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Human Milk Oligosaccharides Inhibit the Adhesion to Caco-2 Cells of Diarrheal Pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*

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ABSTRACT: Breast-fed children, compared with the bottle-fed ones, have a lower incidence of acute gastroenteritis due to the presence of several antiinfective factors in human milk. The aim of this work is to study the ability of human milk oligosaccharides to prevent infections related to some common pathogenic bacteria. Oligosaccharides of human milk were fractionated by gel-filtration and characterized by thin-layer chromatography and high-performance anion exchange chromatography. Fractions obtained contained, respectively, 1) acidic oligosaccharides, 2) neutral high-molecular-weight oligosaccharides, and 3) neutral low-molecular-weight oligosaccharides. Experiments were carried out to study the ability of oligosaccharides in inhibiting the adhesion of three intestinal microorganisms (enteropathogenic *Escherichia coli* serotype O119, *Vibrio cholerae*, and *Salmonella fyris*) to differentiated Caco-2 cells. The study showed that the acidic fraction had an antiadhesive effect on the all three pathogenic strains studied (with different degrees of inhibition). The neutral high-molecular-weight fraction significantly inhibited the adhesion of *E. coli* O119 and *V. cholerae*, but not that of *S. fyris*; the neutral low-molecular-weight fraction was effective toward *E. coli* O119 and *S. fyris* but not *V. cholerae*. Our results demonstrate that human milk oligosaccharides inhibit the adhesion to epithelial cells not only of common pathogens like *E. coli* but also for the first time of other aggressive bacteria as *V. cholerae* and *S. fyris*. Consequently, oligosaccharides are one of the important defensive factors contained in human milk against acute diarrheal infections of breast-fed infants. **(*Pediatr Res* 59: 377–382, 2006)**

Infectious diarrheal diseases constitute a leading cause of infant morbidity not only in developing countries but also in developed areas (1). In fact, in the United States, acute diarrhea represents an important cause of childhood morbidity, especially during the first years of life, determining an economical loss that has been estimated in the order of several millions of dollars (2).

In infants, infections of the gastrointestinal tract are caused by a wide variety of enteropathogens, including bacteria, viruses, and parasites. There is now strong evidence supporting a relationship between breast-feeding and a lower incidence in diarrhea. Breast-feeding offers protection with different mechanisms against diarrhea due to several antiinfective substances present in human milk, such as secretory antibodies, lactoferrin, lysozyme, etc. (3–5).

In the last 15 y, evidence has also emerged on the protective role of another group of substances, oligosaccharides (6–8). They are synthesized in a large number by specific glycosyltransferases present in the mammary gland through the sequential addition to lactose of fucose, galactose, N-acetylglucosamine, and sialic acid. Each single oligosaccharide varies dynamically during the different phases of lactation (9). From the quantitative point of view, oligosaccharides, all together, represent the third component of human milk, besides lactose and lipids (10,11).

Breast-fed infants ingest daily several grams of oligosaccharides and these substances have been found in feces in large quantities (12–14). It follows that most oligosaccharides contained in human milk are not digested by intestinal enzymes, and so are present in large quantity both in the small and large intestine of neonates (15,16).

It is well known that viruses, bacteria, or toxins develop their pathogenic effect through the adhesion to receptors located on the cells of the epithelial surface. Numerous receptors are glycan chains of glycoproteins and glycolipids of the intestinal cell membranes, and human milk oligosaccharides may compete with these receptors in binding pathogenic agents, hindering their adhesion as well as the subsequent pathologic process (7,8,17).

So far, the antiadhesive effect of human milk oligosaccharides has been described only for a few bacteria (18–20). We present the results of experiments carried out *in vitro* on the effect of human milk oligosaccharides in inhibiting the adhesion to Caco-2 cells of enteropathogenic *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris* pathogen bacteria of diarrhea in infancy.

