Bovine Milk Gangliosides: Changes in Ceramide Moiety with Stage of Lactation

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ABSTRACT: The stage of lactation is one of the most important factors that influence milk composition. Changes in fatty acids from triacylglycerols and phospholipids have already been reported. In this study, we looked for a lactational change in the ganglioside lipid moiety since ganglioside contents and patterns vary strongly with stage of lactation. Individual gangliosides from four stages were isolated, methanolyzed to cleave the bonds between individual constituents, and derivatized for gas–liquid chromatography and gas chromatography/mass spectrometry analyses. Ceramide components, both fatty acids (as methyl esters derivatives) and long-chain bases, were identified and quantified. The results pointed to a marked change in ceramide from colostrum to milk that was characterized by a dramatic decrease in saturated and the longest-chain fatty acids as well as an increase in 18:1 and 18:2. The major long-chain base along lactation was a recently described structure, 3-ethoxy-15:0 sphinganine. Other new long-chain base structures appeared in these gangliosides. All these changes suggest differences in the fluidity of the fat globule membrane, reflecting physiological variations in cows with respect to milk production.

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Gangliosides are important constituents of neural membranes, and they also appear in extraneural tissues and body fluids. In milk, they are localized in the milk fat globule membrane (MFGM), where they are assumed to play a role in the defense of the newborn against infection (1). Ganglioside GM1 (GM ganglioside species have one sialic acid, GD have two sialic acids; see Ref. 2) binds cholera toxin although the ability to bind other ligands such as viruses, bacteria and hormones has also been reported (2). These would act as false ligands in the gut, blocking the pathogenic agents and their enterotoxins and preventing newborns from contracting possible infections.

The composition of milk fat is strongly influenced by physiological, seasonal, and nutritional factors (3). The ganglioside content varies with stage of lactation, being very high in colostrum and decreasing in ensuing stages. Nevertheless, the main gangliosides in bovine milk are always GD3 (>60%) and GM3. Several changes in their concentrations with stage of lactation have been reported (4), but until now no reports about changes in the ceramide moiety have been made.

The stage of lactation mainly affects the content of short-chain fatty acids (SCFA) of milk, since their proportions are very low early in lactation and increase over the following weeks. At the same time, the uptake of long-chain fatty acids (LCFA) from adipose stores decreases. A negative correlation between the proportions of several SCFA (C_6–C_{14}) and unsaturated C_{18} compounds has been reported (5).

A theoretical dual origin for milk fatty acids (FA) has been proposed (3): SCFA would be synthesized in the mammary gland from plasma acetate or β-hydroxybutyrate while LCFA would derive directly from circulating lipoproteins. C_{16} FA could have both origins.

Plasma FA have two sources: dietary lipids and those stored in adipose tissue. In nonruminant animals, circulating FA reflect the composition of the food ingested, but in the rumen of cattle, metabolism must be considered. Rumen microorganisms degrade food polysaccharides to produce some volatile FA and metabolic intermediates, such as propionate, butyrate or isobutyrate, which are used to synthesize their own odd-numbered or branched-chain FA. Moreover, rumen bacteria carry out extensive hydrogenation of C_{18} polyunsaturated fatty acids (PUFA), yielding high amounts of 18:0 (6,7). Thus, the true uptake by cows consists mainly of saturated LCFA and small amounts of PUFA, although in general ruminants do not seem to suffer from any lack of essential FA (8). An extensive study of changes in triacylglycerol FA from bovine milk throughout the course of lactation (40 wk) has been carried out (9).

In the present work we determined the possible influence of lactation stage on the ceramide composition of gangliosides. Four different and particularly important stages in lactation were chosen: the two-day colostrum, milk from the midlactation stage (mature milk), and an earlier stage with intermediate features between colostrum and mature milk, known as transitional milk. Finally, we considered late lacta-

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Abbreviations: BSA, bovine serum albumin; C, chloroform; FA, fatty acids; FAME, fatty acid methyl esters; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; GSL, glycosphingolipids; HFBAA, heptafluorobutyric anhydride; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; IgM, immunoglobulin M; LCB, long-chain bases; LCFA, long-chain fatty acids; M, methanol; mAb, monoclonal antibody; MFGM, milk fat globule membrane; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; PIBM, poly(isobutyl methacrylate); PUFA, polyunsaturated fatty acids; RT, retention time; SCFA, short-chain fatty acids; TLC, thin-layer chromatography; VLCFA, very long chain fatty acids.

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tion milk, just before the dry period, when the cow is pregnant again. We obtained gangliosides from all these samples and studied the ceramide components following a recently proposed method (10).

