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Effect of Simulated Gastrointestinal Digestion on Sialic Acid and Gangliosides Present in Human Milk and Infant Formulas

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ABSTRACT: The effects of simulated gastrointestinal digestion upon sialic acid and gangliosides in infant and follow-on formulas and human milk, as well as their bioaccessibility, have been evaluated. The gastric stage is the step that causes a greater decrease in sialic acid and ganglioside contents. The intestinal stage only decreases the total and individual contents of gangliosides. After gastrointestinal digestion, neither sialic acid nor gangliosides were found in the nonbioaccessible fraction. The highest bioaccessibility (100 × content in soluble fraction after gastrointestinal digestion/total content) of sialic acid is found in human milk (87%), followed by infant formula (77%) and follow-on formula (16%). In the case of gangliosides, the highest bioaccessibility is present in the follow-on formula (51%), followed by human milk (29%) and infant formula (5%).

KEYWORDS: Sialic acid, gangliosides, bioavailabity, bioaccessibility, in vitro gastrointestinal digestion, infant formula, human milk

INTRODUCTION

Sialic acid (Sia) and gangliosides (GGs) are bioactive compounds present in biological tissues and fluids. Their most important roles are related to their presence in biological membranes, taking part in cellular recognition and communication and in the adhesion of viruses and bacteria. They currently have also been related to brain development (improvement of learning and memory).[1] As a result of these functions, they are essential during the first stages of life.[2]

Sia and GG could be synthesized de novo in the organism,[1,3] but in newborn infants, because of the immaturity of their metabolic system, the intake of both compounds is essential for correct development.[4] In these first stages, Sia and GG are obtained from human milk (HM) and infant formulas (IFs). To date, the studies on these compounds have focused on the evaluation of Sia and GG contents in HM[5−7] and IFs.[5,6,8] Some authors have studied only total Sia content in HM[9,10] or IFs.[9−12] On the other hand, Laegreid et al. [13] and Pan and Izumi [14] studied total GG content in HM and IFs, and Takamizawa et al. [15] and Iwamori et al. [16] have evaluated GG in HM.

From a nutritional and functional point of view, it is interesting to know not only the Sia or the GG contents in foods but also their bioavailability (the fraction of an ingested nutrient or bioactive compound that is available for use in physiological functions or for storage).[17] In in vivo studies, Sia or GG bioavailability has been estimated using standard solutions. Nohle et al.[18−20] evaluated radiolabeled Sia (^{14}C and ^{3}H or ^{14}C) incorporation to glucidic and/or proteic fractions, as well as its free form in the intestinal tract, blood vessels, and/or urine, after oral and/or intravenous administration to rats, mice, and pigs. These authors estimated that absorption is fastest with the free forms. Park et al.[21] studied GG bioavailability in rats administered a 2 week diet with 20% of fat, of which 0.1% corresponded to a mixture of GG (80% GD_3, 9% GD_{1b}, 5% GM_3, and 6% others). They observed an increase in the total amount of GG in the intestinal mucosa, plasma, and brain, with respect to the control group. In subsequent studies, under identical conditions, they observed an increase in GG in the rat enterocyte membrane microdomain[22] and in the rat retina.[23]

The data on Sia or GG bioavailability in humans are limited; in this context, human studies are time-consuming, costly to perform, and impractical for large-scale applications. Tram et al.[24] studied total Sia contents in newborn infants (5 days postpartum) fed with IFs and HM, showing that the group fed with HM presented 32% more free Sia and 23% conjugated Sia in saliva as compared with those fed with IF. In another study,[25] the oral administration of 140 mg of Neu5Gc (nonhuman form of Sia) in three volunteers induced the presence of this form of Sia in salivary mucin within 48 h. Finally, a postmortem study in newborn infants showed children fed with HM to have a higher brain content of Sia and GG (22% and 32%, respectively) than those fed with IF.[26]

Regarding in vitro bioavailability, the literature is limited to studies of the effect of pH upon Sia, showing that the latter is hydrolyzed (10%) under acidic conditions [similar to gastric digestion (GD)][27] and that hydrochloric acid (present in GD) induces greater losses than sulfuric acid.[28] Kawakami et al.[29] found that the incubation of standards of GGs GM_3 and GD_3 with hydrochloric acid solutions (pH 4 to 1.3) or with aspirated gastric juices decreases their contents, inducing their hydrolysis to lactosilceramide or the formation of a lactone from GD_3 that could be recovered to GD_3 by reincubating the standard at neutral pH values (6.3−7.4) similar to those found in the intestinal tract.

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The first step defining bioavailability is bioaccessibility, which has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption.[17] The determination of the soluble compounds fraction in simulated gastrointestinal digestion allows estimation of the relative availability of compounds from food, and this constitutes a predictive factor of potential Sia or GG bioavailability. No studies on the bioaccessibility of these compounds in foods are known.