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Abbreviations: BHI, brain heart infusion; **CFU,** colony forming units; **2′-FL,** 2′-fucosyllactose; **3-FL,** 3-fucosyllactose; **3′-SL,** 3′-sialyllactose; **6′-SL,** 6′-sialyllactose

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MATERIALS AND METHODS

Human milk oligosaccharide fractions. The adhesion/inhibition tests were performed using human milk oligosaccharide fractions prepared in our laboratory (21). Briefly, a 1000-mL pool of human colostrum was obtained by mixing several samples collected four days after delivery and stored at −80°C until use. Oligosaccharides were isolated according to Kobata *et al.* (22) and fractionated by gel filtration (23). The effluent from a BioGel P-4 column (Bio-Rad, Hercules, CA) was collected in 5.0-mL aliquots. Each of them was characterized by thin-layer chromatography (21) and those with similar pattern were combined, lyophilized, and labeled as fractions A to G on the basis of their different composition. Each fraction was analyzed for total sugar (24) and sialic acid content (25) and characterized by high performance anion exchange chromatography (21). Milk purified oligosaccharides (from Sigma Chemical Co., St. Louis, MO; Bio Carb, Lund, Sweden; and Dextra Laboratories Ltd., Reading, UK) were used both as internal and external standards for the identification of the peaks (9). To use samples with different compositions among all fractions, we chose for our experiments the following: A (acidic fraction: sialyl-oligosaccharides) (Fig. 1*A*); D (neutral fraction: penta-octasaccharides (Fig. 1*B*); F2 (neutral fraction: tri-pentasaccharides and lactose) (Fig. 1*C*). A pool, made up by mixing each fraction (A to G) with weight ratio 1:1, was also used.

Oligosaccharide and monosaccharide standards. Purified oligosaccharide and monosaccharide standards were also used in the adhesion/inhibition tests: a) acidic oligosaccharides: 3′-SL and 6′-SL; b) neutral oligosaccharides: 3-FL, 2′-FL, and lactodifucotetraose; c) monosaccharides: glucose, galactose, sialic acid, N-acetylglucosamine, fucose.

Bacteria and culture conditions. Three bacterial strains were used throughout this study. They included *V. cholerae* ATCC 14034 (O1 classical strain, Inaba serotype) and two strains isolated from infants with diarrhea in Italy—an enteropathogenic (EPEC) serotype O119 *E. coli* strain previously described by The Italian Study Group on Gastrointestinal Infections (26) and *S. fyris* (serotype 4,12:1, v,4,2), an uncommon group B *Salmonella* serotype for which the mechanisms of pathogenicity are uncharacterized, isolated during a small outbreak of diarrhea (unpublished data). Brain heart infusion (BHI) and Luria-Bertani (LB) broth and agar (Difco Laboratories, Detroit, MI) were used for routine growth of bacteria.

Cell line. The human colon carcinoma cell line Caco-2 (ATCC HTB37) (27) derives from a relatively well-differentiated tumor and grows slowly in nude mice; when seeded on either permeable filters or impermeable substrates at high density, Caco-2 cells consistently form well-polarized monolayers joined by tight junction. In postconfluent cultures, monolayers exhibit structural and functional differentiation patterns characteristic of mature enterocytes in which the cell layer is covered with brush border microvilli, simulating intestinal cells (28).

Caco-2 cells were routinely grown in 25 cm^2 plastic tissue culture flasks (Corning Costar, Milan, Italy) at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2 in air. The culture medium was Dulbecco's modified Eagle minimum essential medium (DMEM), containing 25 mM glucose, 4 mM L-glutamine, 3.7 mg sodium bicarbonate (Euroclone, West York, U.K.) per mL, with 1% (vol/vol) nonessential amino acids and supplemented with 10% (vol/vol) fetal calf serum (Euroclone).

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, Milan, Italy) and incubated to obtain, 4 d later, semiconfluent monolayers and, after 15 more days, postconfluent monolayers.

Infection of Caco-2 cells and recovery of adherent bacteria. Infections were performed at different incubation times on the basis of previous reported data (29–32). Briefly, after overnight growth at 37°C in BHI broth (*E. coli* and *S. fyris*) and LB broth (*V. cholerae*), bacterial cells were either subcultured in BHI broth and incubated in a shaker at 37°C for 2 h (*S. fyris* and *V. cholerae*) and then harvested by centrifugation at 5000 rpm or were directly harvested by centrifugation (*E. coli*). Bacterial cells were resuspended in PBS to OD_{540} 0.6 ± 0.02 and diluted in DMEM. Then, 0.5 mL inoculum ($\sim 1 \times 10^8$ CFU/mL) was added to confluent monolayers.

