Glycoconjugate Journal 19, 5–11, 2002

Binding of milk oligosaccharides by several enterotoxigenic *Escherichia coli* strains isolated from calves*

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Milk oligosaccharides have been proposed to play an important role in newborn defense, blocking bacterial adhesion to the intestinal mucosa and preventing infections. Some studies have been performed on human milk oligosaccharides. Here we checked whether bovine milk oligosaccharides would achieve the same protective action against the most common calf enteric pathogens. Seven enterotoxigenic *Escherichia coli* strains, isolated from diarrheic calves, were selected. All strains managed to agglutinate horse erythrocytes, and we therefore used the inhibition of hemagglutination in the presence of oligosaccharides as an indicator of the union between oligosaccharide and bacterial adhesins. Oligosaccharides from different stages of bovine lactation and standard oligosaccharides were assayed. Midlactation milk, in particular that corresponding to the transition period, proved to be the most efficient at inhibiting hemagglutination. The standard oligosaccharides used pointed to the preference of several strains (K99-, F41-, and F17-fimbriated) for α2,6-linked sialic acid. By contrast, B23 fimbriae exhibited higher affinity for α2,3-sialylated isomers and B64 seemed to require *N*-acetylglucosamine for binding.

Our results suggest a general trend for milk oligosaccharides. Probably they participate in the protection of newborn mammals from pathogens.

Keywords: **bacterial adhesion, calves, enterotoxigenic *Escherichia coli*, milk oligosaccharides**

Introduction

The first step in infections due to *Escherichia coli* and other enterobacteria is the attachment to a suitable niche in the intestinal mucosa; this attachment is mediated by different kinds of fimbriae. Fimbriae are filamentous appendages consisting of many copies of an adhesive protein, the so-called adhesin, or several subunits, including adhesins and other proteins involved in fimbria assembly [1].

The fimbrial adhesins are lectins that recognize certain carbohydrate sequences, which determines host and tissue specificity [2]. Thus, although all the enterotoxigenic *Escherichia coli* (ETEC) strains produce heat-stable and/or heat-labile enterotoxins, CFA-fimbriated strains cause diarrhea only in humans [3]; K88 and 987P are common in porcine strains, and K99-fimbriated ETEC are mainly isolated from calves [4].

There are many reports about the carbohydrate specificity of several *E. coli* fimbriae. P fimbriae, which bind human blood group antigen P and glycosphingolipids in the urinary epithelium, recognize the sequence Galβ1-4Gal; F adhesin binds to GalNAcα1-3GalNAc, and S fimbriae to sialyl-galactosides [5].

It is well known that breast-fed infants suffer less enteric diseases than formula-fed infants. Besides milk immunoglobulins, carbohydrates and other glycoconjugates have been shown to play an essential role in the defense of newborns against enteropathogens [6], including several diarrhegenic strains of *Escherichia coli* and their toxins [7]. Milk oligosaccharides have been reported to act as soluble receptors for bacterial adhesins, blocking their binding sites and preventing them from becoming attached to the mucosa cells [8].

Since calf mortality is increased in milk-deprived animals, we hypothesized that bovine milk oligosaccharides could also protect newborn calves from infections. Although the oligosaccharide fraction is much more abundant in human than in bovine milk, and human milk contains a plethora of complex oligosaccharides, many of them fucosylated, the role played by these compounds could be similar in both species [9].

* This paper is dedicated to our master and friend Prof. José A. Cabezas on the occasion of his retirement.

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In bovine colostrum, sialylated oligosaccharides constitute a more abundant fraction than neutral oligosaccharides. More than 1 g/L of sialylated oligosaccharides have been reported [10] versus 147 mg/L of neutral [11]. Only 18 oligosaccharides have been found: sialyllactose represents more than 50% of the total oligosaccharides in colostrum and milk, and lactosamine is the main neutral oligosaccharide in colostrum, although it disappears seven days after calving [12]. Complex oligosaccharides, especially sialylated, undergo a marked decrease, as previously reported by us [13].

The aim of this study was to check the capacity of bovine milk oligosaccharides to bind ETEC fimbriae, comparing them to standard oligosaccharides and monosaccharides. We selected seven bovine ETEC strains that express different fimbriae, including K99. Milk oligosaccharides from different stages of lactation were isolated. Since fimbriae bind specific carbohydrate receptors on the erythrocyte cell surface promoting the hemagglutination, the inhibition of this hemagglutination by free oligosaccharides can be used as an indicator of the interaction between these oligosaccharides and fimbrial adhesins [14].