EXPERIMENTAL PROCEDURES

Samples. Four Spanish-Brown cows were used in this study. Calving took place in December–January. A 300-mL milk sample from each animal was obtained on postpartum day 2 (colostrum), day 15 (transitional milk), day 90 (mature milk), and in the tenth month (late lactation milk). Samples were always obtained from the morning milking, frozen at −20ºC, lyophilized, and homogenized to ensure accurate distribution of the components.

Chemicals. Heptafluorobutyric anhydride (HFBAA) was from Fluka (Buchs, Switzerland) and high-performance liquid chromatography (HPLC)-grade acetonitrile from SDS (Peypin, France). Anhydrous acetonitrile was obtained by addition of calcinated calcium chloride (Prolabo, Paris, France), followed by storage in a closed vessel. The methanolysis reagent was obtained by dissolving anhydrous gaseous HCl (up to 0.5 M) at −50ºC in anhydrous methanol (M) previously redistilled on magnesium turnings. The 25QC3/BP1 column was from SGE trance SARL (Villeneuve St. Georges, France) and the CP-Sil5 CB capillary column was from Chrompak France (Les Ullis, France).

Monoclonal antibodies P3, 14F7, R24, and G1 were a kind gift from Dr. Ana María Vázquez (Centro de Inmunología Molecular, Havana, Cuba). Anti-A2B5 [mouse immunoglobulin M (IgM)] was provided by Roche Molecular Biochemicals (Mannheim, Germany). Anti-9-*O*-acetyl-GD3 (clone JONES, mouse IgG), mouse polyvalent Ig G-, Ig M-, Ig A-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). Poly(isobutyl methacrylate) (PIBM) was from Aldrich Chemical Company (Milwaukee, WI), and *n*-hexane was from Merck (Darmstadt, Germany). Albumin from bovine serum (BSA) was provided by Fluka.

Extraction of gangliosides. Gangliosides were isolated as previously described (4). Briefly, lyophilized milk was homogenized twice with 10 vol of cold acetone (−20ºC) to remove neutral lipids and filtered. The solid residue was successively extracted with 10 vol of chloroform/methanol (C/M) (2:1, 1:2, and 1:1, vol/vol). The combined extracts were evaporated to dryness, taken up in 10 vol of C/M (2:1), and subjected to a Folch partition (4). The upper phases, containing crude gangliosides, were combined and dialyzed against distilled water (volume ratio of sample to dialysate, 1:1000) at 4ºC for 2 d. Water was changed every 6–8 h. After dialysis, the material was lyophilized and dissolved in C/M (2:1).

The ganglioside content was quantified as lipid-bound sialic acids by the resorcinol assay (11). Gangliosides were separated by high-performance thin-layer chromatography (HPTLC) using solvent system A: C/M/0.2% $CaCl_2$ (5:4.5:1, by vol). Individual gangliosides (ganglioside pattern) were analyzed with a dual-wavelength thin-layer chromatography (TLC) densitometer (Shimadzu CS 9000; Kyoto, Japan) after separation by HPTLC. Gangliosides were visualized by spraying the plates with the resorcinol and orcinol reagents.

For the gas chromatography (GC) and GC–mass spectrometry (GC–MS) assays, individual gangliosides were purified by preparative TLC using solvent system B: methyl acetate/C/M/*n*-propanol/0.25% KCl, (25:20:20:20:17, by vol) (12).

Methanolysis and acylation. Ganglioside samples were dried under a nitrogen stream. Methanolysis reagent (300 µL) was added, and closed tubes were incubated for 20 h at 80ºC. Samples were evaporated to dryness under a nitrogen stream and derivatized with 25 µL of HFBAA and 200 µL of acetonitrile for 30 min at 100ºC. After cooling to room temperature, samples were dried and dissolved in an appropriate volume of anhydrous acetonitrile.

GC. Each HFBAA derivative was injected into a Shimadzu GC-14A gas chromatograph equipped with a Ross injector and a 25-m capillary column (25QC3/BP1, 0.5-mm film phase). The injector and flame-ionization detector temperatures were 260ºC. The temperature program was 1.2ºC/min from 100 to 140ºC, and then 4ºC/min up to 240ºC. This temperature was maintained for 10 min. The carrier gas (helium) pressure was 0.8 bar. This program allowed a good separation of monosaccharides into their different isomers; it has been used to identify each ganglioside before the GC–MS analyses (10).