After 30 min (*V. cholerae*), 90 min (*E. coli*), or 120 min (*S. fyris*) of incubation at 37°C in 5% CO_2, 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 or LB agar and incubating for 36–48 h at 37°C. Results were expressed as percentages of the initial inoculum.

Bacteria associated with Caco-2 monolayers were also evaluated by Gram staining. Stained monolayers grown on slides (SlideFlask, Nunc GmbH & Co., Weisbaden, Germany) were examined microscopically. The percentage of Caco-2 cells with associated bacteria was determined by counting all Caco-2 cells in 10 random microscopic areas: a positive result was scored when there was at least one bacterial cell per Caco-2 cell. The number of cell-associated bacteria was determined by examining 100 cells.

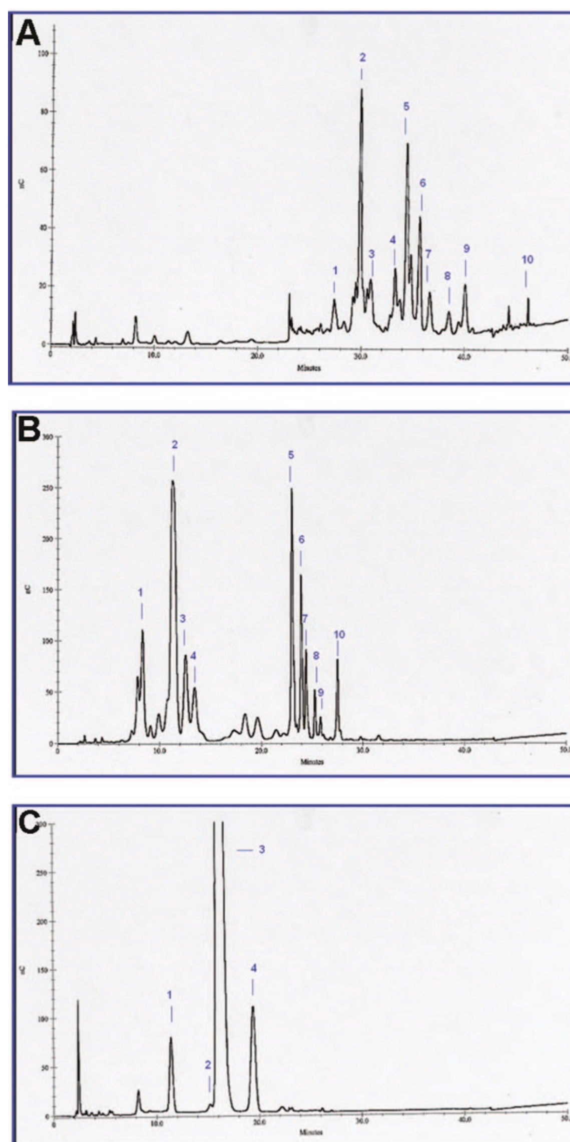

Figure 1. (*A*) High-performance anion exchange chromatography (HPAEC) of 1 mg/mL solution of fraction A; major peaks with the concentration expressed as mg/mL (in parenthesis): 1) N-acetyl-neuraminic acid (0.005); 2) monofucosyl-monosialyllacto-N-neohexaose (0.032); 3) monosialyl-monofucosyllacto-N-tetraose (0.015); 4) monofucosyl-monosialyllacto-N-hexaose (0.018); 5) sialyllacto-N-neotetraose (c) (0.072); 6) 6′-sialyllactose (0.045); 7) sialyllacto-N-tetraose (a) (0.025); 8) 3′-sialyllactose (0.015); 9) sialyllacto-N-tetraose (b) (0.025); 10) disialyllacto-N-tetraose (0.059). (*B*) HPAEC of fraction D: 1) difucosyllacto-N-hexaose II (0.028); 2) difucosyllacto-N-hexaose I (0.035); 3) lacto-N-fucopentaose III (0.044); 4) lacto-N-fucopentaose II (0.040); 5) lacto-N-fucopentaose I (0.159); 6) monofucosyllacto-N-hexaose II (0.056); 7) lacto-N-neotetraose (0.072); 8) lacto-N-neohexaose (0.008); 9) lacto-N-tetraose (0.083); 10) lacto-N-hexaose (0.006). (*C*) HPAEC of fraction F2: 1) 3-fucosyllactose (0.034); 2) lactodifucotetraose (0.032); 3) lactose (0.592); 4) 2′-fucosyllactose (0.264).