Materials and methods

Bacterial strains and media

Seven bovine ETEC strains, expressing six different fimbriae, were kindly provided by the Laboratorio de Referencia de *Escherichia coli* (Facultad de Veterinaria, Lugo, Spain). The characteristics of each strain are listed in Table 1 [15]. Bacteria were grown in Mueller-Hinton broth (Difco, Detroit MI) and incubated for 3–5 days at 37°C. Then, they were incubated on Minca-Is agar plates at 37°C for 16 hours, as previously reported [16]. This medium is appropriate for fimbriae expression in all the strains.

Milk oligosaccharides

Milk was obtained from six Spanish-Brown cows on day 2 (colostrum), day 7 (transitional milk), day 90 (mature milk), and day 270 (late lactation milk) after calving. All samples were frozen and lyophilized. Milk from the six cows was pooled and total oligosaccharides were purified from defatted milk. Lactose and proteins were precipitated at 4°C with 2 vol. of ethanol, as described [17]. After centrifugation (2000 g, 15 min), supernatants were evaporated to eliminate the remaining ethanol. Oligosaccharide content was determined using the phenol-H_2SO_4 method [18].

Table 1. Enterotoxigenic *E. coli* strains

Strain	*Serogroup*	*Adhesin*
K99-12	O8	K99
F41-15	O20	F41
K99-4	O101	K99 + F41
CCB1	O101	F17 (Att25)
CCB22	O139	B16 (Vir)
CCB33	O21	B64
CCB37	O123	B23

Standard oligosaccharides and monosaccharides

Glucose (Glc), galactose (Gal), mannose (Man), *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) were from Sigma Chemical Co. (St. Louis, MO). Lactose was from Merck (Darmstad, Germany). Lactosamine (LN), NeuAcα2-3Galβ1-4Glc (3′-sialyllactose: 3′SL), NeuAcα2-6Galβ1-4Glc (6′-sialyllactose: 6′SL), NeuAcα2-6Galβ1-4GlcNAc (6′-sialyllactosamine: 6′SLN), NeuAcα2-8NeuAcα2-3Galβ1-4Glc (disialyllactose: DSL) were also from Sigma. NeuAcα2-3Galβ1-4GlcNAc (3′-sialyllactosamine: 3′SLN), was from Glyko (Upper Heyford, UK).

HPLC separation

Milk sialyloligosaccharides were analyzed using a NH_2-bonded silica column (Carbohydrate Analysis 3,9 × 300 mm column from Waters Co., Milford MA), as described [19]. Isocratic elution was kept up for 15 minutes using the solvent mixture acetonitrile/ 15 mmol/L KH $_2PO_4$, pH 5.2 (75:25). Then, elution was made by a linear gradient over 45 minutes from 75:25 to 50:50. The flow rate was 1 mL/min. Detection was accomplished using an UV detector at 206 nm (Absorbance Detector 2487 from Waters Co.). Individual oligosaccharides were identified by comparison of their retention times with those of standard oligosaccharides.

Hemagglutination assays

Hemagglutination tests were carried out at 4°C on sterile V-shaped 96-well plates (Nalge Nunc International, Roskilde, Denmark). Horse erythrocytes were supplied by the Laboratorio de Grupos Sanguíneos del Servicio de Cría Caballar (Córdoba, Spain). A 5% erythrocyte suspension in PBS (pH 7.2, 150 mmol/L) was used. Bacteria were also resuspended in PBS. 25 μL of PBS or oligosaccharide dilution were added to 25 μL of bacterial suspension and gently mixed. After 5 minutes, 25 μL of erythrocyte suspension was added to each well. The plates were kept at 4°C for 2 hours after which agglutination was read by the naked eye.

Statistical assays

Oligosaccharide differences among stages were tested by one-way ANOVA using the SPSS 9.0.1 program for Windows (SPSS Inc., Chicago, IL).

