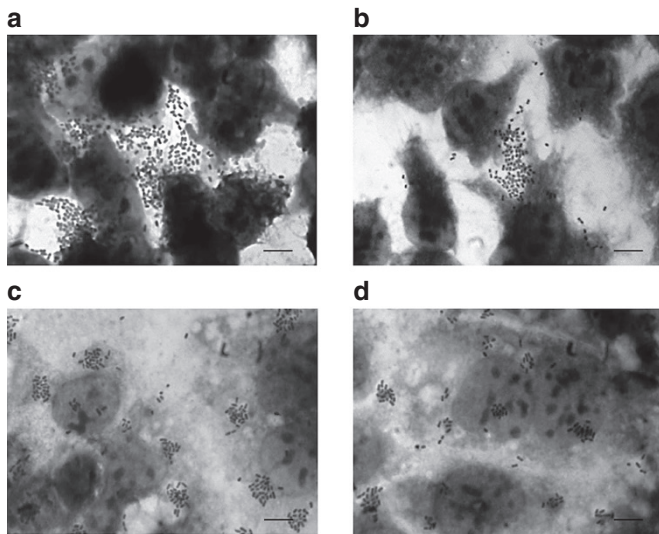


Figure 2. Standard light microscopy of Giemsa-stained cell monolayers infected with *E. coli* O119. Adhesion to Int-407 monolayers in the (a) absence and (b) presence of glycosaminoglycans (GAGs): a significant reduction in the adhesion of *E. coli* O119 was observed in the presence of GAGs. Adhesion to Caco-2 monolayers in the (c) absence and (d) presence of GAGs: no effect of GAGs on *E. coli* O119 adhesion to Caco-2 monolayers was observed in the presence of GAGs. Magnification = $\times 1000$. Bar = 0.01 mm.

adult, respectively) of the two cell lines. In fact, the human colon carcinoma cells Caco-2, widely used to study the adhesion of intestinal pathogens to human cells and cytotoxicity of enterobacteria, are physiologically closer to the natural targets of the pathogens as, in culture, they exhibit the differentiated features of enterocytes, such as cell polarity and apical brush border (27). On the other hand, Int-407, also used in adhesion experiments (28), are human embryonic intestinal cells having, as a consequence, possible different molecular structure(s) of receptor(s) compared with Caco-2.

The human milk GAGs tested are effective on the *S. ftyris* and *E. coli* O119 though with different degrees of intensity. It has been shown that GAGs are also effective in inhibiting the HIV virus binding to the GP-120 receptor (9). Moreover, Hill *et al.*

(8) recently demonstrated that human milk hyaluronan inhibits the adhesion of *S. typhimurium* to cells. From the above results, it is derived that human milk GAGs have the capacity to act separately on different specific receptors. This effect may be related to their heterogeneous composition, structure, and properties of each single polysaccharide component. Our present results regarding the ability of human milk GAGs to inhibit the adhesion of enteropathogens to intestinal cells, as previously reported for human milk oligosaccharides, are a further demonstration that the anti-infective effect of human milk is the result of the presence of bioactive (macro)molecules able to protect newborns from the adhesion of some common pathogenic bacteria at gastrointestinal level.

On the basis of these results, further investigations will be performed using different GAG concentrations as well as single purified component present in human milk complex GAGs to better understand their possible physiological role and their involvement in the inhibition of bacteria adhesive mechanism.

METHODS

GAGs Isolation

Human milk GAGs were obtained from a pool of samples collected during the first month of lactation and stored at -20°C until use. Purified free GAGs were obtained and isolated as previously described in details (10). Briefly, single aliquots of human milk were defatted with acetone, and after centrifugation and drying at 60°C , pellet was solubilized in adequate buffer and treated with a proteolytic enzyme. After boiling for 10 min and centrifugation, free GAGs were precipitated with three volumes of ethanol. The precipitate was recovered by centrifugation, dried at 60°C and purified on a column packed with anion-exchange resin. Column fractions positive to uronic acid assay (29) and containing free GAGs were collected and further precipitated with ethanol. Finally, after centrifugation and drying, the free purified complex human milk GAGs were analyzed for purity and composition and used for adhesion experiments.

The composition of purified GAGs was assessed by agarose-gel electrophoresis (30) and disaccharide determination after specific enzymatic treatment (10). The purity of the complex GAGs was greater than 85% as evaluated by uronic acid assay (29) with the remaining formed of salt and a very low content (<1%) of proteins/peptides. Human milk oligosaccharides were found absent due to the adopted protocol useful for the purification of GAGs, in particular the purification on strong-anion exchange resin and several precipitations with organic solvent (not shown).

Bacteria and Culture Conditions

Two enterobacterial strains, both isolated from infants with diarrhea in Italy, were used: enteropathogenic *E. coli* ser. O119 (31) and *Salmonella enterica* subsp. *enterica* ser. Fyris (4,12:1, v,4,2 serotype), here named *S. fyris*. Both strains were previously used in inhibition studies with oligosaccharides (14). Enteropathogenic *E. coli* strains infect human populations throughout the world and remain one of the primary causes of acute infantile gastroenteritis in developing countries (32). In particular, enteropathogenic *E. coli* strains of the O119 serogroup have been found in association with infantile diarrhea in many countries (33). *S. enterica* subsp. *enterica* is an invasive, facultative intracellular pathogen of both animal and man with the ability to colonize various niches in diverse host organisms (34). The pathogenesis of infections by *S. enterica* subsp. *enterica* requires adhesion to cell surfaces, and a large number of adhesive structures can be found (35). Brain heart infusion (BHI) broth and agar (Difco Laboratories, Detroit, MI) were used for the routine growth of bacteria. Strains were maintained in glycerol at -70°C and subcultured twice before testing.