*GC–MS analyses.*The GC separation was performed on a Carlo Erba GC 8000 gas chromatograph (Milan, Italy) equipped with a 60 m × 0.32 mm CP-Sil5 CB low-bleed/MS capillary column, 0.25 mm film phase. The temperature of the Ross injector was 280ºC, and the temperature program was as follows: 90ºC for 3 min, then 5ºC/min up to 260ºC. The temperature was held at 260ºC for purposes of cleaning. This column was coupled to a Finnigan Automass II mass spectrometer (mass limit 1000). Analyses were performed in the electron impact mode (ionization energy 70eV; source temperature 150ºC) (10).

Immunostaining assay. Immunostaining on HPTLC plates was performed as previously reported (13). Gangliosides from each stage of lactation were chromatographed with solvent system A. Once dried, the plates were soaked with 0.1% PIBM in *n*-hexane for 75 s and kept overnight at room temperature for drying. The plates were blocked with 1% BSA in Tris-0.1 N HCl for 30 min. They were then incubated with each monoclonal antibody (mAb) at room temperature for 2 h. After washing with phosphate-buffered saline (PBS), plates were incubated with conjugated biotin (1:2000 in 1% BSA in PBS) for 1 h 30 min, and then with streptavidin-alkaline phosphatase (1:1000 in the same buffer) for 1 h 30 min before developing with the substrate.

Statistical assays. In order to find statistically significant differences among the four stages of lactation, an analysis of variance test was applied in each case.

RESULTS

Ganglioside content. The ganglioside contents at each stage of lactation, expressed as lipid-bounded sialic acids [mg/kg of milk, expressed as means ± SD (n = 4 cows); three replicates were made per cow and sample time], were as follows: colostrum, 3.5 ± 1.7; transitional milk, 1.2 ± 0.5*; mature milk, 0.9 ± 0.4*; and late lactation milk, 1.8 ± 1.4. The asterisks indicate statistically significant differences (P < 0.05) that were found between colostrum and transitional and mature milk but not between colostrum and late lactation milk nor among the other stages. No statistically significant differences among cows were found. The ganglioside content was high in colostrum but then slowly decreased until day 90, after which it increased again in late lactation milk.

Several gangliosides were separated on HPTLC (solvent system A) as resorcinol- and orcinol-positive spots and named G1 to G6 according to their mobility (increasing polarity). They were identified by co-migration with authentic standards and the ganglioside pattern previously found in cow's milk (4). Gangliosides were also analyzed by the HPTLC-overlay method, using specific mAb.

Ganglioside G1 co-migrated with standard GM3. G1 reacts with mAb 14F7 [specific for *N*-glycolylneuraminic acid (NeuGc)-containing GM3]. After mild hydrolysis (formic acid, pH 2) of the ganglioside, both *N*-acetylneuraminic acid (NeuAc) and NeuGc were found as sialic acid moieties by TLC. These data suggest that G1 is a mixture of NeuGc- and NeuAc-containing GM3. With TLC, G2 was found in the monosialoganglioside region, and it reacted with the JONES anti-*O*-acetyl GD3 mAb. This mAb only detected one band in colostrum and transitional milk but two bands in mature and late lactation milk. G2 was identified as *O*-acetyl GD3. Only traces of G2 were found in the first stages of lactation, but a clear increase was detected as lactation progressed.

G3 showed a mobility pattern identical to that of GD3. R24, a mAb with a high degree of specificity against GD3, reacted strongly with G3. The structure of G3 was therefore assumed to be GD3. Additionally, NeuAc and NeuGc were detected in the TLC analyses after hydrolysis, suggesting the presence of NeuGc- and NeuAc-containing GD3. G1 and G3 also showed a positive reaction with mAb P3, which is specific for NeuGc-containing gangliosides. The staining was very prominent in colostrum and weaker in the other stages, suggesting that the content of NeuGc in gangliosides decreases during the course of lactation.

G5 was located in the trisialoganglioside region of the chromatogram. G5 also reacted with the anti-A2B5 mAb (specific for GT3 as well as for *O*-acetyl GT3). According to this, G5 was identified as GT3. G4 and G6 were tentatively designated on the basis of their mobility on TLC plates and previous data (14). G4 could be a monosialoganglioside with a branched oligosaccharide chain, and G6 might be a trisialoganglioside with the same branched oligosaccharide chain. A specific mAb against GM1, called G1, was also used. No reaction was found in any stage of lactation, pointing to the notion that cow's milk does not contain GM1.