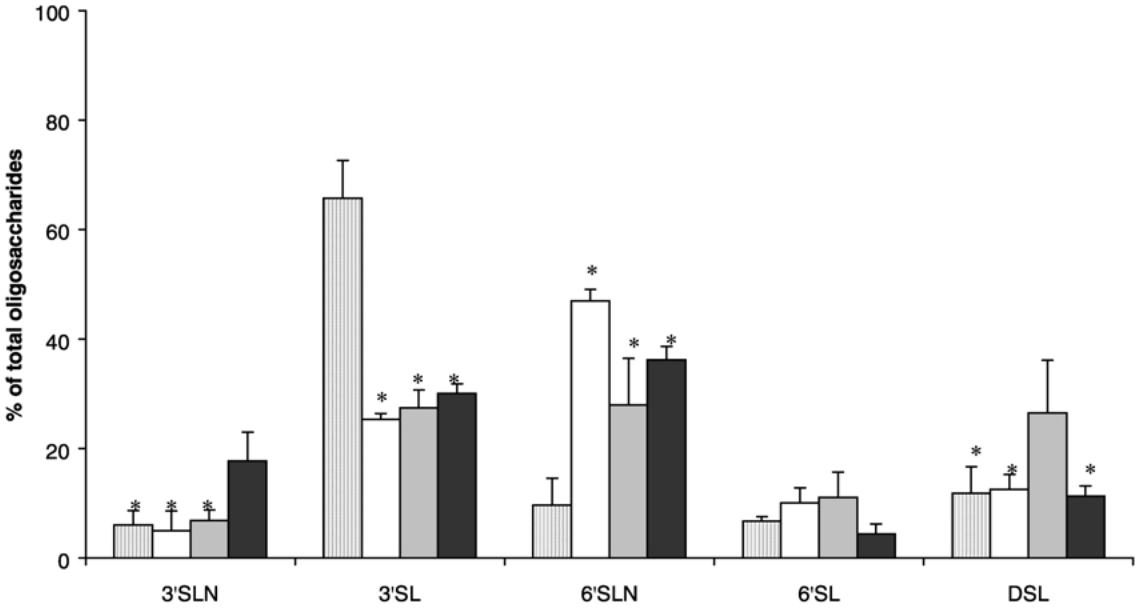

Figure 1. Oligosaccharide pattern of bovine milk. Stripped bars: colostrum, white bars: transitional milk, shaded bars: mature milk, black bars: late lactation milk. Bars represent mean ± SD of oligosaccharide injections from five separate extractions of the same pool of milk. Statistically significant differences (*, $p < 0.001$) are shown. 3′SL: 3′-sialyllactose, 6′SL: 6′-sialyllactose, 3′SLN: 3′-sialyllactosamine, 6′SLN: 6′-sialyllactosamine, DSL: disialyllactose.

Results

Horse erythrocyte hemagglutination mediated by the ETEC strains studied

K99-fimbriated ETEC have been reported to agglutinate erythrocytes from several species, including horses and humans [20]. Since horse erythrocytes have a specific receptor for K99 fimbrial adhesins, the ganglioside NeuGc-GM3 (about 90% of total ganglioside content) [21], horse erythrocyte agglutination has been selected as a positive control of the process. This assay was a very simple and effective method to assess agglutination. Erythrocytes from twelve horses belonging to different blood groups were tested. Bacterial growth in a Petri dish was taken up in 1 mL of PBS. 50 μL of this bacterial suspension (about 5×10^9 colony-forming units (CFU) agglutinated 50 μL of the erythrocyte suspension, in both the presence and the absence of mannose. No differences among individual blood groups were found. The rest of the strains, except B23, agglutinated horse erythrocytes at similar concentrations of bacteria. Three times the amount of B23 was necessary to achieve the agglutination.

Hemagglutination mainly depends on the expression of fimbriae since the non-fimbriated bacteria failed to agglutinate erythrocytes.

Bovine milk sialylated oligosaccharides

In order to identify the sialyloligosaccharides present in the samples, total oligosaccharides from each stage of lactation were injected in the HPLC apparatus. Under the conditions used, only acidic compounds were resolved and detected. The sialyloligosaccharide pattern for each stage is shown in Figure 1. Oligosaccharide quantification was carried out from the calibration curves of individual standard oligosaccharides using the Millenium software (Waters) coupled to the HPLC system. Although all the oligosaccharides were detected in all stages, the individual percentages changed in a statistically significant way. For instance, α2-6 sialylated trisaccharides were more abundant than α2-3 isomers in midlactation milk.