Cell Lines

The human colon carcinoma Caco-2 (ATCC HTB37) (36) and the human embryonic intestine Int-407 (ATCC CCL6) cell lines were used (37). Cells were routinely cultured in 50 ml (25 cm²) plastic tissue culture flasks (Corning Costar, Milan, Italy) in an atmosphere with 5% CO₂ at 37 °C in Modified Eagle Medium (Gibco, Grand Island, New York) supplemented with 1% (v/v) L-glutamine (Gibco), 1% (v/v) non-essential amino acids (Gibco), and 10% (v/v) fetal calf serum (Gibco). Confluent cell monolayers were trypsinized and adjusted to a concentration of 2.5×10^5 cells/ml in culture medium; 1-ml cell suspension was dispensed into each 22-mm well of a 12-well tissue culture plate (Corning Costar) and incubated to obtain confluent monolayers.

The range of cell passage numbers used for the different experiments was 28–45 for Caco-2 cells and 312–320 for Int-407 cells.

Determination of Cell Viability

The effect of GAGs on Caco-2 and Int-407 monolayers was evaluated by using the Trypan blue exclusion assay able to give a measure of the cell viability (38). Briefly, Caco-2 and Int-407 monolayers were grown on SlideFlask and then incubated with GAGs dissolved at a concentration of 1.5 mg/ml, as described earlier. Control cultures received culture medium without GAGs. At the end of 1-h infection period, the monolayers were washed, stained with 0.4% Trypan Blue solution (Gibco) at room temperature for 30 min, and examined under light microscopy at 20× magnification. The viable cell ratio was calculated as follows: viable cell ratio (%) = (unstained cell number / total cell number) × 100. Cell viability was determined as the percentage of the ratio of the number of unstained cells (viable) to the total number of unstained (viable) and stained (nonviable) cells.

Agglutination Assay

E. coli O119 and *S. fyris* strains were used in agglutination assays with GAGs, according to Facinelli *et al.* (39). Briefly, after overnight growth in BHI broth at 37 °C, bacterial cells were harvested and suspended in PBS to an optical density of 1.0 ± 0.1 at 540 nm. Agglutination tests were carried out on sterile U-well microtiter plates (Greiner Labortechnik, Frickenhausen, Germany). In each well, 50 µl of cell suspension was added to 50 µl of GAGs dissolved at a concentration of 1.5 mg/ml. As a control, 50 µl of PBS was added to 50 µl of GAGs and to 50 µl of each cell suspension. Plates were incubated at 37 °C overnight and then visually inspected.

Cell Adhesion Experiments

Preliminary adhesion experiments were performed with two test strains at different incubation times on the basis of previously reported data (14). Briefly, after overnight growth at 37 °C in BHI broth, bacterial cells were either subcultured in BHI broth and incubated in a shaker at 37 °C for 2 h (*S. fyris*) and then harvested by centrifugation at 5,000 rpm or were directly harvested by centrifugation (*E. coli* O119). Bacterial cells were resuspended in PBS to OD₅₄₀ 0.6 ± 0.02 , diluted in Modified Eagle Medium, and added (0.5 ml, approximately 1×10^8 CFU/ml) to confluent monolayers. After incubation for 90 min (*E. coli*) or 120 min (*S. fyris*) at 37 °C in 5% CO₂, cells were washed

three times with PBS to remove nonadherent bacteria and then lysed in Triton X-100 (0.1% in cold sterile water) to release adherent bacteria. CFU of bacteria were counted by plating suitable dilutions of the lysates on BHI agar and incubating for 36–48 h at 37 °C. Results were expressed as percentages of the initial inoculum. Adhesion experiments were performed in triplicate and repeated at least three times.

E. coli O119 and *S. fyris* Adhesion to Cell Monolayers in the Presence of GAGs

Adhesion experiments were performed by assessing the rate of recovery of adherent bacteria from infected Caco-2 or Int-407 cells in the presence of GAGs. Briefly, after washing, monolayers were covered with 250 µl of Modified Eagle Medium containing GAGs dissolved at a concentration of 1.5 mg/ml and incubated for 1 h at 37 °C in 5% CO₂. At the end of the incubation period, 250 µl of bacterial inoculum (approximately 2×10^8 CFU/ml) were added. After incubation at 37 °C in 5% CO₂, cells were washed three times with PBS, and adherent bacteria were recovered as described earlier. Results were expressed as percentages of the initial inoculum. Adhesion experiments were performed in triplicate and repeated at least three times.

Bacteria associated with Int-407 and Caco-2 monolayers were also evaluated by Giemsa staining. Stained monolayers grown on slides (SlideFlask, Nunc GmbH, Weisbaden, Germany) were examined microscopically (Leica DMRB microscope, Wetzlar, Germany) using the ×100 oil-immersion objective.

Statistical Analysis and Ethical Approval

Each cell adhesion assay was performed in triplicate and repeated at least three times. All data are presented as mean ± SD. Significant differences between analyzed groups, i.e., cell adhesion in the absence and presence of GAGs, were calculated with the paired Student's *t* test by using GraphPad software. *P* values less than 0.05 were considered statistically significant.

The study protocol was approved by the ethics committee of the Italian Association of Human Milk Donor Banks.

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