University of Groningen

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Publication date:
2023

[Link to publication in University of Groningen/UMCG research database](#)

Quantitation of bioactive components in infant formulas: Milk oligosaccharides, sialic acids and corticosteroids

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A B S T R A C T

Human milk is considered the optimal food for infants with abundant nutrients and bioactive components, which play key roles in infant health and development. Infant formulas represent appropriate substitutes for human milk. There are many brands of infant formula with different ingredient sources and functions on the market. The present study aims to quantify important bioactive components, i.e., milk oligosaccharides (MOS), sialic acids (Sia) and corticosteroids, in different infant formulas and compare these to human milk. In total, 12 different infant formulas available on the Dutch market were analyzed in this study. The concentrations of MOS and Sia were characterized by UHPLC-FLD and LC-MS, while corticosteroids were determined using established UHPLC-MS/MS methods. Among infant formulas, 15 structures of oligosaccharides were identified, of which 2′-Fucosyllactose (2′FL), 3′-Galactosyllactose (3′GL) and 6′-Galactosyllactose (6′GL) were found in all infant formulas. The oligosaccharide concentrations differed between milk source and brands and were 3–5 times lower than in human milk. All infant formulas contained Sia, *N*-acetylneuraminic acid (Neu5Ac) was dominant in bovine milk-based formulas, while *N*-glycolylneuraminic acid (Neu5Gc) was major in goat milk-based formula. All infant formulas contained corticosteroids, yet, at lower concentrations than human milk. Insight in concentrations of bioactive components in infant formula compared to human milk may give direction to dietary advices and/or novel formula design.

1. Introduction

Human milk is considered to be the most suited nutrition for infants (Ballard & Morrow, 2013). Breastfeeding impacts on short- and long-term aspects of infant health and development: protecting against diabetes and infections, preventing allergies and diarrhea, as well as enhancing cognition and learning ability (de Weerth et al., 2022). Modern infant formula is an adequate substitute for human milk. Infant formulas are available as powder to mix with warm water and as ready-to-feed liquid (Martin et al., 2016). Infant formulas on the Dutch market have three major ingredient sources: bovine milk is the predominant source, followed by soy-based formula and recently goat milk-based formulas have become available. There are also some specialized formulas on the market, using hydrolyzed protein from bovine milk or rice (Martin et al., 2016). Although mother's milk changes significantly in composition during the first six months (Ballard & Morrow, 2013), stage 1 infant formulas are used for the period 0–6 months (DiMaggio et al., 2019). Infant formula producers make continuous efforts to improve the

composition of infant formulas to mimic human milk in nutritional as well as bio-functional properties (Li et al., 2021).

Human milk not only contains macro- and micro-nutrients, but also contains abundant bioactive components (Ballard & Morrow, 2013; de Weerth et al., 2022), like human milk oligosaccharides (hMOS) (Bode, 2012), sialic acids (Sia) (Wang et al., 2001) and hormones (Hollanders et al., 2017). Human milk oligosaccharides are quantitatively major bioactive components in human milk, offering benefits for breastfed infants, such as immune system maturation (Triantis et al., 2018), brain (Wang, 2009) and gastrointestinal development (Cheng et al., 2020). The concentration and structures of oligosaccharides in human milk are more abundant and complex than in milk of domesticated dairy animal species and consequently in infant formulas (Albrecht et al., 2014; Li et al., 2021; Urashima et al., 2018). Galactooligosaccharides (GOS) and fructooligosaccharides (FOS), are often added to infant formulas as prebiotic hMOS substitutes, although other direct effects of GOS/FOS on formula-fed infants are still not completely elucidated (Bode et al., 2016). Some modern infant formula with added HMOs, particularly 2′-fucosyllactose (2′FL) and lacto-*N*-neotetraose (LNnT), have reached the market (Hegar et al., 2019; Puccio et al., 2017; Vandenplas et al., 2018).

