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Sialoglycoconjugate content of milk replacers for neonatal calves

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Abstract

Sialoglycoconjugate contents of several milk replacers (MR) currently used in Spain have been determined. The ingestion of these compounds by calves fed bovine milk or MR is also discussed. Total sialic acids and glycoprotein-, oligosaccharide-, casein-, and lipid-bound sialic acids and free sialic acid were determined. High sialic acid contents in all the fractions studied, including total sialic acids, were found. *N*-acetylneuraminic acid was found to be the major sialic acid in MR. However, MR-D and H had a high *N*-glycolylneuraminic acid (NeuGc) content in all fractions (15-40%), while E and F only had a high NeuGc content in the glycoprotein fraction. Five different sialyloligosaccharides -3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine, and disialyllactose, which are the most abundant oligosaccharides in bovine milk- were detected in the MR. Samples B to I contained high amounts of these oligosaccharides. The content of individual gangliosides was very similar to that of bovine milk, with GD3 as the major ganglioside. Although MR are not formulated as regards their sialic acid content, high amounts of sialic acid-containing glycoconjugates with high levels of NeuGc, a sialic acid critical in the adhesion of some *Escherichia coli* strains to calf intestinal epithelium were detected. The ingestion of MR analyzed in this work could protect newborn calves from several enteric pathogens.

Additional key words: gangliosides, milk oligosaccharides, sialic acids.

Resumen

Contenido de sialoglicoconjugados de algunos lactorreemplazantes usados en la alimentación de terneros recién nacidos

En este trabajo se analizó el contenido de sialoglicoconjugados de diferentes lactorreemplazantes (MR) usados habitualmente en España. Además, se ha comparado la ingesta de estos compuestos por terneros alimentados con leche materna o MR. Se determinó el contenido de ácidos siálicos totales, ácidos siálicos libres y ácidos siálicos unidos a glicoproteínas, a oligosacáridos y a caseína, habiéndose encontrado una elevada cantidad de los mismos en todas las fracciones estudiadas. El ácido *N*-acetilneuramínico es el ácido siálico mayoritario de todos los MR. Sin embargo, los MR-D y H tienen un elevado contenido de ácido *N*-glicolilneuramínico (NeuGc) en todas las fracciones estudiadas (15-40%). Por el contrario, los MR-E y F sólo tienen grandes cantidades de NeuGc en la fracción de glicoproteínas. En cuanto al contenido de oligosacáridos sialilados, se encontraron cinco diferentes (3'-sialillactosa, 6'-sialillactosa, 3'-sialillactosamina, 6'-sialillactosamina y disialillactosa), que son a su vez los oligosacáridos sialilados más abundantes de la leche bovina. La distribución de los gangliósidos individuales que se ha encontrado es muy similar a la de la leche bovina, siendo GD3 el ganglósido más abundante. Aunque los MR no han sido formulados teniendo en cuenta su contenido de ácidos siálicos, se encontraron en ellos grandes cantidades de sialoglicoconjugados con una elevada proporción de NeuGc, un ácido siálico muy importante en la adhesión de algunas cepas de *Escherichia coli* al epitelio intestinal de los terneros. La ingestión de los MR analizados en este estudio podría proteger a los terneros recién nacidos frente a patógenos entéricos.

Palabras clave adicionales: ácidos siálicos, gangliósidos, oligosacáridos de la leche.

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Abbreviations used: CBSA (casein-bound sialic acid), FSA (free sialic acid), GBSA (glycoprotein-bound sialic acid), HPLC (high-performance liquid chromatography), HPTLC (high-performance thin-layer chromatography), LBSA (lipid-bound sialic acid), mAb (monoclonal antibodies), MR (milk replacer), NeuAc (*N*-acetylneuraminic acid), NeuGc (*N*-glycolylneuraminic acid), SD (standard deviation), TLC (thin-layer chromatography), TSA (total sialic acids).