Infection inhibition studies. In inhibition experiments, Fraction A, Fraction D, Fraction F2, and the pool of milk oligosaccharides were used; lactose and monosaccharides (glucose, galactose, sialic acid, N-acetylglucosamine, fucose), which are components of oligosaccharide molecules, were also tested.

Confluent monolayers were washed three times with DMEM, covered with 250 μL of DMEM containing oligosaccharides (pool, fractions, or purified

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standards) and incubated for 1 h at 37°C in 5% CO_2. The concentration of both pool and fraction oligosaccharides was 1, 5, and 10 mg/mL. Single purified standards were used at the concentrations found in human milk of the most common genotype mothers (active Secretor and Lewis genes): 2′-FL = 2.5 mg/mL; 3-FL = 0.5 mg/mL; lactodifucotetraose = 0.5 mg/mL; 3′-SL = 0.1 mg/mL 6′-SL = 0.3 mg/mL (6,9,33). At the end of the incubation period, 250 μL bacterial inoculum (approx. 2×10^8 CFU/mL) was added. After different times of incubation at 37°C in 5% CO_2, cells were washed three times with PBS and adherent bacteria were recovered as described above.

Adhesion inhibition experiments were performed by assessing the rate of recovery of adherent bacteria from infected Caco-2 cells. Before infection, cell monolayers were separately incubated with lactose, monosaccharides (glucose, galactose, sialic acid, N-acetylglucosamine, fucose), the pool, the A, D, and F2 fractions, and 3′-SL, 6′-SL, 3-FL, DFL, and 2′-FL standards.

Statistical analysis. All experiments were performed as three or five independent assays. Assays were performed in triplicate per experimental run. The percentage of inhibition was calculated by the equation: (control – test)/control × 100. Results were expressed as mean value and 95% confidence interval (CI).

RESULTS

In the absence of oligosaccharides, bacteria associated with Caco-2 monolayers were evaluated by determining CFU per milliliter and by microscopic examination of Gram-stained monolayers. Counts of CFU indicated that bacteria associated with the monolayers ranged from 14.9% (*V. cholerae*) to 32.1% (*E. coli*) and 23.4% (*S. fyris*). In Gram-stained preparations, approximately 50% of Caco-2 cells with associated bacteria were observed (Figs. 2*a* and 3*a*).

The results of inhibition experiments are reported in Table 1. Each single monosaccharide tested (glucose, galactose, sialic acid, N-acetylglucosamine, fucose), which human milk oligosaccharides are made up of, did not significantly inhibit the adhesion to Caco-2 cells of any of the three tested strains; in fact, their percentage of inhibition ranged from –3.1 to 6.25.

Oligosaccharides as a whole (pool) were effective in inhibiting the adhesion of *V. cholerae* and *E. coli* O119, but not of *S. fyris*. As for the inhibition effect of the different fractions tested, *E. coli* O119 was significantly inhibited by all fractions, acidic (A) as well as neutral (D, F2) (Fig. 2); between acidic oligosaccharide standards, 3′-SL showed the highest degree of inhibition, whereas among neutral oligosaccharides, only 3-FL was effective.

Also, *S. fyris* was inhibited by the acid fraction (A) as well as by the neutral one (F2) (Fig. 3), essentially made up of low-molecular-weight oligosaccharides and lactose; whereas the neutral high-molecular-weight fraction (D) and pool did not show any inhibitory capacity. Among the acid and neutral low-molecular-weight oligosaccharide standards, only 6′-SL and 3-FL showed a limited percentage of inhibition.