Sialic acids are abundant in human milk and have been implicated in brain development during the first year of life (Liu et al., 2022; Schnaar et al., 2014). Sialic acid, bound to hMOS, glycoconjugates or in free form, has been linked to beneficial properties (Liu et al., 2022). Sia has three representative forms, including *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxynonulosonic acid (Kdn). Recent evidence showed that exogenous Sia could improve learning and memory ability in pre-clinical studies (Sakai et al., 2006; Wang et al., 2007). Human milk contains almost exclusively Neu5Ac and most of Sia is bound to hMOS (Wang et al., 2001). In contrast, bovine and goat milk-based infant formulas contain both Neu5Ac and Neu5Gc, the majority of Sia in bovine and goat milk is linked to glycoproteins (Wang et al., 2001).

Furthermore, human milk contains corticosteroids (Hollanders et al., 2017). Corticosteroids are stress responsive hormones excreted into the blood stream. Recent studies showed that corticosteroids in breast milk can cross the neonatal intestinal epithelial barrier into their plasma and the brain, and are suggested to be involved in the regulation of infants' metabolism, gut maturation, behavioral imprinting and neuro-development (Hollanders et al., 2017). There is, however, limited knowledge on corticosteroid composition of infant formulas. Considering the potential functions of corticosteroids there is reason to study the concentrations naturally occurring in infant formula.

These bioactive components are one of the reasons why World Health Organization (WHO) recommends mothers exclusive breastfeeding for the first six months and continued partial breastfeeding for at least two years (Bhandari & Chowdhury, 2021). Globally, only 44% of infants under six months are exclusively breastfed (Bhandari & Chowdhury, 2021). Moreover, WHO European Region reported that less than 25% of infants are exclusively breastfed during the first six months (Theurich et al., 2019). Among the survey data and national reports in the Netherlands, 80% of infants are solely breastfed at birth, but only 39% of infants are exclusively breastfed for six months (Theurich et al., 2019). Although breastfeeding is highly recommended, many parents switch to infant formula at an early stage.

In view of increasing evidence on the myriad of complex functions in infant development of these bioactive compounds like hMOS, sialic acid and corticosteroids in human milk, there is reason to evaluate their presence and quantities in infant formula. To date, there are very limited studies to monitor bioactive components in infant formulas. Therefore, the purpose of this study is to quantify the concentration of milk oligosaccharides (MOS), Sia and corticosteroids in infant formulas and compare these between infant formulas and human milk. Here, we bought 12 stage-1 infant formulas available in the supermarket and web shops in the Netherlands. There are nine term infant formulas and one ex-pre-term formula based on bovine milk, one term formula based on goat milk, and one "vegan" hypoallergenic formula based on rice protein. Using UHPLC-FLD, UHPLC-MS and HPLC-MS/MS analyses several bioactive factors in infant formulas were analyzed quantitatively.

2. Material and methods

2.1. Materials

Quantitative oligosaccharide standards 2′FL, 3FL, 3′SL, LNT, LNnT, LNFP I, and LND I and qualitative standard LSTa, were obtained from Elicityl Oligotech (Crolles, France). Quantitative standards 3′GL, 4′GL, 6′GL and 6′SL and qualitative standard LNFP V were obtained from Carbosynth/Biosynth B.V. (Lelystad, the Netherlands). Quantitative standards LNFP II, LNFP III, DLSNT, and DFL, as well as qualitative standards DFLNHa, LSTb, LSTc, 3S3FL and DFLNnH were obtained from IsoSep ab (Tullinge, Sweden). Qualitative standards for LNFP VI and MFLNH III were obtained from Dextra Laboratories UK (Reading, United Kingdom). Laminaritriose internal standard was obtained from Megazyme (Wicklow, Ireland) and maltotriose calibration standard was obtained from Merck Netherlands BV (Amsterdam, the Netherlands). A human milk quality control (QC) sample was kindly provided by Nestlé Research to validate milk oligosaccharide results.

Cerilliant® corticosteroids from Supelco (11-deoxycortisol, 21-deoxycortisol, 11-deoxycorticosteron, corticosterone, cortisol, cortisol-2,3,4-$^{13}C_3$, cortisone, and cortisone-2,3,4-$^{13}C_3$) and Sigma-Aldrich standards (11-deoxycortisol-2,3,4-$^{13}C_3$, 21-deoxycortisol-9,11,12,12-d4, 11-deoxycorticosteron-2,3,4-$^{13}C_3$ and corticosterone-9,11,12,12-d4) were purchased from Merck Netherlands BV (Amsterdam, the Netherlands).