Thus, the aim of the present study was to evaluate the effect of simulated gastric and gastrointestinal digestion upon Sia (Neu5Ac and Neu5Gc) and GG (total and individual forms) present in HM and IFs and to estimate the bioaccessibility of these compounds in the matrices described.

MATERIALS AND METHODS

Reagents and Equipment. All reagents used were analytical grade. Digestive enzymes and bile salts were purchased from Sigma-Aldrich (St. Louis, MO): pepsin (porcine: catalog no. P-7000), pancreatin (porcine: catalog no. P-1750), and bile extract (porcine: catalog no. B-8631). Working solutions of these enzymes were prepared immediately before use. Also from Sigma-Aldrich were bovine brain GGs type III and GD_3 standards, resorcinol (minimum 99%), sodium hydrosulfite, and 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB). Acetonitrile and methanol used for high-performance liquid chromatography (HPLC) were purchased from J. T. Baker (HPLC grade) (Deventer, The Nederlands. Milli-Q water was supplied by Millipore (Barcelona, Spain). Chloroform, methanol, acetone, fuming HCl, copper(III) sulfate pentahydrate, sodium hydrogen carbonate, 2-mercaptoethanol, and HPTLC silica gel plates (10 cm × 10 cm) were purchased from Merck (Darmstadt, Germany). Calcium chloride (95%) and potassium chloride were purchased from Panreac (Barcelona, Spain).

Spectra/Por dialysis membrane (MWCO 3500) was purchased from Iberlabo (Madrid, Spain), Dowex 1 × 8 and DEAE-Sephadex (40−125 μm) were purchased from Fluka (St. Louis, MO), Microcon Ultracel-YM 10 filter devices were purchased from Millipore (Bedford, MA), and solid phase extraction cartridges Sep-Pak C18 (surface area 309 m^2/g) were purchased from Waters (Milford, MA).

The densitometer used for the quantification of GGs was a dual-wavelength Shimadzu CS-9000 TLC densitometer (Kyoto, Japan). Polytron PT2000 (Kinematica, Bohemia, NY) was used for homogenization. The determination of Sia was performed using a Waters HPLC system comprising a Waters 600 quaternary pump, Waters 474 fluorescence detector, Waters 717 plus autosampler, Waters 600 degas system, and Jones Chromatography 464 oven. The column used was a LiChrosorb RP-18 (250 mm × 4.6 mm, 5 μm), with a LiChrosorb RP-18 (5 μm) guard column (both from Merck). The shaking water bath used for in vitro digestion was an SS40-2 (Gran Instruments, Cambridge, United Kingdom). The lyophylizer used was a CT4 from EtoSic (Maryland, MA).

Samples. A powder IF and a liquid follow-on formula (FF) from the same commercial source were evaluated. Both formulas present a casein/whey ratio of 40/60. We also analyzed a pool of HM (from fourth to seventh month of lactation) kindly provided by a single woman.

Simulated Gastrointestinal Digestion. The simulated gastrointestinal digestion described by Perales et al.,[30] consisting of two sequential steps (gastric and intestinal), was applied to samples with slight modifications in the pH value to more closely simulate the physiological conditions of newborn infants (gastric pH 4.0 vs 2.0 and intestinal pH 6.5 vs 5.0). Briefly, two aliquots of 10 g of IF dissolved in Milli-Q water or 100 mL of FF or HM was adjusted to pH 4.0 with 6 M HCl. The pH was checked after 15 min and if necessary readjusted to 4.0 and made up to 100 g with Milli-Q water. Then, an amount of freshly prepared pepsin solution sufficient to yield 0.02 g pepsin/g sample was added and incubated in a shaking water bath at 37 °C/120 strokes per minute for 2 h. One aliquot of digest was maintained in ice for 10 min to stop pepsin digestion (GD). GD should be lyophilized prior to determine Sia and GG.

To continue with the intestinal stage using the other aliquot, the pH of the GD was raised to pH 6.5 by dropwise addition of 1 M $NaHCO_3$, checked after 15 min, and, if necessary, readjusted to pH 6.5. Then, an amount of freshly prepared pancreatin−bile salt solution sufficient to provide 0.005 g of pancreatin and 0.03 g bile salt/g sample was added, and incubation was continued for an additional 2 h. To stop intestinal digestion, the sample was kept for 10 min in an ice bath. The pH was then adjusted to 7.2 by dropwise addition of 0.5 M NaOH. The resulting sample was centrifuged for 1 h at 3262*g* and 4 °C. The supernatant (bioaccessible fraction, BF) was set aside the precipitate and lyophilized. Sia and GG were determined in both fractions (BF and the precipitate).