Ceramide content. Individual gangliosides were separated by TLC (solvent system B) and analyzed by GC–MS. FA and long-chain bases (LCB) of the different individual gangliosides were determined (data not shown). Table 1 shows the FA average content of gangliosides from the different stages. Values are means of the FA content of the different individual

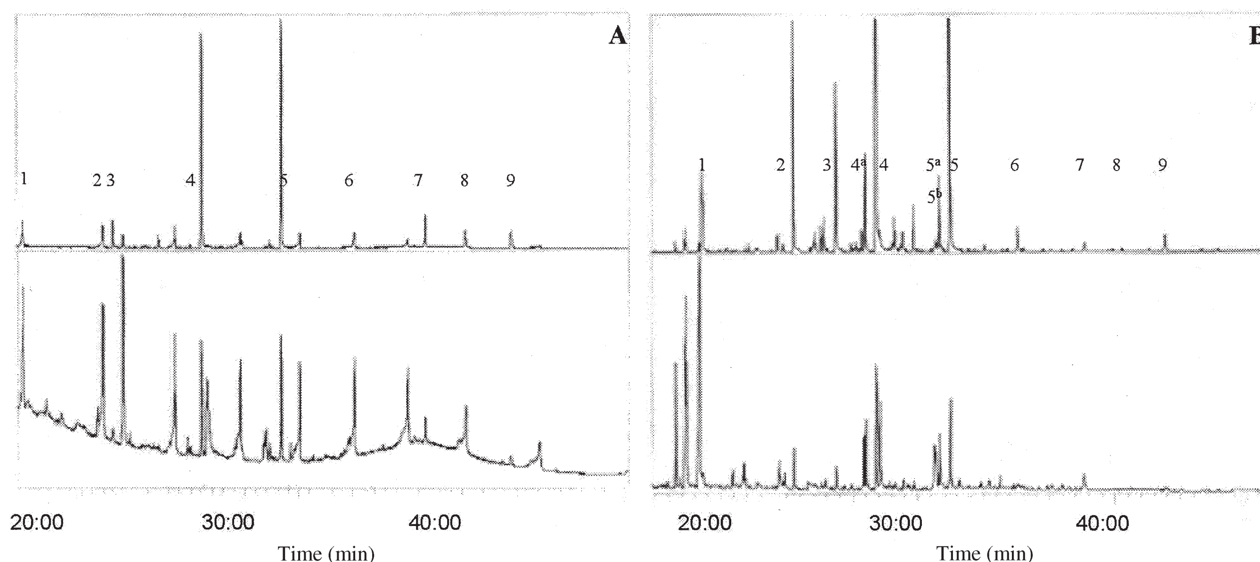

FIG. 1. Gas chromatogram of fatty acid methyl ester content of colostral (A) and transitional (B) gangliosides. Samples were methanolyzed (20 h at 80°C) and derivitized with heptafluorobutyric anhydride (30 min at 100°C). A Carlo Erba GC 8000 gas chromatograph coupled to a Finnigan Automass II mass spectrometer was used. The gas chromatograph was equipped with a 60 m × 0.32 mm CP-Sil5CB low-bleed/MS capillary column. Peak 1, 12:0; 2, 14:0; 3, 15:0; 4, 16:0; 4a, 16:1; 5, 18:0; 5a, 18:1; 5b, 18:2; 6, 20:0; 7, 22:0; 8, 23:0; 9, 24:0.

TABLE 1
Content[a] (%) of Fatty Acids and Long-Chain Bases (LCB) of Gangliosides from the Different Stages of Lactation