Introduction

The composition of milk is specifically adapted to provide the essential nutritional and immunological elements required by the newborn. Sometimes, newborns may circumstantially be fed with milk formulas or replacers when breastfeeding is not possible. Since bovine milk has become an important food for humans, dairy farms are forced to use MR for feeding neonatal calves. MR are designed with regard to major components, such as proteins, lipids, and carbohydrates, which must be manufactured from different sources in order to obtain more economic products (Heinrichs *et al*., 1995). However, little or no attention has been paid to other minor components such as hormones, vitamins, growth factors or glycoconjugates. Glycoconjugates have been proposed to play an important role in the defense of newborns against infection, acting as putative receptors for certain bacteria, and thereby blocking their pathogenic effects in the small intestine (Newburg, 1999). Antibiotics have been extensively used in MR with much success against infectious diseases, but some concern has arisen about the risk of antibiotic resistance and the presence of such compounds in food intended for human consumption. For this reason, several prebiotic products have been tested as alternative anti-infectives, such as different oligosaccharides (Quigley *et al*., 1997, 2002; Heinrichs *et al*., 2003) and serum glycoproteins (Morril *et al*., 1995; Nollet *et al.*, 1999; Quigley *et al*., 2002). The use of probiotic bacterial has also been described (von Buenau *et al*., 2005)

Due to the very extensive practice of the use of MR feeding in calves, it seems that knowledge of the sialoglycoconjugate composition of these products is of considerable value, in view of the recognized protective role of these compounds. This paper describes the sialoglycoconjugate composition of several MR that are currently used in Spain and compares sialoglycoconjugate ingestion by calves fed bovine milk and calves receiving MR.

Material and methods

Chemicals

3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine and 6'-sialyllactosamine were purchased from Glyko (Upper Heyford, UK). Disialyllactose was purchased from Sigma (St. Louis, Mo, USA).

Sephadex G-50, DEAE-Sephadex A-25, Dowex 2x8, orcinol, resorcinol, ninhydrin, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) were from Sigma (St. Louis, Mo, USA) and the sulfophosphovanillin test, thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) plates, *n*-hexane and acetonitrile were from Merck (Darmstadt, Germany).

Monoclonal antibodies (mAb) P3, 14F7 and R24 were a kind gift from the Centro de Inmunología Molecular (Havana, Cuba). Anti-A2B5 [mouse immunoglubulin M (IgM) was provided by Roche Molecular Biochemicals (Manheim, Germany). Anti-9-*0*-acetyl-GD3 (clone JONES, mouse IgG), mouse polyvalent IgG-, IgM- IgA-biotin conjugated, ExtrAvidin-alkaline phosphatase, and FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate /nitro blue tetrazolium were from Sigma (St. Louis, MO, USA). Poly isobutyl methacrylate (PIBM) was from Aldrich Chemical Company (Milwaukee, WI, USA). Albumin from bovine serum (BSA) was provided by Fluka (Buchs, Switzerland).

Milk replacers

Nine MR that are currently used in Spain from three different companies were analysed. MR-A is a colostrum replacer and all others are MR to be administered to calves after colostrum consumption.

Analitycal procedures

MR were reconstituted in distilled water (0.1 g mL^{-1}). Total lipids were determined by the sulfophosphovanillin Merkotest. Proteins and total carbohydrates were determined by a modification of the Lowry procedure as described by Markwell *et al*. (1978) and by the phenol sulphuric acid assay as described by Dubois *et al*. (1956), respectively.

Sialoglycoconjugate fractions (oligosaccharides, glycoproteins and casein) were obtained from skimmed samples (defatted by centrifugation at 3,000xg, 30 min at 4ºC and filtered through glass wool) as described in a previous paper (Martín *et al.,* 2001a). Analyses of the total sialic acid content (TSA) of MR and that of the fractions were also carried out as described (Martín *et al.,* 2001a). Free sialic acids (FSA) were determined by the thiobarbituric acid procedure in the oligosaccharide fraction after purification by ion-exchange chromato-

graphy on Dowex 2x8 (Aminoff, 1961). Identification of sialic acid types was performed on silica gel 60 HPTLC plates using propanol/ 0.57 M aqueous ammonia, 7:3 by vol., as solvent. They were visualized with resorcinol reagent (Svennerholm, 1957). The relative amount of each sialic acid was determined by scanning densitometry (Puente and Hueso, 1993).