2.2. Infant formulas & milk samples

Commercial infant formulas (n = 12) were bought from the consumer market (Table 1): Nestlé Nan Complete Comfort (Nestlé S.A.; NCC); Nestlé Nan Optipro Standard 1 (Nestlé S.A.; NOS); Nutrilon Profutura 1 (Nutricia NV, Zoetermeer, the Netherlands; PFU); Nutrilon Omneo 1 (Nutricia NV; OMN); Nutrilon ready to feed (Nutricia NV; RDY); Nutrilon Ex-Premature ready to feed (Nutricia; PRE); Hipp HA Combiotik 1 (Hipp GmbH, Pfaffenhofen, Germany; HAC); Holle Bio 1 (Holle baby food AG, Riehen, Switzerland; BIO); Novolac NovaRice (Menarini Benelux N.V./S.A., Machelen, Belgium; RIC) were ordered online at NewPharma.nl. Nutrilon Standard 1 (Nutricia NV; NST); Hero Baby Standard 1 (Hero Baby, Breda, the Netherlands; HST) and Kabrita 1 (Ausnutria Nutrition BV, Zwolle, the Netherlands; KAB) were bought in the supermarket. Formula were prepared using demineralized water that was first heated to 95 °C for 10 min, and then cooled to 40 °C for preparations. Formulas NOS, HAC, BIO, RIC, KAB, NST and HST were prepared by adding 3 flat scoops, provided with the product, to ~ 90 mL of demineralized water. Products NCC, PFU and OMN were provided in pre-weighed sachets and added entirely to ~ 90 mL warm water. The water was measured in a measuring cup with similar accuracy as a baby bottle, to simulate home preparation. The prepared formulas were aliquoted and kept at −20 °C for further analysis.

Human milk samples (n = 40; 1 month *postpartum*) from the Lifelines NEXT prospective birth cohort (Warmink-Perdijk et al., 2020) were used as a reference for milk oligosaccharide and sialic acid analyses. In this cohort, human milk was collected from mothers in northern Netherlands. Human milk samples were collected at five time points *postpartum* (2, 4, 8, 12 and 26 weeks). Informed consent was obtained from all individuals included in the study. The Lifelines NEXT study was approved by the ethics committee of the University Medical Center Groningen, document number is UMCG METc2015/600.

Human milk was collected at second feeding after midnight with at least 2 h in between feedings. Mothers were asked to collect breast milk from preferably the right breast that had been emptied completely in the feeding preceding the sample collection. Aliquots of milk (15 * 2 mL) were collected from each mother. Mothers were then asked to fill in the

Table 1
Characteristics of infant formulas.

Infant formula	Brand	Country of Manufacture	Forms	Source	Stage
Nestlé Nan Complete Comfort (NCC)	Nestlé	Switzerland	Power	Cow milk	0–6 months
Nestlé Nan Optipro Standard 1 (NOS)	Nestlé	Switzerland	Power	Cow milk	0–6 months
Nutrilon Standard 1 (NST)	Danone	Netherlands	Power	Cow milk	0–6 months
Nutrilon Profutura 1 (PFU)	Danone	Netherlands	Power	Cow milk	0–6 months
Nutrilon Omneo 1 (OMN)	Danone	Netherlands	Power	Cow milk	0–6 months
Nutrilon 1 ready to use (RDY)	Danone	Netherlands	Liquid	Cow milk	0–6 months
Nutrilon Ex-Premature ready to feed (PRE)	Danone	Netherlands	Liquid	Cow milk	Pre-term infants from 0 months
Hipp HA Combiotik 1 (HAC)	Hipp	Germany	Power	Cow milk	0–6 months
Hero Baby Standard 1 (HST)	Hero Baby	Netherlands	Power	Cow milk	0–6 months
Holle Bio 1 (BIO)	Holle	Germany	Power	Cow milk	0–6 months
Kabrita 1 (KAB)	Kabrita	Netherlands	Power	Goat milk	0–6 months
Novolac NovaRice (RIC)	Novolac	France	Power	Rice	0–36 months

accompanying information sheet, including date and time of collection. The collected milk samples were subsequently stored at −20 °C in the home freezer before transfer to the lab for storage at −80 °C until analysis. The frozen samples were thawed once for analysis.