The neutral high-molecular-weight fraction D was effective in inhibiting adhesion of *V. cholerae*, whereas a lower inhibition was obtained by the acid fraction (A) on the same pathogen.

It must be pointed out that none of the low-molecular-weight oligosaccharides (fraction F2 and five purified standards) showed an inhibitory effect on *V. cholerae*.

Moreover, the analysis of the results shows that the acid fraction A had an antiadhesive effect on the three pathogenic strains studied (with different degrees of inhibition). On the contrary, the neutral high-molecular-weight fraction D significantly inhibited the adhesion of *E. coli* O119 and *V. cholerae* but not that of *S. fyris*; whereas the neutral low-molecular-weight fraction F2 was effective toward *E. coli* O119 and *S. fyris* but not *V. cholerae*.

DISCUSSION

The adhesion of a bacterium to the host cell is the basic requirement to carry on the pathogenic effect. 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 Walls interactions, and charge and dipole attraction (34,35), suggesting that if a surface on the glycan is complementary to the protein-combining site, water can be displaced and binding occurs. Most combining sites of adhesins interact with oligosaccharides, mainly made of up to 5 monosaccharidic units (8). Usually, adhesion takes place with the nonreducing end of the oligosaccharidic chain, even though different adhesins can interact with sugars placed in more internal positions of molecules (36). As a consequence, the affinity between a pathogenic agent and the cell receptor is the result of a multiple interaction.

Receptors present on the surface are made up of oligosaccharidic residues of glycoproteins and glycolipids of cell membranes. 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 pathogenic effect as a consequence (8).

It has been proved that also human milk oligosaccharides have a protective effect, based on the same mechanism. Such

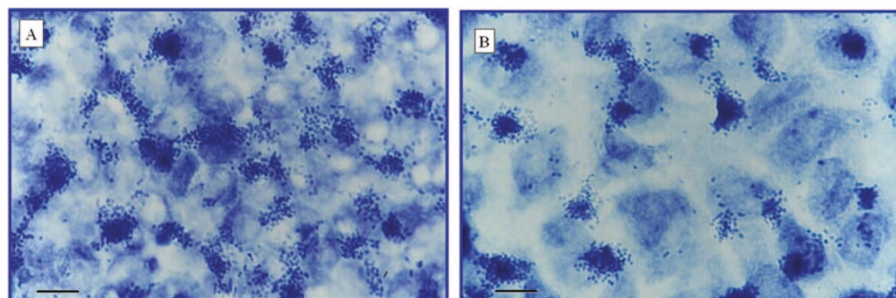

Figure 2. Adherence of enteropathogenic *E. coli* strain 0119 to Caco-2 cells in the absence of human milk oligosaccharides (*A*) and in the presence of fraction D oligosaccharides (*B*). (Magnification: ×1000; scale bar: 25 mm.)

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Table 1. *Percentage inhibition (95% CI) of bacterial adhesion to Caco-2 monolayers by human milk oligosaccharides**

Sugars	Mean of % inhibition (95% CI)		
	E. coli	*V. cholerae*	*S. fyris*
Lactose	1.80 (−1.36, 5.02)	1.35 (−3.83, 6.52)	1.21 (−1.46, 3.88)
Pool	**38.10** (37.02, 39.17)	**31.23** (24.16, 38.31)	3.09 (−1.22, 7.41)
Fraction A†	**36.10** (30.24, 41.92)	**16.13** (3.62, 28.64)	**20.33** (8.23, 32.43)
Fraction D‡	**42.20** (38.03, 46.40)	**31.54** (22.44, 40.64)	0.99 (−2.24, 4.22)
Fraction F2§	**33.30** (30.32, 36.20)	1.17 (−1.14, 3.80)	**25.00** (20.95, 29.05)
Standard 3′-SL	**28.70** (25.02, 32.40)	2.34 (−2.34, 7.02)	3.25 (−1.47, 7.96)
Standard 6′-SL	**16.20** (13.69, 18.62)	0.51 (−3.43, 4.45)	**16.45** (15.03, 17.87)
Standard 3-FL	**30.20** (21.94, 38.53)	1.11 (−3.61, 5.82)	**16.59** (14.03, 19.16)
Standard DFL	2.10 (−0.27, 4.53)	1.56 (−1.15, 4.28)	2.14 (−3.73, 8.01)
Standard 2′-FL	1.80 (−3.24, 6.76)	1.91 (−1.07, 4.88)	2.10 (−0.67, 4.87)