Extraction of gangliosides

Gangliosides were prepared according to Puente *et al*. (1992) and quantified as lipid-bound sialic acid (LBSA) by the resorcinol procedure (Svennerholm, 1957). They were also separated on silica gel 60 HPTLC plates using the following solvent system: chloroform/methanol/water (50:45:10, by vol.) containing 0.02% $CaCl_2$. Individual gangliosides (ganglioside pattern) were analysed in a dual-wavelength densitometer (Shimadzu CS 9000, Kyoto, Japan) after separation by HPTLC and development with resorcinol reagent. Immunostaining on silica gel 60 HPTLC plates to identify individual gangliosides was performed as previously reported (Martín *et al*., 2001b). Different mAb against gangliosides were used.

Determination of the sialyloligosaccharide content

Isolation of total oligosaccharides was performed as described by Kobata (1972), with several modifications. Briefly, samples (2 g in 20 mL of distilled water) were defatted by centrifugation (3,000xg, 30 min, 4ºC) and filtered through glass wool. The filtrates were mixed with two volumes of ethanol and allowed to stand overnight at 4ºC. Most proteins and lactose precipitated and were removed by centrifugation at 0ºC. Ethanol was eliminated by rotary evaporation.

A sialyloligosaccharide fraction was prepared according to Parkinnen and Finne (1987) with several modifications. The crude oligosaccharide fraction was first purified by molecular exclusion chromatography on a Sephadex G-25 column to eliminate residual proteins and peptides. Ninhydrin (0.3 g ninhydrin in 95 mL butanol) -positive fractions were discarded. Orcinol (0.1% orcinol in 20% H_2SO_4) -positive fractions were collected together and kept at –20ºC until use.

Sialyloligosaccharides were separated by ion-exchange chromatography on a DEAE-Sephadex A-25 (acetate form) column (30 x 1.5 cm; 25 mL bead volume) after purification on Sephadex G-25. The column was equilibrated with 1 mM pyridine acetate, pH 5.2, and the sample was applied in the same solvent. Neutral oligosaccharides were eluted with 250 mL of 1 mM pyridine acetate. Sialyloligosaccharides were eluted with 100 mM pyridine acetate. Fractions were evaporated to dryness, taken up in distilled water and lyophilized.

Sialylated oligosaccharide contents were analyzed by high performance liquid chromatography (HPLC) according to the method previously described by Michalski (1995). Analyses were performed on a Waters apparatus (Waters, Milford, MA), using a NH_2-bound silica column (Carbohydrate Analysis, 300 x 3.9 mm, Waters). HPLC elution of samples was performed using a gradient of acetonitrile and 15 mM KH_2PO_4 adjusted to pH 5.2; isocratic elution with 25% 15 mM KH_2PO_4 for 10 min, linear gradient up to 50% 15 mM KH_2PO_4 for 50 min, maintaining these conditions for 15 min. Flow rate was 1 mL min^{-1}. Oligosaccharides were detected at 206 nm and identified by comparison of their retention times with those of standard oligosaccharides injected previously. Sometimes, individual standards were injected together with the sample and the resulting chromatographic profile was compared with the native sample profile. Oligosaccharide quantification was carried out from the calibration curves of standard oligosaccharides using Millenium software (Waters) coupled to the HPLC system. All assays were carried out in triplicate.

Several sialyloligosaccharide standards from human milk and bovine colostrum of known concentration were injected to obtain calibration curves on the HPLC apparatus.

Sialyloligosaccharides were also separated by HPTLC on silicagel 60 plates using butanol/ ethanol/ water/ acetic acid/ pyridine 5:50:15:1.5:5 by vol.

Results

Tables 1 and 2 show respectively the general composition (protein, lipids and carbohydrates) and the glycoconjugate-bound sialic acid distribution [TSA, glycoprotein-bound-sialic acids (GBSA), oligosaccharide-bound sialic acids (OBSA), FSA, casein-bound sialic acids (CBSA) and LBSA] of the different MR.

Glycoprotein-bound sialic acid (Table 2) varied from 256.9 to 615.5 μg g^{-1}. MR-A had the highest content, probably due to its high protein content, including con-

Table 1. General composition of the different milk replacers (mg g^{-1} dry weight milk replacer)

Milk replacer	Protein	Lipids	Carbohydrates
A	829.4±15.9	108.1±4.4	79.1±4.6
B	220.7±2.0	190.8±4.4	493.7±27.9
C	254.9±5.2	165.8±7.2	367.8±5.6
D	228.4±1.5	148.8±4.7	422.3±9.7
E	302.3±1.5	205.8±3.8	517.7±9.8
F	206.5±3.1	206.8±6.9	409.1±37.0
G	190.3±11.7	201.2±20.5	461.0±13.7
H	178.7±15.1	210.0±30.4	497.1±24.8
I	169.1±17.8	242.2±1.9	493.1±27.4

Date are means ± SD of three different experiments

siderable amounts of immunoglobulins that contain sialic acids.