Fatty acids	Colostrum	Transitional milk	Mature milk	Late lactation milk
12:0	2.46 ± 4.3	1.08 ± 1.4	0.75 ± 0.7	1.03 ± 1.6
13:1	—	0.01 ± 0.02	—	—
13:0	—	0.43 ± 0.7	0.06 ± 0.1	—
14:1	—	0.35 ± 0.3	0.07 ± 0.1	—
14:0	6.86 ± 2.4	6.88 ± 1.5	6.21 ± 1.7	4.37 ± 2.5
15:1	—	0.09 ± 0.2	—	—
15:0[b]	3.36 ± 2.2	5.54 ± 2.3	3.63 ± 1.3	2.43 ± 1.4
16:1	0.73 ± 1.4	9.01 ± 2.1	4.08 ± 3.2	2.82 ± 2.6
16:0	37.94 ± 4.3	26.61 ± 3.5	35.59 ± 6.4	45.26 ± 17.7
17:1	—	0.60 ± 1.05	—	—
17:0[b]	1.98 ± 2.5	3.49 ± 1.9	2.45 ± 0.6	2.74 ± 2.1
18:2	—	10.00 ± 4.6	3.54 ± 2.6	6.17 ± 4.4
18:1	1.32 ± 2.8	11.56 ± 1.2	14.12 ± 3.6	8.04 ± 8.9
18:0	27.92 ± 6.8	18.46 ± 3.1	22.59 ± 2.8	21.96 ± 5.2
19:0	0.09 ± 0.2	0.17 ± 0.2	0.03 ± 0.05	0.07 ± 0.1
20:0	—	1.12 ± 1.1	1.13 ± 1.3	0.41 ± 0.4
21:0	—	0.48 ± 0.6	—	—
22:1	0.91 ± 2.2	—	—	—
22:0	15.03 ± 7.1	2.65 ± 1.9	3.81 ± 1.6	2.88 ± 3.7
23:0	—	0.11 ± 0.2	0.34 ± 0.4	0.04 ± 0.06
24:1	—	—	0.57 ± 1.1	—
24:0	1.39 ± 2.1	0.93 ± 0.2	0.91 ± 0.6	1.59 ± 1.8
25:0	—	0.36 ± 0.3	0.14 ± 0.2	0.10 ± 0.1
26:0	—	0.07 ± 0.1	—	0.09 ± 0.2
Long-chain bases				
8-Me-3-*O*-ethoxy-C_{14} spha	3.36 ± 3.9	5.40 ± 1.9	4.36 ± 2.4	2.96 ± 0.6
3-*O*-Ethoxy-C_{15} spha	62.82 ± 10.3	53.20 ± 12.0	43.57 ± 2.59	65.25 ± 9.6
3-*O*-Ethoxy-C_{17} spha	19.07 ± 9.5	19.45 ± 11.2	19.75 ± 7.7	13.47 ± 6.6
C_{18} sphingosine	9.53 ± 4.4	19.22 ± 4.3	31.35 ± 5.3	16.33 ± 6.9
C_{20} phytosphingosine	5.22 ± 1.1	2.73 ± 1.8	0.98 ± 0.25	1.99 ± 2.5

[a]Values, expressed as mean ± standard deviation, are means of the fatty acid and LCB contents of the different gangliosides from each stage of lactation.
[b]Including the branched isomers. Spha, sphinganine.

gangliosides from each stage of lactation. FA were identified as fatty acid methyl esters (FAME) by the typical ions at *m/z* 74 and *m/z* 87 (Fig. 1). Each FAME could be identified by its retention time (RT) as well as by its molecular ion.

Several FA (C_{12}–C_{26}) were identified by MS analysis, including odd-numbered chains. No FA below C_{12} was detected, although these are very common in milk fat (triacylglycerols). The most abundant were C_{16} and C_{18}, whose proportions were over 65% of total saturated FA in gangliosides. The FA 22:0 was well represented in colostrum, but its percentage was below 4% in the other stages.

Monounsaturated FA—from 13:1 to 24:1—were also detected. Palmitoleic (16:1) and oleic (18:1) acids represented 69.9, 64.47, 81.32, and 63.7% of the total unsaturated FA from colostral, transitional, mature, and late lactation gangliosides, respectively. The only PUFA found was 18:2.

The saturated FA content in gangliosides changed over the course of lactation. Significant differences were found between colostrum and transitional ($P < 0.01$), mature ($P < 0.01$), and late lactation ($P < 0.05$) milk. Statistical assays revealed no differences among the last three stages, although a tendency to increase was observed, as seen in Figure 2. C_{16}

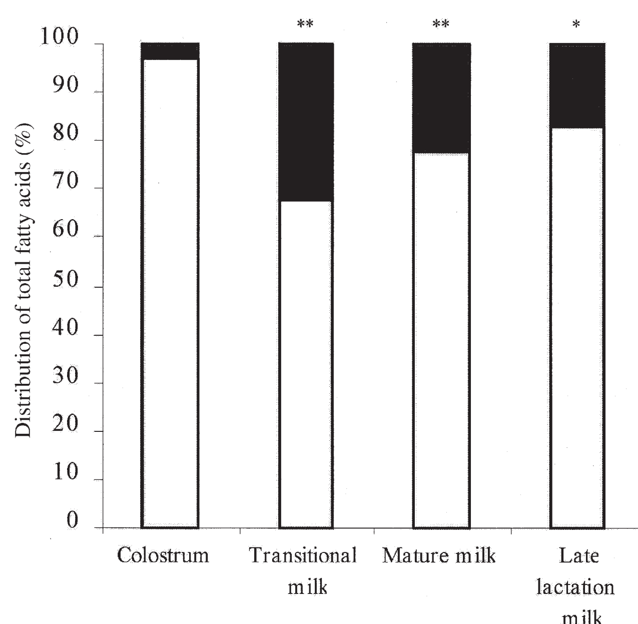

FIG. 2. Distribution of saturated (□) and unsaturated (■) fatty acids. Statistically significant differences from colostrum are shown: *$P < 0.05$, **$P < 0.01$.