* The percentage of inhibition is not significant when its confidence interval contains the zero; significant values are expressed in bold.
† Acidic oligosaccharides.
‡ Neutral high-molecular-weight oligosaccharides.
§ Neutral low-molecular-weight oligosaccharides.

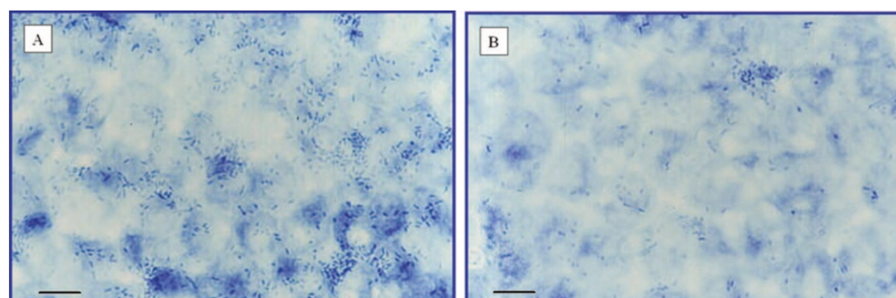

Figure 3. Adherence of *S. fyris* to Caco-2 cells in the absence of human milk oligosaccharides (*A*) and in the presence of F2 fraction oligosaccharides (*B*). (Magnification: ×1000; scale bar: 25 mm.)

effect was observed on viruses and toxins as well as on bacteria (7,8,36,37). In particular, as regards the capacity of human milk oligosaccharides to inhibit adhesion of pathogenic germs, data available up to now concern few bacteria only (*Streptococcus pneumoniae*, *E. coli*, *Helicobacter pylori*, *Campylobacter jejuni*, *Listeria monocytogenes*) responsible for infections at the expense of respiratory, digestive, and urinary systems. As for *S. pneumoniae*, Anderson *et al.* (18) proved in 1986 that lacto-N-tetraose (LNT) and lacto-N-neotetraose (LNnT) can inhibit the adhesion to buccal epithelial cells. The same positive effect of LNnT was also confirmed on lung tumoral cells and animal models (38). On the contrary, Barthelson *et al.* (39), using conjunctival cells, observed a reduced effectiveness of LNnT in inhibiting some *Streptococcus* strains, while its 6′ sialyl-derivative was very effective. Hence, it may be stated that *Streptococcus* can be inhibited in different conditions both by neutral and acid oligosaccharides.

Data available so far about the activity of human milk oligosaccharides toward *E. coli* concern both enteropathogenic and uropathogenic strains. Coppa *et al.* (40) observed that a mixture of neutral oligosaccharides, mainly tri-tetrasaccharides, inhibited the adhesion of uropathogenic *E. coli* to human uro-epithelial cells, while, as far as enteropathogenic *E. coli* is concerned, Cravioto *et al.* (19) showed that fucosyl-tetra-pentasaccharides were able to inhibit its adhesion to HEp-2 cells. Our present study confirms the capacity of fucosyl-oligosaccharides containing fractions (D, F2) to inhibit the adhesion of enteropathogenic *E. coli* O119; in addition, 3-FL, among neutral low-molecular-weight oligosaccharides, acidic oligosaccharides *in toto* (A fraction), 3′-SL, and 6′-SL were also able to reduce adhesion. The difference between the data observed by Cravioto *et al.* (19) and ours may be due to different systems used both for the strains of enteropathogenic *E. coli* and the types of cells.