Oligosaccharide-bound sialic acid (Table 2) varied from 851 to 5,154 μg g^{-1}, MR-A having the highest content. This oligosaccharide fraction probably includes the κ-caseinoglycomacropeptide insoluble in 12% TCA and present in the whey derivatives from cheese manufacturing when this ingredient was used.

The CBSA content (Table 2) was also determined. MR-E, F and I formulated as "zero type" (without casein), had a very low (5 to 6%) CBSA content. Since those MR do not have casein, the sialic acids found in this fraction probably belong to contaminating proteins other than casein precipitated at pH 4.6. MR-A also had the highest content. It has been also detected a high content of FSA.

The sialic acid types present in the different fractions were also determined. NeuAc and NeuGc were detected in all fractions (Table 3). MR-D and H had a high NeuGc content in all fractions (15-40%). MR-E and F only had a high NeuGc content in the glycoprotein fraction. MR-C and D from the same commercial supplier had a very similar composition in all fractions. However, samples E and F, also from the same commercial supplier, had large differences in their TSA, GBSA and OBSA contents, even though the ingredients used by the supplier to formulate both MR were also very similar.

Five different sialyloligosaccharides -3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine and disialyllactose- were detected in the MR (Table 4). MR-A had a very low content in these oligosaccharides (254.6 μg g^{-1}); however, MR-B to I contained between 680 and 1,666 μg g^{-1}. The content of individual oligosaccharides was very similar among the MR belonging to the same supplier, -C and D, E and F, H and I but different from that of others. In general, the samples had high levels of 3'-sialyllactose and 6'-sialyllactose, the two sialylated oligosaccharides most abundant in bovine milk. 3'-sialyllactose predominated in MR-B and D, and 6'-sialyllactose in E, F and I. MR-G had the highest content (1,666 μg g^{-1}) and a different composition, with a very high content in 3'-sialyllactose, followed by an unusually high content in 6'-sialyllactosamine.

Finally, the individual ganglioside distribution of MR has also been determined (Table 5). Seven different gangliosides designated G1 to G7 according to their mobility (increasing polarity) were detected by HPTLC. They were identified by co-migration with authentic standards and analyzed by the HPTLC-overlay method using specific mAb (for ganglioside nomenclature see Martín *et al.,* 2003). Ganglioside G1 co-migrated with standard NeuAc-GM3 and reacted with mAb 14F7 (specific for NeuGc-containing GM3). The data suggest that G1 could be a mixture of NeuGc- and NeuAc-containing GM3. G2 reacted with the Jones anti-*O*-acetyl GD3 mAb. G2 was identified as *O*-acetyl GD3. G3 showed a mobility pattern identical to that of GD3 and reacted strongly with

Table 2. Glycoconjugate-bound sialic acid distribution of the different milk replacers (mg g^{-1} dry weight milk replacer)

Milk replacer	TSA[a]	GBSA[b]	OBSA[c]	FSA[d]	CBSA[e]	LBSA[f]
A	13,593.3±1251.4	6,151.5±86.0	5,154.3±107.5	2,038.9±160.6	1,045.1±35.0	41.4±2.2
B	3,395.7±176.9	1,108.8±18.8	1,640.9±85.9	510.7±17.5	300.5±8.7	38.2±2.5
C	1,498.7±58.9	595.6±30.0	621.1±17.9	230.8±9.5	306.0±15.1	5.1±0.2
D	1,512.0±91.0	511.0±16.5	777.0±21.8	205.6±12.4	383.3±7.6	5.3±0.4
E	5,198.3±348.8	659.6±26.1	3,504.5±94.9	675.8±45.3	22.8±7.3	15.1±1.9
F	2,458.3±110.8	335.8±28.5	1,541.7±37.7	356.4±17.2	16.9±1.3	18.5±1.5
G	3,465.4±102.7	654.5±30.7	2,126.2±48.1	537.1±17.9	32.4±1.8	3.7±0.0
H	1,970.8±114.4	621.0±32.3	923.0±31.4	315.3±13.7	379.7±3.1	4.1±0.0
I	2,119.4±25.6	256.9±15.1	1,273.1±95.4	349.7±20.6	15.5±1.9	ND[g]