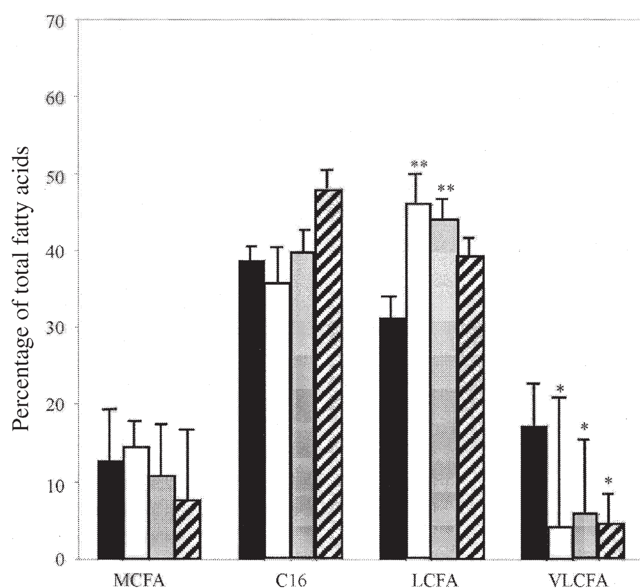

FIG. 3. Lactational variation of fatty acids: medium-chain fatty acids (MCFA, C_{12}–C_{15}), C_{16} (includes 16:0 and 16:1), long-chain fatty acids (LCFA, C_{17}–C_{21}), and very long chain fatty acids (VLCFA, C_{22}–C_{26}). Statistically significant differences from colostrum are shown: $*P < 0.05$, $**P < 0.01$). Closed bar, colostrum; open bar, transitional milk; shaded bar, mature milk; striped bar, late lactational milk.

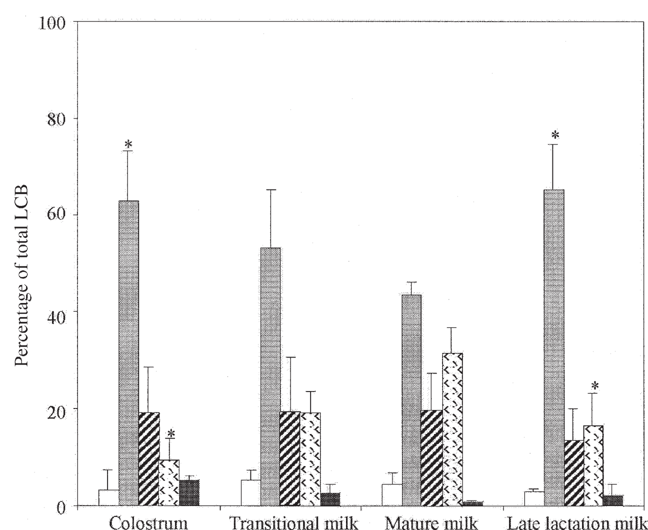

FIG. 4. Lactational variation of long-chain bases (LCB). Statistically significant differences from mature milk ($*P < 0.01$) are shown. Open bar, 9-Me-3-*O*-ethoxy-C_{14} sphinganine; shaded bar, 3-ethoxy-C_{15} sphinganine; striped bar, 3-ethoxy-C_{17} sphinganine; patterned bar, 18:1 sphingosine; closed bar, 20:0 phytosphingosine.

was the most abundant FA in all gangliosides for each stage, followed by 18:0.

Very long chain fatty acids (longer than C_{20}, VLCFA) decreased over the course of lactation, since in colostrum the proportion of C_{18} FA was twice that of VLCFA but 10-fold higher in the rest of the stages (Fig. 3). A similar trend was found for C_{16}. In fact, the sum of C_{16} and C_{18} increased during the course of lactation from 67.9% in colostrum to 84.2% in the tenth month postpartum. The most radical changes occurred in the first 2 wk after calving: 22:0 decreased dramatically from 15% (on day 2) to 2.6% (on day 15) while unsaturated C_{16} and C_{18} increased to the same extent (palmitoleic, 0.73 to 9.01%; oleic, 1.32 to 11.56%).

The acids 15:0 and 17:0 were the only branched-chain FA detected in our analyses. They never represented more than 1.5% of the total, and they did not appear in all gangliosides. The branched C_{15} was characterized by the molecular ion at *m/z* 256 (corresponding to the C_{15} FAME) and by an ion at M − 29, indicating that it belonged to the anteiso series. The branched 17:0, whose molecular ion appeared at *m/z* 284, was also an anteiso branched FA.