Sia (Neu5Ac and Neu5Gc) Determination. The Neu5Ac and Neu5Gc contents were determined by HPLC−fluorimetry.[31] Briefly, 0.1 g of nondigested (ND) IF or lyophilized FF or HM or their respective GD, BF, and the precipitates (obtained after gastrointestinal digestion) were dissolved in 0.5 mL of distilled water and hydrolyzed with 2.5 mL of 0.05 M H_2SO_4, and the mixture was incubated at 80 °C for 1 h. The hydrolysate was cooled to room temperature, centrifuged (1000*g*, 5 min, 4 °C), and purified by ion-exchange chromatography using a Dowex 1 × 8 column, HCO_2^- form (2 mL of gel). Four hundred microliters of purified sample was ultracentrifuged (13000*g*, 15 min, 4 °C). Then, 50 μL was derivatized with 50 μL of DMB reagent (8 mM DMB, 1.5 M acetic acid, 14 mM sodium hydrosulfite, and 0.8 M 2-mercaptoethanol) (2.5 h, 50 °C, dark, in block heater). Neu5Ac and Neu5Gc were determined by HPLC from 40 μL of derivatized sample, under the following conditions: temperature, 30 °C; mobile phase water: methanol:acetonitrile 85:7:8 (v/v/v); flow, 0.9 mL/min; detection, λ_{ex} = 373 nm, λ_{em} = 448 nm. For quantification, external calibration (12.5−150 ng of Neu5Ac; 1−8 ng of Neu5Gc) was made. Total Sia was calculated as the sum of Neu5Ac and Neu5Gc.

GGs Determination. *Lipid Extraction.* Powdered IF, lyophilized FF, HM (5 g), or their respective GD, BF, or precipitate (10 g) was dissolved in 10 volumes (w/v) of cold acetone and stirred for 45 min at 4 °C to remove neutral lipids.[32] The homogenate was filtered through a sintered glass funnel. The process was repeated, and the solid residue was left overnight at room temperature for complete elimination of the acetone.

The solid residue obtained was successively extracted with 10 volumes (w/v) of $CHCl_3$:MetOH [1:1 overnight, 2:1, and 1:2 (v/v), 45 min each one]. After centrifugation, all supernatants were pooled, evaporated to dryness by rotavaporation (40 °C), and kept at 4 °C until analysis.

GGs Extraction. The lipid extract was dissolved in 10 volumes (w/v) of $CHCl_3$:MetOH (2:1, v/v). Insoluble material was discarded by centrifugation (1000*g*, 10 min, 4 °C), and the supernatant was subjected to Folch's partition.[33] The upper aqueous phases were pooled and evaporated to dryness by rotavaporation (40 °C). The crude GGs fraction was dissolved in cold water (4 °C) and dialyzed exhaustively with a Spectra/Por membrane (MWCO 3500) against distilled water at 4 °C for 3 days. The dialysate was then lyophilized and resuspended in 1 mL of $CHCl_3$:MetOH (2:1, v/v), and the insoluble material was discarded by centrifugation (100*g*, 10 min, 4 °C). In samples not digested, the GG could be quantified and identified in the collected supernatant.

GGs Purification of Digested Samples. In the case of GD, BF and precipitates, the extracts of GG were evaporated and dissolved in 5 mL of $CHCl_3$:MetOH:water (30:60:8) (v/v/v) and passed through 10 mL of

Table 1. Sia (mg/L) in ND, GD, and BF of HM, IF, and FF[a]

		Neu5Ac	Neu5Gc	total	bioaccessibility %
IF[a]	ND[1]	182.2 ± 8.5	3.3 ± 0.4	185.5 ± 8.5	
	GD[2]	152.2 ± 0.8	3.5 ± 0.2	155.7 ± 0.8	
	BF[2]	139.8 ± 2.0	2.9 ± 0.1	142.7 ± 2.0	76.9 ± 2.5
FF[b]	ND[1]	199.7 ± 7.5	4.9 ± 0.3	204.6 ± 7.5	
	GD[2]	31.4 ± 3.4	1.3 ± 0.1	32.7 ± 3.4	
	BF[2]	31.2 ± 1.3	1.4 ± 0.1	32.6 ± 1.3	15.9 ± 0.1
HM[c]	ND[1]	299.9 ± 7.8	2.1 ± 0.2	302.0 ± 7.5	
	GD[2]	243.5 ± 6.8	1.8 ± 0.1	245.3 ± 6.8	
	BF[2]	260.0 ± 12.1	2.2 ± 0.1	262.2 ± 12.1	86.8 ± 1.9

[a] Bioaccessibility = 100 × BF/total content (ND). Different superscript letters indicate differences in total Sia content among samples; different superscript numbers indicate differences in total Sia content among digestive stages ($p < 0.05$). Results are expressed as the mean ± standard deviation of the analyses of three aliquots for each one of the three digestions done for each sample ($n = 9$).