[a] TSA, total sialic acids. [b] GBSA, glycoprotein-bound sialic acids. [c] OBSA, oligosaccharide-bound sialic acids. [d] FSA, free sialic acids. [e] CBSA, casein-bound sialic acids. [f] LBSA, lipid-bound sialic acid. Data are means ± SD of three different experiments. [g] ND, not determined

Table 3. NeuGc content of the different sialoglycoconjugate fractions of milk replacers. NeuGc content is expressed as percentage of the total sialic acid content (NeuAc + NeuGc) of the samples

	A	B	C	D	E	F	G	H	I
TSA[a]	3.3	11.2	11.4	34.5	8.4	12.9	17.5	20.6	18.4
GBSA	12.0	11.4	10.3	24.9	19.8	25.3	19.4	14.8	23.1
OBSA	2.4	3.3	11.3	40.0	6.2	10.9	15.6	20.9	20.4
CBSA	14.3	23.6	11.3	19.4	ND[b]	ND	ND	11.9	ND
LBSA	12.2	traces	traces	traces	traces	traces	17.2	17.1	18.6

[a] TSA, GBSA, OBSA, CBSA and LBSA, see Table 2. [b] ND, not determined. Data are means of two different experiments

R24, a mAb with a high degree of specificity against GD3. It was assumed to be GD3. Additionally, G3 showed a positive reaction with mAb P3, which is specific for NeuGc-containing gangliosides. Analysis suggests the presence of NeuGc- and NeuAc-containing GD3. G4 reacted with Jones mAb and A2B5 (specific for GT3 as well as for *O*-acetyl GT3) and was assumed to be *O*-acetyl GT3. G6 was located in the trisialoganglioside region and also reacted with the anti-A2B5 mAb and was identified as GT3. G5 and G7 were not identified. In previous works (Takamizawa *et al*., 1986; Martín *et al*., 2001b), they were tentatively designated on the basis of their mobility on HPTLC plates as a monosialo- and a trisialoganglioside sharing the same branched oligosaccharide chain. G3 (GD3) is the most abundant ganglioside, accounting for 45 to 79% of the total lipid-bound sialic acid content, followed by G6 (GT3). However, G1 (GM3) was not found, or only in trace amounts. The ganglioside profile of the samples suggests that bovine milk fat, but no ovine or caprine milk fat, was used in the formulation of the MR.

Discussion

MR are formulated to provide neonatal calves with a sufficient carbohydrate, protein and lipid content to support animal growth and health. In the past, they were mainly based on milk ingredients but the high prices of milk products are now leading to the use of other animal and vegetable sources to replace the most expensive components, mainly proteins. These include spray-dried animal plasma proteins, by-products of cheese manufacture, protein concentrates of oleaginous seeds, and blended dry fat (edible lard, tallow and grease and coconut and palm oils).

The values for protein, lipids and carbohydrates are very similar to those indicated by the suppliers with minor differences, probably due to the methodology used in the determinations. Proteins, carbohydrates and lipids are also in the range recommended for MR (BAMN, 1997), except for the MR-A, with a very high protein content, also probably due to its particular design: a colostrum substitute that includes a high amount of immunoglobulins. However, the carbohydrate (7.9%) and lipid (10.8%) contents seem to be low. Considering a colostrum intake of 3 L in the first feeding, a calf fed with MR-A would have a protein and lipid intake 20% and three-fold lower, respectively, and a carbohydrate intake three-fold higher than a calf fed maternal colostrum. In fact, MR-A is probably a colostrum supplement for use when the quantity or the quality of maternal colostrum is not adequate rather than a colostrum substitute. Nutrient ingestion was cal-

Table 4. Content of the five most abundant sialyl oligosaccharides found in milk replacers (μg g^{-1} dry weight milk replacer). Data are means ± SD of three different experiments