Taking into consideration the three most abundant gangliosides (GM3, GD3, and GT3), we observed a within-group variability of certain FA: in colostrum, 18:0 varied from 38.1 (in GM3) to 18.8% (in GD3), and 22:0 represented 8.6% of the total in GT3 but represented 27.9% of FA in GD3. Transitional gangliosides were more homogeneous, like those of mature milk, although linoleic acid represented 4.68 and 16.13% of FA from transitional GD3 and GT3, respectively, and palmitic acid varied from 29.9 (in GD3) to 42.2% (in GT3) in mature milk. The most extreme values were found in 16:0 from late lactation gangliosides: from 25.2% in GD3 to 69.4% in GT3, and oleic acid represented 21.6% of total FA from GD3 although it was not detected in the most polar gangliosides.

By GC–MS, LCB were separated as heptafluorobutyric derivatives. In the electron impact mode, sphingosines (monounsaturated and dihydroxylated bases) gave a typical fragment ion at *m/z* 290, allowing unambiguous identification of two isomers at RT of 1.001 and 1.010 (relative to 18:0). C_{20} phytosphingosine was found at RT 1.100. Three new LCB, recently reported (15), were predominant in milk gangliosides (Fig. 4). They gave a typical ion at *m/z* 510 corresponding to the substituted first three carbons. The most abundant one is 3-ethoxy-C_{15} sphinganine, whose spectrum is shown in Fig-

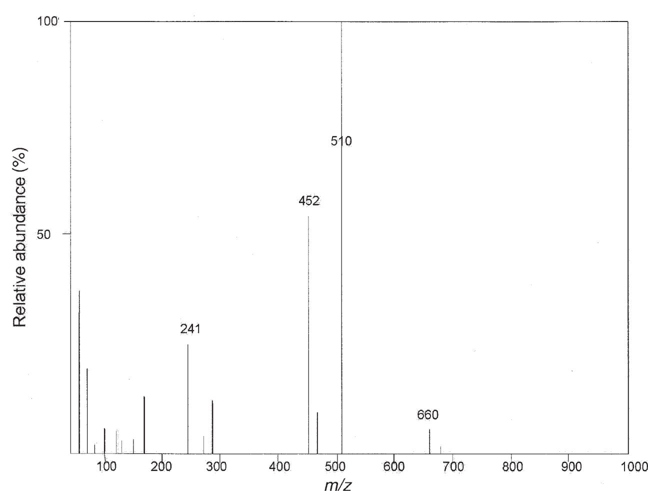

FIG. 5. Electron impact spectrum showing the characteristic ion sequence of 3-ethoxy-C_{15} sphinganine.

ure 5. Table 1 also shows the LCB average content of gangliosides from the different stages of lactation. Values are means of the LCB content of the different individual gangliosides from each stage of lactation.

DISCUSSION

Lipids are the most variable component in milk. The most abundant lipids are triacylglycerols, representing 97–98% of the total, followed by phospholipids and cholesterol. Glycosphingolipids (GSL) represent only 0.6% and are localized in the MFGM, although a small fraction of them is dispersed in the aqueous phase. Glucosyl- and lactosyl-ceramide are the main GSL in bovine milk (16), and GD3 is the most abundant ganglioside.

Our results are in agreement with a previous report demonstrating a decrease in the ganglioside content of the milk as lactation progresses (4). Nonstatistically significant variations among individual cows were observed. The individual ganglioside content (ganglioside pattern) revealed six different gangliosides. G1, G3 and G5 were identified as GM3, GD3, and GT3, respectively, and they account for 63–83% of total gangliosides depending on the stage of lactation. Several authors have also reported that about 80–90% of the gangliosides in bovine milk (expressed as lipid-bound sialic acid) consist of hemato-series gangliosides, such as GM3, GD3, and GT3. G4 and G6 were designated as a monosialo- and a trisialoganglioside, respectively, with a branched oligosaccharide chain, as previously reported (14). Since no specific monoclonal antibodies are available against these two gangliosides and since the amount of the latter was insufficient to determine their structure, their identification remains unclear. G2 was identified as *O*-acetyl GD3. We detected one band in colostrum and transitional milk but two bands in mature and late lactation milk. By using TLC, two bands in the region between GM3 and GM1 corresponding to alkali-labile gangliosides and reacting with JONES were observed in buttermilk (17). These were identified as 7,9-*O*-diacetyl GD3 (higher mobility) and a mixture of 7- and 9-*O*-acetyl GD3 (lower mobility). These data suggest that our G2 ganglioside could be a mixture of *O*-acetylated derivatives of ganglioside GD3.