DEAE-Sephadex resin at a rate of 1 mL/min. The resin was washed with 75 mL of $CHCl_3$:MeOH:water (30:60:8) (v/v/v), and the sample was eluted with 65 mL of 0.8 M $CHCl_3$:MeOH:sodium acetate (30:60:8) (v/v/v). The elute was dried by evaporation and dialyzed as previously described.[34]

Then, solid phase extraction with Sep-Pack C18 cartridges was performed.[35] Briefly, cartridges were conditioned with 60 mL of MetOH, $CHCl_3$:MetOH (1:2) (v/v), and MetOH, successively. The dialyzed sample dissolved in 2 mL of MetOH:water (1:1) (v/v) was passed through the cartridge, and the eluted sample was passed again through the same cartridge. The latter was washed with 25 mL of water and eluted with 25 mL of MetOH, followed by 25 mL of $CHCl_3$:MetOH (1:2) (v/v). The elute was dried by evaporation with a nitrogen stream to room temperature, and the GG extract was dissolved in 1 mL of $CHCl_3$:MetOH (2:1) (v/v) and stored at a −20 °C until analysis.

GG Quantification and Identification. Total GGs were determined as lipid-bound Sia (LBSA) by the resorcinol procedure of Svennerholm.[36] Identification of GGs was performed by HPTLC on precoated silica gel plates (10 cm × 10 cm) with the following solvent system: $CHCl_3$:MeOH:0.5% (w/v) aqueous $CaCl_2$, 55:45:10 (v/v/v).[32] GGs and standards (mixture of bovine brain GGs type III and GD_3 standard) were visualized by spraying the plates with the resorcinol reagent.[36] The relative percentages of GGs (GG pattern) were determined by comparison with standards using a dual-wavelength TLC densitometer after separation by HPTLC in the same solvent system at $\lambda = 580$ nm. The individual content of each GG was calculated from the percentage obtained by densitometry, and the total content was evaluated by spectrophotometry.

Statistical Analysis. Throughout the text, results are expressed as the mean ± standard deviation of the analyses of three aliquots for each one of the three digestions done for each sample. To evaluate the influence of sample (IF, FF, and HM) and digestion stage (ND, GD, and BF) upon total Sia and GG contents, two-factor analysis of variance (ANOVA) and an least significant difference (LSD) test were performed, assuming $\alpha = 0.05$. Statistical analyses were performed with the Statgraphics Plus 5.0 statistical package (Statistical Graphics Corp., Rockville, MD).

RESULTS AND DISCUSSION

Sia. Contents of Neu5Ac, Neu5Gc, and total Sia (as the sum of both forms) in IF, FF, and HM and their respective GD and BF are shown in Table 1. After simulated digestion, the decrease in total Sia contents was 23.1, 84.1, and 13.2% for IF, FF, and HM, respectively. Thus, the greatest bioaccessibility corresponded to HM, followed by IF and FF.

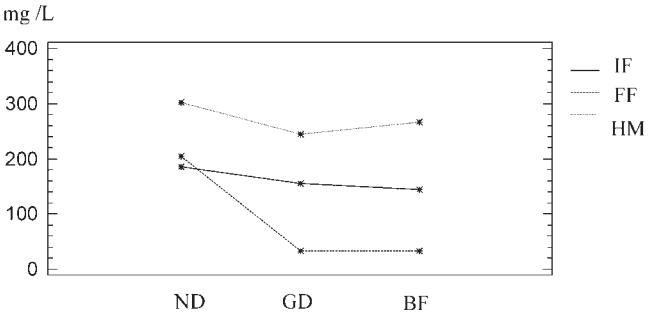

Figure 1. Interaction plots of the factors sample (IF, FF, and HM) and digestive stage (ND, GD, and BF) over total Sia content.

Statistically significant differences have been found among samples (see Table 1). Independently of the sample, the digestive stage significantly reduced the total content of Sia, without differences between GD (mean value, 144.4 mg/L) and BF (mean value, 148.1 mg/L). In the nonsoluble fraction, no Sia forms (Neu5Ac or Neu5Gc) were found; thus, the decrease in Sia should be related to degradation.

Furthermore, interaction was observed between the analyzed factors (samples and digestive stages) (see Figure 1). The behavior of sample FF differed from that of the others—FF being the sample showing most losses as a consequence of the gastric stage. The decrease in total Sia content in the gastric stage was 23.1 and 13.2% for IF and HM, respectively, versus 84.1% for FF.

To our knowledge, there are no previous studies on the influence of the stages of gastrointestinal digestion upon Sia. Only the influence of acidic conditions upon Sia has been studied. Svennerholm[28] evaluated the stability of Neu5Ac and Neu5Gc during hydrolysis with different concentrations of H_2SO_4 (0.05, 0.1, and 0.2 N), temperatures (80−90 °C), and times (1 and 2 h), recording losses of 5−11 and 5.7−5.8% for Neu5Ac and Neu5Gc, respectively. The minimum loss is reached with 0.1 N H_2SO_4, at 80 °C for 1 h, which have been the conditions used in the present study. During the determination of Sia, this effect is corrected by treatment of the standards under the same conditions. On the other hand, the authors pointed out the fact that HCl induces greater losses than H_2SO_4, attending to previous studies. Thus, the decrease in total Sia content is not only due to a low pH in the gastric stage but is also related to the presence of HCl.