Sialyl oligosaccharide	A	B	C	D	E	F	G	H	I
3' SLN[a]	45.2±3.6	84.7±3.7	ND[b]	80.6±3.7	73.4±4.7	84.5±4.2	62.2±2.1	65.5±3.7	167.2±12.5
3' SL[c]	84.7±5.0	455.5±19.0	282.2±10.5	493.5±16.7	168.3±2.1	269.3±8.9	861.9±39.7	274.6±10.5	271.8±7.8
6' SLN[d]	ND	39.7±1.2	13.6±1.6	16.0±2.4	60.7±7.4	41.9±2.5	434.2±16.8	41.2±0.8	11.6±1.0
6' SL[e]	48.2±5.6	249.8±8.2	371.6±7.4	440.9±8.9	591.4±8.4	699.7±5.2	88.9±3.0	275.1±12.0	439.6±15.8
DSL[f]	76.5±3.0	191.0±6.8	13.0±2.3	59.6±0.9	343.6±7.9	313.3±9.6	218.8±12.8	67.8±4.9	145.7±4.8

[a] 3'-sialyllactosamine; [b] ND, not detectable; [c] 3'-sialyllactose; [d] 6'-sialyllactosamine; [e] 6'-sialyllactose; [f] disialyllactose.

Table 5. Distribution of individual gangliosides (ganglioside pattern) of milk replacers

Gangliosides	A	B	C	D	E	F	G	H
G1	traces	ND	ND	2.5±0.8	4.0±0.1	ND	traces	traces
G2	4.5±0.4	3.5±0.5	3.4±0.8	6.5±0.2	2.7±0.1	2.8±0.2	4.1±0.1	6.2±0.1
G3	78.5±0.6	62.6±2.9	43.9±7.1	57.0±1.3	45.1±1.7	53.0±0.3	58.0±0.1	62.4±0.6
G4	ND	2.8±0.8	10.9±3.4	5.3±0.7	6.2±0.2	5.2±0.6	2.6±0.1	3.2±0.1
G5	4.3±0.4	2.1±0.3	5.0±0.3	ND	1.4±0.2	2.2±0.9	1.8±0.1	1.2±0.1
G6	10.1±1.1	23.3±1.1	31.7±1.4	26.6±0.5	31.3±1.5	24.3±1.3	33.7±0.1	27.0±0.6
G7	4.6±1.9	5.7±1.8	9.5±1.5	2.2±1.1	9.4±0.3	12.5±2.3	ND	ND

Gangliosides were designated G1 to G7 according to their mobility on HPTLC plates. Mobility decrease from G1 to G7. Percentage of each ganglioside (expressed as lipid-bound sialic acid) in the total lipid-bound sialic acid content. Data are means ± SD of three different experiments. ND, not detectable

culated taking into consideration the MR feeding protocol recommended by the commercial suppliers and the recommendations of the Bovine Alliance Management Nutrition (3 L of colostrum in the first feeding, 4 L d^{-1} of milk in the first and second weeks of life and 3-4 L d^{-1} of milk in the third week) (BAMN, 1997). MR-B to I provide calves with more proteins (20 to 25%) and carbohydrates (40%) and a lower amount of lipids (15%) than the ingestion of mature milk.

However, MR are not formulated with regard to their sialic acid content. In this sense, it could be surmised that sialic acid intake by calves could be lower than in those fed maternal colostrum or milk. Surprisingly, the TSA content of the MR was high (1.5 to 13.5 mg g^{-1} MR), with the highest value for MR-A. When a calf was fed with MR-A it received five-fold more sialic acid than a calf fed maternal colostrum. When calves were fed MR-B to I after colostrum ingestion in the 1st week after birth, they received similar or slightly more sialic acids than calves fed maternal milk. The differential intake increased in the 2nd and 3rd weeks after birth, being five-fold higher in the latter.

The MR had more OBSA than GBSA, in contrast with bovine milk which has more GBSA than OBSA (Martín *et al*., 2001a). FSA are abundant in bovine (16% of TSA, Martín *et al*., 2001a) and ovine milk (15%, unpublished data) and are also probably present in derivatives from cheese manufacturing. Since bovine milk has only low levels of NeuGc (5 to 10%), it seems that those MR had been formulated using caprine or ovine milk derivatives, which have been demonstrated to contain high levels of NeuGc (unpublished data from our group). This is also supported by the sialic acid types found in the CBSA fraction. Since bovine casein does not contain NeuGc, the samples seem to have been formulated with ingredients containing ovine or caprine casein, which contains large amounts of this sialic acid (Alais and Jollès, 1961). It appears that the MR were formulated with whey derivatives or lactoserum from different animal sources (bovine, ovine, caprine) and at variable ratios.