We also detected several NeuGc-containing gangliosides (G1 and G3) by the HPTLC-overlay method. The staining was very strong in colostrum but weaker in the other stages, suggesting that the content of NeuGc in milk gangliosides decreases as lactation progresses. These data are consistent with other observations previously reported by our group (18). We did not detected ganglioside GM1 in bovine milk. This result is in contrast with a previous study reporting the presence of GM1 in bovine milk (19). However, our results are in agreement with the individual ganglioside content of milk and the biosynthetic pathways of these gangliosides. Milk gangliosides mainly consist of hemato-series gangliosides (GM3, GD3, and GT3). Other minor gangliosides, such as *O*-acetyl GD3 and *O*-acetyl GT3, share the same biosynthetic pathway, and there are also monosialo- and trisialogangliosides with a branched chain. Most researchers have failed to detect any ganglioside of the classical a-pathway of ganglioside synthesis in milk. Thus, the presence of GM1 in cow's milk would be unlikely. If it were present, this would in trace amounts, below the limit of detection by TLC immunostaining.

Regarding the ceramide content, it was found that MFGM gangliosides were richer in VLCFA (47.4%) than in 16:0 (25.6%) (20). Nevertheless, Palmquist and Schanbacher (21) found lower amounts of VLCFA (3.7%) than 16:0 (22.6%) in MFGM lipids. Except for the colostrum, our results are in agreement with the latter authors. According to several investigators (3), the proportion of the longest FA ($>C_{16}$) decreases as the mammary gland assumes responsibility for the major part of FA synthesis and as uptake from plasma ceases. Since MFGM comes from mammary gland cells, changes in the FA content of MFGM should reflect changes in mammary gland metabolism. Although we found no differences in the global content of LCFA, which persisted at around 45–50% throughout lactation, a marked decrease in the amounts of VLCFA was detected in the postcolostrum stages. Studying lactational changes in several phospholipids, some authors (22) have reported that 40–50% of sphingomyelin moieties have FA chains longer than C_{20} (VLCFA) whereas other workers (23) have described the same characteristic in MFGM cerebrosides. However, these latter authors also showed that skim milk cerebrosides are richer in C_{16} than in VLCFA. All these differences could be due to the influence of dietary fat intake (21).

The presence of branched-chain FA in milk lipids has been reported (24). These branched-chain FA would be of ruminal origin, since this kind of FA constitutes more than 20% of the total in rumen bacteria and plays an important role in membrane fluidity (25). The same source has been reported for odd-numbered FA.

Although there was an increase in the C_{18} sphingosine percentage in mature milk, the 3-ethoxy-C_{15} sphinganine predominated throughout lactation. This LCB has never been reported in milk gangliosides, nor has 3-ethoxy-C_{17} sphinganine and 9-methyl-3-ethoxy-C_{14} sphinganine. Nevertheless, C_{18} sphingosine had been reported as the most abundant LCB in previous studies (14,26). Since those results had been obtained by GC, differences are probably due to the methodology employed.

To determine the biological significance of these changes in ceramide, membrane fluidity must be addressed. Changing both FA and LCB could control the fluidity of the membrane. In colostrum, the presence of high amounts of VLCFA and saturated FA would suggest a thick structure. In other stages of lactation the increase of unsaturated FA content points to a more fluid membrane. The increase of the length of LCB leads to a larger hydrophobic volume and affects the membrane organization (27). Gangliosides with longer LCB contribute to membrane thickening. Thus, the physiological changes in FA synthesis would be balanced by the proportions of the longest LCB.

The role of milk gangliosides in the protection of newborns against infection has been discussed for a long time (2). Milk

gangliosides could act as alternative receptor analogs for bacteria and/or toxins in the gut, preventing bacterial infection. The orientation of the carbohydrate moiety of membrane gangliosides may be influenced by the composition of ceramide. Furthermore, the ceramide moiety is essential in the lateral phase separation of gangliosides leading to the formation of ganglioside-enriched microdomains. This feature could be important in the presentation and/or accessibility of the ganglioside binding site (epitope) (28). Specific ceramide moieties involved in *Escherichia coli* binding have been reported. NeuGc-GM3 with highly hydroxylated ceramide was found to bind *E. coli* K99 more strongly than nonhydroxylated forms (29). Adhesion is mediated by electrostatic interactions between the bacterial adhesin and the carboxyl group of sialic acid (30). Changes in the position of this group could lead to variations in the adhesion properties, modulating the inhibitory activity of gangliosides.

A detailed knowledge of the ceramide composition of milk gangliosides and their interactions with bacteria could be useful to develop synthetic analogs, possibly easier to obtain than natural compounds.

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