Inhibition of hemagglutination by milk oligosaccharides

We first assayed the capacity of milk oligosaccharides to inhibit hemagglutination. Several dilutions of bacterial suspension were tested in order to determine which is the minimal number of CFU (colony-forming units) required to hemagglutinate horse erythrocytes for each strain. To check whether there were differences among the stages, amounts of oligosaccharide (from 25 μg up to 1200 μg in 25 μL) from each stage were assayed against the whole set of adhesins (see Table 2). The amount of bacteria added per well (25 μL) was the minimum required to agglutinate horse erythrocytes.

Transitional and mature milk oligosaccharides proved to be the most efficient at inhibiting the agglutination produced by all the strains and colostrum was the least efficient, since higher amounts of colostrum oligosaccharides were required to inhibit the hemagglutination produced by all the ETEC strains assayed. Late lactation milk was less efficient at inhibiting than midlactation milk (transition and mature milk). Transitional milk oligosaccharides showed the best inhibition with K99, B16 and B64.

Table 2. Minimum amounts of milk oligosaccharides required for complete inhibition of horse erythrocyte agglutination by ETEC strains

		Amount of oligosaccharide (μg)			
Adhesin	*CFU*[1,2]	*Colostrum*	*Transitional milk*	*Mature milk*	*Late lactation milk*
K99	5.3×10^8	1000	25	75	500
F41 + K99	5.7×10^8	1200	75	200	500
F41	2.5×10^8	500	75	75	500
F17	7.8×10^7	1200	75	75	300
B16	4.2×10^9	1000	25	25	300
B23	1.1×10^{10}	1200	75	500	1000
B64	2.1×10^9	500	25	75	500

[1]CFU: Colony-forming units.
[2]Minimum amount of bacteria required to hemagglutinate horse erythrocytes. These results correspond to four experiments carried out on different days. Each assay was made by triplicate. The same volume (25 μL) of bacterial suspension, oligosaccharides and erythrocyte suspension was used.

Inhibition of hemagglutination by standard oligosaccharides and monosaccharides

In order to determine whether the position of the sialic acid and the length of the carbohydrate chain could determine the degree of binding to bacterial adhesins, we attempted a comparison of the inhibitory capacity of a set of pure oligosaccharides and monosaccharides, including the sialylated ones found in milk. Since a small amount of lactose was detected in these samples by thin-layer chromatography, as described [22], we also assayed the capacity of lactose to inhibit hemagglutination. To complete the set, we assayed lactosamine since this is the main neutral oligosaccharide in bovine milk.

The different strains showed different affinities for oligosaccharides (Table 3). In many cases, the addition of an oligosaccharide reduced the hemagglutination mediated by the bacterial fimbriae, showing that oligosaccharides bind and block adhesins.

6′SL was a better inhibitor than 3′SL in the cases of F41 and F17. Although 3′SL and 6′SL behaved in a similar way against K99 and (F41 + K99), 6′SLN inhibited better than 3′SLN. Thus, α2,6-linked sialic acid seems to be more efficient than the α2,3-linked type at inhibiting hemagglutination mediated by adhesins K99, F41, and F17. By contrast, B23 showed a stronger affinity for 3′-sialylated forms (3′SL and 3′SLN).

B64 could exhibit a preference for GlcNAc since the oligosaccharides containing the Galβ1-3/4GlcNAc sequence inhibited hemagglutination quite well. Although sialyllactose exerted no inhibition, lactose was inhibitory at the same concentration tested for the rest of the oligosaccharides.

The presence of a second sialic acid (in DSL) increased the inhibition capacity in all the strains except the K99- and B16-fimbriated strains.

Single monosaccharides also inhibited hemagglutination mediated by F41, F17, B16 and B64 (Table 4). K99-mediated hemagglutination was inhibited by NeuGc; this was as expected because NeuGc has been reported to be essential in the binding of this strain to ganglioside NeuGc-GM3. However, none of the sialylated oligosaccharides from these samples contained NeuGc.

Table 3. Hemagglutination by ETEC strains in the presence of different oligosaccharides[1]

Adhesin	*CFU*[2]	*PBS*	*3′SL*	*6′SL*	*3′SLN*	*6′SLN*	*DSL*	*LN*	*Lac*[3]
K99	7.3×10^7	+	±	±	+	±	+	±	+
F41+K99	2.6×10^7	+	−	−	±	−	+	+	+
F41	1.4×10^7	+	±	−	±	−	−	+	±
F17	6.9×10^6	+	+	±	+	±	−	+	+
B16	2.9×10^7	+	±	±	±	±	+	±	±
B23	2.2×10^7	+	±	+	±	+	−	+	±
B64	4.3×10^7	+	+	+	±	±	−	−	−

−: no agglutination; ±: less agglutination than control (PBS); +: agglutination similar to control; ++: more agglutination than control.
[1]3′SL: 3′-sialyllactose, 6′SL: 6′-sialyllactose, 3′SLN: 3′-sialyllactosamine, 6′SLN: 6′-sialyllactosamine, DSL: disialyllactose, LN: lactosamine; Lac: lactose.
[2]Estimated CFU/μg of oligosaccharides (25 μg/well). Results correspond to four experiments carried out on different days.
[3]Lack of inhibition was assayed up to 1.4 mol/L (25–500 μg/well).