The MR analyzed in this work point to an elevated sialic acid level in all the fractions studied, even though they were not formulated with regard to their sialic acid contents. However, the combination of the different ingredients used to prepare MR results in sialoglycoconjugate levels exceeding the initial amount found in mammalian milks. Whey-based raw materials are very rich in all milk glycoconjugates and oligosaccharides and increased use of such materials will result in high contents of these micronutrients in MR. Furthermore, since it has been proposed (Gustafsson *et al*., 2005) that glycoproteins or glycolipids expressing multiple sets of specific carbohydrate epitopes could be more protective against pathogens than the same carbohydrate epitope expressed in a single oligosaccharide, MR should probably be formulated to include high contents of GBSA rather than of OBSA.

The biological significance of milk sialic acid-containing glycoconjugates remains unclear. It has been suggested that they could influence the functional development of the neonatal intestine, the absorption of nutrients, and the postnatal development of the intestinal immune system (gut-associated lymphoid system, GALT) and also that they could exert a defensive role, acting as false soluble receptors for microorganisms and microbial toxins (Dai *et al*., 2000).

The binding of several microorganisms (bacteria and viruses) to different sialoglycoconjugates has been described (Newburg, 1999; Dai *et al*., 2000). It has also been demonstrated that milk glycoconjugates inhibit bacterial adhesion to erythrocytes and other cellular sur-

faces and that specific glycoconjugates inhibit specific pathogens (Karlsson, 1998; Dai *et al*., 2000). NeuGc-GM3 and other NeuGc-containing gangliosides have been reported to be the main receptors for *Escherichia coli* K99 adhesins in the intestinal epithelium of calves (Teneberg *et al*., 1994). K99 is the most common fimbria expressed in enterotoxigenic *E. coli* strains isolated from calves. Since MR have large amounts of NeuGc-containing glycoconjugates, including gangliosides, calves fed these MR could benefit from the protective effect of these compounds against *E. coli* K99. Martín *et al.* (2002 and 2003) reported the binding of bovine milk gangliosides and sialyloligosaccharides to enterotoxigenic *E. coli* strains isolated from calves. Bovine κ-caseinoglycomacropeptide has been found to bind and protect from *Vibrio cholerae* and *E. coli* enterotoxins; to inhibit bacterial and viral adhesion to their hosts; to promote bifidobacterial growth, and to modulate the immune system response (Brody, 2000). These beneficial effects could be extrapolated to ovine or caprine κ-caseinoglycomacropeptide, mainly taking into account that they also have *N*-acetylgalactosamine, galactose and NeuAc, but also high amounts of NeuGc (Alais and Jollès, 1961). In this sense, a successful treatment of experimental colibacillosis mediated by *E. coli* K99 and F41 in calves with glycoprotein glycans from bovine and porcine plasma has already been reported (Mouricout *et al*., 1990; Nollet *et al*., 1999). The sialic acid moiety, mainly NeuGc, seems to be critical in the process. Thus the ingestion or administration of sialoglycoconjugates protect human and animals from infection by microorganisms, and indeed some of the abovementioned sialoglycoconjugates were found in the MR studied here.

Even though the MR studied here were not formulated as regards their micronutrient content but instead on the basis of the content of fat, protein and carbohydrates (macronutrients), a high glycoconjugate-bound sialic acid content has been found in all fractions studied. This means that calves fed these MR have sialic acid intakes higher than those fed maternal milk or colostrum. Furthermore, a high relative proportion of these sialic acids is NeuGc, which has been described as the specific receptor for *E. coli* K99, one of the most common pathogens causing diarrhea in neonatal calves. The ingestion of large amounts of NeuGc may be beneficial for the healthy growth of calves.

MR also contain several sialyloligosaccharides, namely 3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine and disialyllactose, previously reported in bovine milk that could also contribute to active defense against pathogens, acting as false receptors for microorganisms and their toxins.

In addition, the results might induce the dairy food industries to formulate their milk replacers taking into account not only the costs of ingredients but also their sialoglycoconjugate content.

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