On the other hand, Schauer and Corfield[27] reported losses of 10% in total Sia content under acidic conditions—this percentage being close to that obtained in the present study for IF and HM. Differences could be related to interday variability (5.7% Neu5Ac and 4.3% Neu5Gc).

Under the conditions used to simulate the gastric stage (pH 4 and 37 °C), Sia could be present in open and hemiacetal forms in equilibrium, and both could suffer dehydration—the open form yielding a highly reactive conjugate system (Michael's acceptor) and the hemiacetal form an acetal that could not react with DMB.[37]

GGs. Prior to GG determination and identification, purification of the GG from GD and BF of the analyzed samples must be optimized. For this purpose, GD and BF seeded in the HPTLC plates were previously processed as a common sample and purified by DEAE-Sephadex or by DEAE-Sephadex plus solid

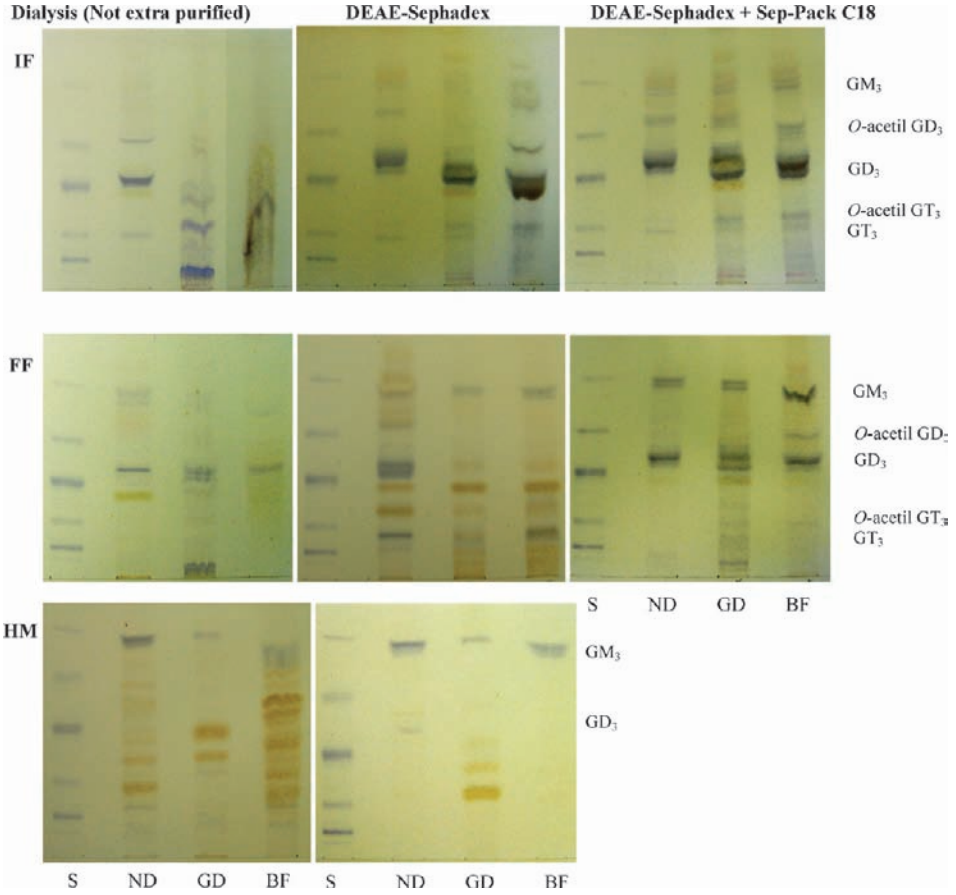

Figure 2. Identification of GGs by HPTLC of samples IF, FF, and HM, ND, and their respective GD and BF vs a standard (S), with different purifications (DEAE-Sephadex and Sep-Pack C18).

Table 2. Total GGs Content and Individual Pattern of GGs (Content and Relative Percentage) of Samples IF, FF, and HM, ND, and Their Respective GD and BFs[a]

	sample	ND	GD	BF	bioaccessibility%
total LBSA (μg/L)	IF	2172.3 ± 64.9	290.2 ± 22.2	115.2 ± 19.2	5.3 ± 0.9
	FF	235.2 ± 4.4	157.1 ± 4.4	118.7 ± 4.1	50.5 ± 1.7
	HM	2306.0 ± 31.2	1013.2 ± 64.2	661.3 ± 27.0	28.7 ± 1.2