Table 4. Hemagglutination by ETEC strains in presence of different monosaccharides

Adhesin	*CFU*[1]	*PBS*	*Glc*	*Gal*	*NeuAc*	*NeuGc*
K99	7.3×10^7	+	+	+	+	−
FK	2.6×10^7	+	+	+	+	±
F41	1.4×10^7	+	−	−	+	+
F17	6.9×10^6	+	−	−	+	+
B16	2.9×10^7	+	−	−	±	−
B23	2.2×10^7	+	+	+	+	−
B64	4.3×10^7	+	−	−	±	−

−: no agglutination; ±: less agglutination than control (PBS); +: agglutination similar to control; ++: more agglutination than control.
[1]Estimated CFU/μg of oligosaccharides.

Discussion

Milk and colostrum contain different antimicrobial factors that protect the newborn from the most common infections and are crucial for the survival. Immunoglobulins are the main factor transferred in milk, but there are other compounds that cooperate in the newborn's defense, including proteins, glycolipids and carbohydrates [23]. Since the inhibition of the attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* to host cells by human milk oligosaccharides was described [24], many studies have addressed the protective role of milk oligosaccharides. In this sense, fucosyloligosaccharides have been reported to block the adhesins of *Campylobacter jejuni* and those of enteropathogenic *E. coli* as well as heat-stable toxin from ETEC [23], and 3′SL inhibits the adhesion of *Helicobacter pylori* [25].

To exert their protective role, milk oligosaccharides should remain undigested in the gastrointestinal tract. Recent studies have shown that several digestive enzymes, such amylase or lactase, have minor effects on milk complex oligosaccharides [26,27] Fucose and sialic acid could avoid their degradation by digestive enzymes. In the preruminant calf, intestinal lactase is a very active enzyme, efficiently digesting lactose but not sialylated oligosaccharides. Thus, the proposed role in the youngest calves could be the same as that found for humans. However, when calves develop into ruminants (about 30 days postpartum), it is likely that ruminal flora would metabolize the most of the soluble carbohydrates [28].

We first studied the oligosaccharide content and pattern at four stages of lactation. Carbohydrates are a very abundant component in human milk, but in bovine milk they constitute a minor and much more simple fraction of glycoconjugates. The carbohydrate content in milk increases as lactation progresses (from 18% to 43% of dry matter) due to an increase in the amount of lactose. Nevertheless, oligosaccharides decrease markedly. As previously mentioned, lactosamine disappears completely after the colostrum stage and acidic oligosaccharide content is also reduced. We identified the five major sialyloligosaccharides whose percentages varied throughout lactation. Briefly, 3′sialyllactose decreased significantly in transition and following stages, whereas α2,6-sialylated isomers increased with respect to α2,3 in these stages.

Since oligosaccharides in bovine milk are such a minor fraction, only those from colostrum had been previously studied and assayed as antibacterial agents. Here we assayed the capacity of milk oligosaccharides from the four stages of lactation to bind different strains of bovine ETEC producing the most common fimbriae. K99 was found in 80% of isolates from diarrheic calves, and in many cases, these strains coexpressed F41 and/or F17 fimbriae. Other bovine strains expressing new adhesins (B23 and B64) were also included in this study. Midlactation milk oligosaccharides proved to be the most efficient at inhibiting hemagglutination and colostral oligosaccharides were the least efficient. Besides a potential and non-specific blocking by lactose, these results pointed to a stronger affinity of adhesins for α2,6-sialylated isomers, which are more abundant than α2,3 isomers in midlactation milk. This trend was also observed when isolated oligosaccharides were assayed.