Recently, further pathogenic agents have been reported toward which human milk oligosaccharides are effective as antiadhesive agents; in particular, by using different epithelial cell lines, adhesion of *H. pylori* was inhibited by sialyl-oligosaccharides, mainly 3′-SL. The effectiveness of such oligosaccharides on *H. pylori* was also confirmed by using animal models (rhesus monkeys) (41).

Two more bacteria were studied to evaluate the effect of human milk oligosaccharides: *L. monocytogenes* and *C. jejuni*. The adhesion of *L. monocytogenes* to Caco-2 was inhibited by neutral high- and low-molecular-weight oligosaccharides (mostly fucosylated) (21) and that of *C. jejuni* was inhibited by fucosyl-oligosaccharides, using both *in vitro* and *in vivo* systems (42).

Data reported in this work are the first about the effect of human milk oligosaccharides on *S. fyris* and *V. cholerae* strains and show that the antiadhesive effect on *S. fyris*—even though rather weak—was obtained by acid and neutral low-molecular-weight oligosaccharides, whereas inhibition on *V. cholerae* took place mainly in the presence of neutral high-molecular-weight oligosaccharides.

It may hence be inferred that human milk oligosaccharides on the whole are effective in inhibiting adhesion of the dif-

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ferent pathogenic agents to receptors of epithelial cells. This is to be related to the fact that bacterial adhesins are numerous and structurally different as well as to the receptors present on the epithelial cell surface. Therefore the germ/host cell adhesion is the result of a multiple interaction and a high binding affinity through a joint action of several receptors ("Velcro effect") (8,36). Actually, when considered separately, oligosaccharides may have a weak bond, whereas they have an especially strong bond as a whole. This could explain the different percentage of inhibition observed in the different pathogenic strains, depending on oligosaccharides tested. In fact, it must be considered that each fraction studied is made up of a mixture of oligosaccharides, and if it was possible to carry on tests using few specific pure standards of acidic or neutral low-molecular-weight oligosaccharides, it was not possible to determine the efficacy of the remaining oligosaccharides contained in the mixture. On the contrary, each fraction tested, made up of a mixture of several oligosaccharides, was effective on the various pathogens, even though with different degrees of intensity. For example, D-fraction (made up of neutral fucosylated high-molecular-weight oligosaccharides) was able to inhibit both enteropathogenic *E. coli* O119 and *V. cholerae*; on the other hand, from the literature, it appears that fucosylated oligosaccharides have always been effective on *C. jejuni* (19). *L. monocytogenes* (21), and another strain of enteropathogenic *E. coli* (30). Such phenomenon could be explained by the fact that the mixture is made up of oligosaccharides, separately acting on different specific receptors. This proves also that a microorganism has a share of adhesins similar to those of another germ, and, therefore, both have common oligosaccharidic receptors. This explains why one single oligosaccharide contained in human milk can—at least to some extent—inhibit the adhesion of several pathogenic germs, as our tests showed for 3-FL toward *E. coli* O119 and *S. fyris*. We therefore believe that the protective effect of human milk oligosaccharides is not due to the action of single oligosaccharides acting on single bonds but is the result of the contemporary and joint action of several oligosaccharides that can act on various bonds, both of one single germ and different germs, considering also that a high-molecular-weight oligosaccharide with a branched structure can have several bond sites with one single adhesin. Moreover, a careful analysis of the structure of the nonreducing ends of oligosaccharides, used in our experiment, shows a very heterogeneous monosaccharide composition; therefore, it is impossible to identify a specific monosaccharide sequence able to inhibit the adhesion.

In conclusion, the literature and our own results make it possible to assert that human milk oligosaccharides can carry on a protective role toward the most common intestinal infections of breast-fed newborns and infants (43). These results may represent a stimulus to further research such as *in vivo* and/or *ex vivo* studies, as recently carried out by Ruiz-Palacios *et al.* (42).

Oligosaccharides, then, enter the complex defence system made up of various substances and mechanisms protecting breast-fed infants against infections. It follows that there are further reasons to recommend prolonged breast-feeding, mainly in developing countries where intestinal infections are still a significant cause of morbidity and mortality.

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