	sample	% relative	LBSA (μg/L)	% relative	LBSA (μg/L)	% relative	LBSA (μg/L)	bioaccessibility%
GM_3	IF	10.25 ± 3.03	222.4 ± 65.8	4.42 ± 1.78	12.8 ± 5.2	6.51 ± 2.04	7.8 ± 2.4	3.5 ± 1.1
	FF	36.04 ± 2.84	86.5 ± 6.8	25.97 ± 1.28	41.6 ± 2.0	40.89 ± 2.22	49.1 ± 2.7	56.8 ± 3.1
	HM	97.48 ± 0.09	2232.2 ± 2.1	95.18 ± 3.91	970.8 ± 39.8	97.14 ± 2.24	718.8 ± 16.6	32.2 ± 0.7
O-acetyl GD_3	IF	9.62 ± 1.92	208.8 ± 41.7	5.97 ± 4.49	17.3 ± 13.0	11.36 ± 2.12	13.6 ± 2.5	6.5 ± 1.2
	FF	4.84 ± 1.56	11.6 ± 3.7	6.23 ± 0.83	10.0 ± 1.3	10.74 ± 2.34	12.9 ± 2.8	111.2 ± 24.1
GD_3	IF	72.73 ± 1.22	1578.2 ± 26.5	79.48 ± 1.45	230.5 ± 4.2	71.39 ± 1.37	85.7 ± 2.1	5.4 ± 0.1
	FF	56.49 ± 0.17	135.6 ± 0.4	61.78 ± 0.78	98.8 ± 1.2	44.56 ± 0.97	53.5 ± 1.2	39.5 ± 0.9
	HM	2.52 ± 0.92	57.7 ± 21.1	4.82 ± 2.13	49.2 ± 21.7	2.86 ± 1.07	21.2 ± 7.9	36.7 ± 13.7
O-acetyl GT_3	IF	1.23 ± 0.45	26.7 ± 9.8	6.71 ± 0.77	19.5 ± 2.2	6.83 ± 0.62	8.3 ± 0.7	31.1 ± 2.6
	FF	1.17 ± 1.01	2.8 ± 2.4	2.49 ± 0.37	4.0 ± 0.6	ND	ND	0
GT_3	IF	6.18 ± 2.76	134.1 ± 59.9	3.43 ± 0.49	9.7 ± 1.4	3.92 ± 0.64	4.7 ± 0.8	3.5 ± 0.6
	FF	1.48 ± 0.72	3.6 ± 1.7	3.53 ± 0.03	5.6 ± 0.1	3.81 ± 0.14	4.6 ± 0.02	127.8 ± 0.5

[a] Bioaccessibility = 100 × BF/total content (ND). Results are expressed as the mean ± standard deviation of the analyses of three aliquots for each one of the three digestions done for each sample (n = 9).

phase extraction with Sep-Pack C18, followed by developing and staining with resorcinol (see Figure 2).

The samples of BF without extra purification contain a large amount of brown color interfering compounds that preclude

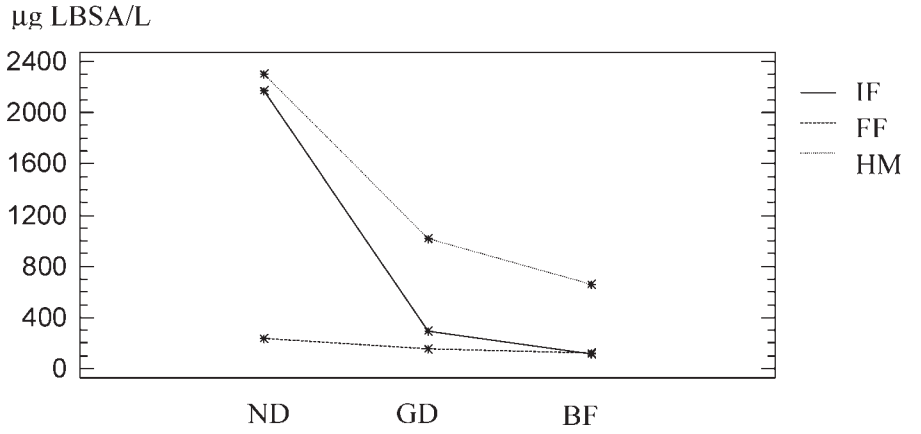

Figure 3. Interaction plots of the factors sample (IF, FF, and HM) and digestive stage (ND, GD, and BF) over total GG content.