K99 has been reported to recognize the sequence NeuGcα2,3Galβ1,4Glc in the ganglioside NeuGc-GM3 but, as we showed here, it had a certain affinity for NeuAc-lactose and NeuAc-lactosamine. It has been reported [20] a stronger affinity for α2,6-linked sialic acid, as we found in the present study. Regarding isolated sialic acid, NeuGc inhibited hemagglutination but NeuAc did not. Since NeuGc has been found in milk oligosaccharides only in tiny amounts [12], it must be concluded that the observed inhibition is not so much due to type of sialic acid itself, but to the whole structure of the compound and the sialic acid linkage type.

F41, an adhesin belonging to the K88 family, has been assayed both isolated and coexpressed with K99. A positive cooperation between the two kinds of fimbriae (F41 and K99) in the binding of double-fimbriated microbes to calf intestine mucins has been reported [29]. GalNAc and, to a lesser extent GlcNAc, Gal and Fuc have been reported to inhibit F41 hemagglutination [30]. In many reports, sialic acid has been found to hinder the adhesion of F41$^+$ strains [30]. However, we here found an interaction between α2,6-sialylated disaccharides and F41 fimbriae. As expected, the double-fimbriated strain seemed to interact with more compounds than the single-fimbriated K99 and F41 strains.

F17 (F17a = Att25) belongs to an adhesin family formed by F17b (F17-like), F17c (20K) and F17d (F111) [31]. Although F17 has been reported to bind GlcNAc but not sialic acids, another member of its family, F17b, binds to highly sialylated proteins [32]. In the present study, F17 failed to bind isolated sialic acids but did show a certain affinity for α2,6-sialylated oligosaccharides and DSL.

To our knowledge, this is the first report about possible B16, B23 and B64 receptors. B16 showed a very weak affinity for the individual oligosaccharides assayed and a stronger interaction with monosaccharides. B23 seemed to prefer α2,3- or α2,8-linked sialic acids to α2,6-linked type. B64 seemed to

require the presence of N-acetylhexosamine, although lactose also inhibited the hemagglutination mediated by this strain.

The prevalence of α2,6 sialyloligosaccharides and DSL could increase the capacity for blocking these bacterial adhesins, since oligosaccharides from midlactation were stronger inhibitors of hemagglutination. Surprisingly, colostral oligosaccharides proved to be poorer inhibitors for these strains. However, since the amount is higher than in midlactation, the whole effect could be similar.

During the first days of life, newborn calves cannot synthesize antibodies by themselves and depend completely on those transferred from the mother. Thus, colostrum is richer in immunoglobulins than the later secretions of the mammary gland. In this context, the passive defense carried out by non-specific agents, such as oligosaccharides, could be merely a coadjuvant factor. Since the immunoglobulin levels of colostrum decrease rapidly in the first 24 h after calving [33], the contribution of oligosaccharides to anti-microbial defense may increase. At day 15 postpartum (transitional milk), the total oligosaccharide fraction has been shown to be an efficient inhibitor of adhesion mediated by the most common fimbriae of bovine ETEC strains. After this stage, maturation of the gastrointestinal tract and the existence of a functional rumen prevent any protective action of oligosaccharides. From a nutritional point of view, the Bovine Alliance on Management and Nutrition [34] recommends that calves should not be weaned before 4–6 weeks of age. According to our results, these recommendations can also be accepted from a protective point of view, since transitional milk oligosaccharides were very effective in the inhibition of the bacterial adhesion.

Several authors have reported the significance of human milk oligosaccharides in the newborn defense against pathogens. This milk contains high amounts of complex oligosaccharides, mainly fucosylated. However, little is known about the role of these compounds in other mammals. Our results in bovine milk oligosaccharides are complementary to those obtained in human milk. They are also able to bind enteric pathogens isolated from calves. These results pointed out to a general trend for milk oligosaccharides. Probably they participate in the protection of newborn mammals from pathogens.

Acknowledgments

We would like to thank Dr. J. Blanco, from the Spanish Reference *E. coli* Laboratory (Lugo, Spain), who provided us all the strains and some information about bacterial culture. We also thank Dr. J.L. Vega, from the Blood Groups Laboratory (Córdoba, Spain), who kindly sent us horse erythrocytes on many occasions. We are also grateful to N. Skinner (from the Servicio de Idiomas, Universidad de Salamanca) for revising the English version of the manuscript.

This work was supported by a grant of the Programa de Apoyo a Proyecto de Investigación de la Junta de Castilla y León, España (SA093/01).

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Received 14 March 2002; revised 25 October 2002;
accepted 4 November 2002