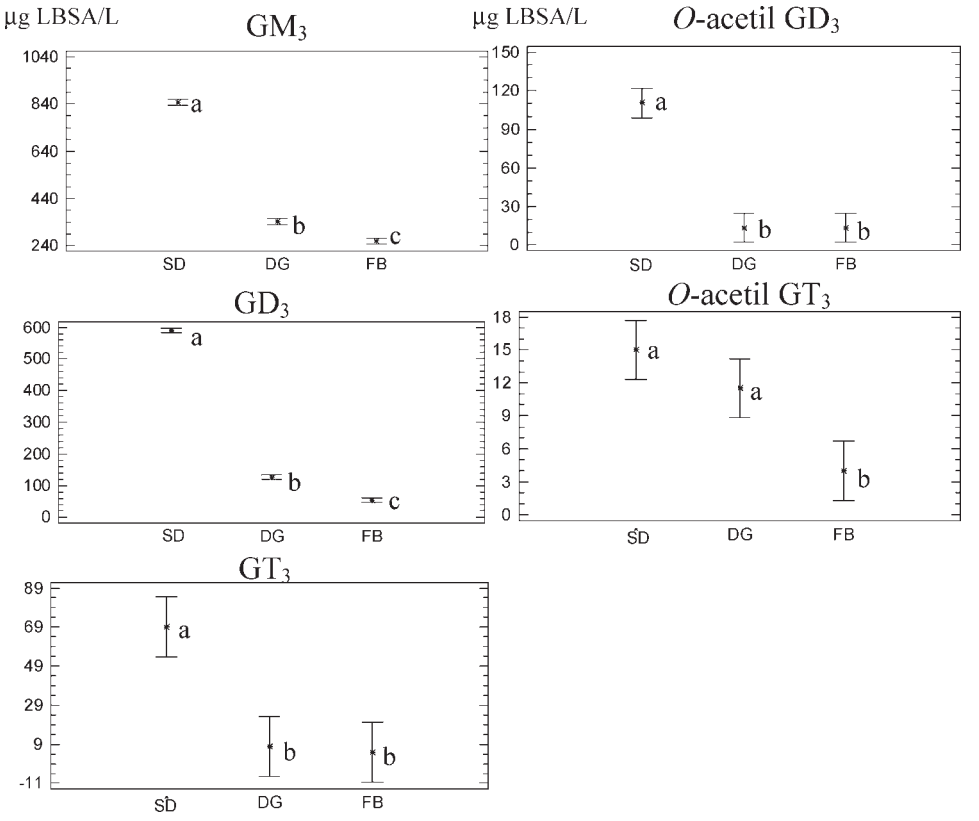

Figure 4. Two-factors ANOVA (digestive stage factor) over individual GGs content (μg LBSA/L). LSD test. Different letters indicate significant differences among digestive stages.

measurement of the HPTLC plates by densitometry. In GD, these compounds do not appear, but the resolution is not clear—probably due to solid particles in the extract. Purification with DEAE-Sephadex increases the resolution, although a zone of blue bands drawn from the deposit appears, especially in IF, that could be hydrolyzed or free Sia. Moreover, many yellow bands appear in GD, probably corresponding to lactosylceramide or other neutral glycosphingolipids (see Figure 2). Purification with Sep-Pack C18 increases the resolution of the bands in HPTLC (see Figure 2).

After both extra purifications, 5 GGs are shown in samples IF and FF; as compared with standards and previous studies,[38] these could be identified (in order of decreasing polarity or increasing Rf) as GT_3, *O*-acetyl GT_3, GD_3, *O*-acetyl GD_3, and GM_3. In HM, only GD_3 and GM_3 are present.

Table 2 shows the total content, the relative percentages, and individual contents of GG, in samples IF, FF, and HM ND, as well as in their respective GD and BF. As in the case of Sia, no GG were found in the nonsoluble fraction.

The statistical analysis showed significant differences for both factors (sample and digestive stage) and revealed interaction between them (see Figure 3). This difference could be due to the liquid or solid nature of the samples (liquid showing the highest bioaccessibility) related to the fact that GG remains in emulsions

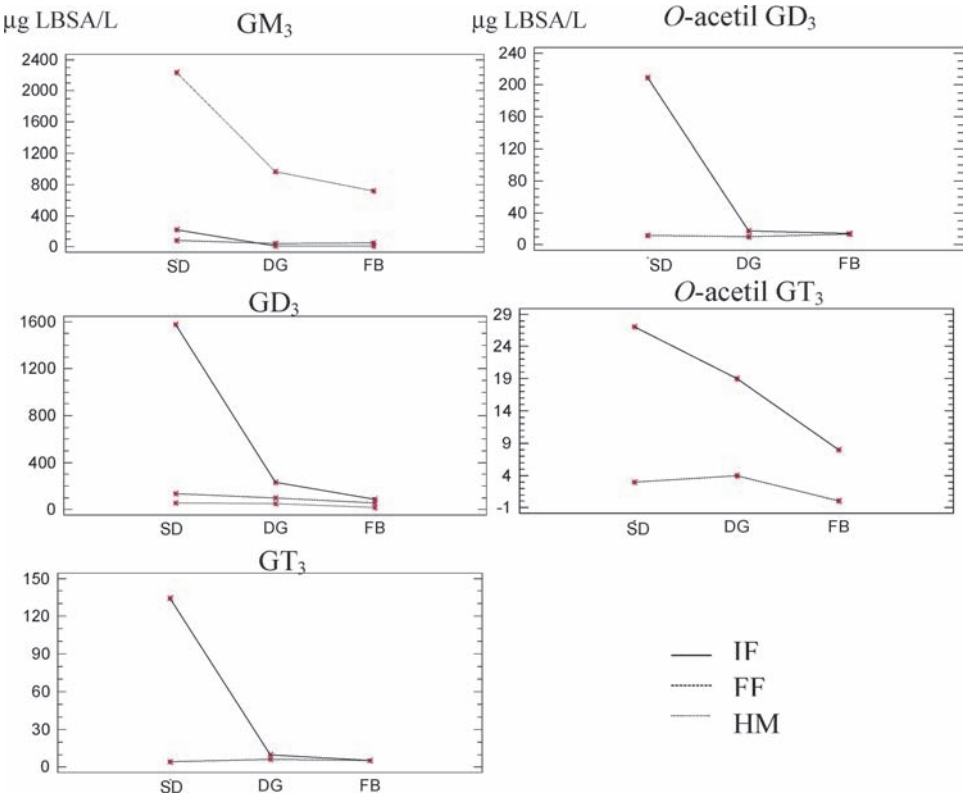

Figure 5. Interaction plots of the factors sample (IF, FF, and HM) and digestive stage (ND, GD, and BF) over individual GG content (μg LBSA/L).

and liquid sample contains emulsifiers in its composition that may affect the effect of the in vitro gastrointestinal digestion. The gastric stage decreases the GG total content more than the intestinal stage. In this sense, the gastric step decreased the total content with respect to the ND sample by 86.6, 33.3, and 55.3% in IF, FF, and HM, respectively. In the case of the intestinal stage, the decrease with respect to the gastric stage was 58.6, 25.5, and 27.5% in IF, FF, and HM, respectively. Sample FF showed the highest bioaccessibility, followed by HM and IF.

The effect of simulated gastrointestinal digestion was the same in relation to both the individual content of GG and the total content, apart from *O*-acetyl GT_3 (see Figure 4). Significant interactions were also found between the sample and the intestinal stage. The effect of simulated gastrointestinal digestion depends on the individual amount of GG, GM_3 being the most affected in HM and GD_3, *O*-acetyl GD_3, GT_3, and *O*-acetyl GT_3 in sample IF (see Figure 5).

The literature yields no data on the effect of digestion upon GG. Kawakami et al.[29] studied the effect of acidic pH (1.3−4) using different HCl solutions and gastric juices over GM_3 and GD_3 standards, observing a decrease in the contents of both GG when the pH was lowered. Incubation with gastric juice produces reductions similar to those obtained with pH 1.3. In the case of pH 4 (used in our study for simulating the gastric stage), the above authors recorded decreases of 10.9 and 12.7% for GM_3 and GD_3, respectively; using gastric juices, the decreases in GM_3 and GD_3 were 75 and 94.5%. Our reductions are closer to the results, which the aforementioned authors obtained with gastric juices (GM_3: 96.5, 43.2, and 67.8% for IF, FF, and HM, respectively; GD_3: 94.6, 60.5, and 63.3% for IF, FF, and HM, respectively). They found GM_3 to be completely degraded to lactosylceramide, while GD_3 was converted to a lactone and lactosylceramide. Figure 2 shows yellow and brown bands in the samples without the extra purification that could be the lactosylceramide formed during digestion of the samples, but GD_3 lactone is not present.

Finally, Kawakami et al.,[29] after incubation under acidic conditions, reincubated the samples to pH 6.3−7.4, observing that from lactones previously formed GD3 could be recovered. This pH is similar to that used in the intestinal step of this study (pH 6.5), but in our case, a higher decrease in its amount is observed. This fact could be due to the presence of digestive enzymes, which can rupture GG emulsions, making their hydrolysis easier.

Summarizing the results, the main conclusions are that in the simulated gastrointestinal digestion of infant and FFs and HM, the gastric stage is the step that causes a greater decrease in Sia and GGs contents. Only in the case of GGs, the next step (intestinal digestion) also decreased the total and individual contents, and HM shows the highest Sia bioaccessibility, followed by IFs and FFs. In the case of GGs, the order of bioaccessibility changes—the greatest corresponding to the FF, followed by HM and the IF.

Because of the importance of these compounds, as commented in the introduction, the decrease of contents induced by the gastrointestinal digestion, especially notable in IFs, could be avoided by the elaboration of this formulas with enriched in Sia and GGs raw materials. However, the enrichment should be done carefully, since recently the European Food Safety Agency has refuted the claims about the beneficial effects of Sia in learning and memory in humans associated to WPC, due to the lack of studies revealing a cause−effect relationship.[39]

Of note is the fact that this is the first time data have been obtained on the bioaccessibility of Sia and GGs in infant food.

Although bioaccessibility is the first stage to study bioavailability, it must be evaluated to continue with studies of bioactivity. In this context, further studies involving different kinds of simulated digestions more similar to human physiological conditions are needed, to improve the knowledge about bioaccessibility of these compounds.

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