2.3. Milk oligosaccharides quantification

Milk oligosaccharides in infant formulas and one reference Lifelines NEXT human milk sample at 1 month *postpartum* were labelled with 2-aminobenzamide (2-AB) and then were separated by UHPLC-FLD according to previously published protocol (Austin & Bénet, 2018). Briefly, homogenized milk samples (40 µL) were mixed with the same volume of internal standard (laminaritriose, 0.5 µmol/mL). Mixed sample (20 µL) was transferred to a new microtube, and added labelling solution (2-aminobenzamide [0.35 M] and 2-picoline borane complex [1.0 M] in dimethylsulfoxide containing acetic acid [30%], 200 µL). Then, the mixed solution was placed in an oven at 65 °C for 2 h. After cooling for 15 min at 4 °C, 600 µL 75% acetonitrile was added. The labelled oligosaccharides were separated and quantified by UHPLC system (Waters Chromatography B.V., Etten-Leur, The Netherlands). The system performance was validated using real hMOS standard curves and a QC sample provided by Nestlé Research. All hMOS were detected within 15% of the established values in the Nestlé Research laboratory. The LOD and LOQ of the standards were reconfirmed on our system. The columns were an Acquity BEH Glycan (1.7 mm, 2.1 × 150 mm) and VanGuard BEH amide (1.7 mm, 2.1 × 50 mm) (Waters, The Netherlands). Eluent A was acetonitrile and eluent B was 50 mM ammonium formate. All oligosaccharides were quantified against maltotriose calibration curve with known purity, assuming equimolar

response factors as previously established (Austin & Bénet, 2018).

2.4. Milk oligosaccharides purification and mass spectrometry analysis

The labelled oligosaccharides were purified using a graphitized carbon SPE column pre-activated and conditioned with acetonitrile and water. The SPE column was activated by 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA) (3 times 1 mL). Milli-Q water containing 0.05% TFA (3 times 1 mL) was used to condition the column. Then, samples of labelled oligosaccharides (0.5 mL) were loaded on the column. After washing 3 times with 1 mL milli-Q water containing 0.05% TFA, the oligosaccharides were eluted with 40% acetonitrile containing 0.05% TFA (3 times 1 mL). The 3 mL fractions from the SPE column was collected and dried to 1 mL under Nitrogen (N_2) for LC-MS analysis of individual MOS.

The samples were measured by an HPLC instrument (Shimadzu 20 series) followed an Orbitrap Velos Pro high-resolution mass spectrometer (Thermo-Fisher Scientific, USA). MS acquisition was performed in a positive electrospray mode (the spray voltage was 3.5 kV) lenses were set to a range of *m/z* 200 to 2000 detection range at a resolution of 60,000 FWHM. The source temperature was 325 °C and the ion transfer tube temperature was 350 °C. Data were analyzed with MZmine 3 software.

2.5. Sialic acids quantification

Sialic acids in infant formulas and 40 Lifelines NEXT human milk samples collection at 1 month *postpartum* were derivatized by 1,2-diamino-4,5-methylenedioxybenzene (DMB) and then were analyzed by UHPLC-FLD as previously described (Spichtig et al., 2010). In short, milk sample (20 µL) was diluted five times with Milli-Q water. Mixtures of 20 µL infant formula were hydrolyzed by adding the same volume of 1 M formic acid (total Sia) at 85 °C for 1 h. For total Sia, the hydrolyzed sample was mixed with 40 µL of DMB reagent (7 mM DMB, 1.4 M acetic acid, 18 mM sodium hydrosulfite, 0.75 M 2-mercaptoethanol) and heated at 85 °C for 50 min. For free Sia, the native sample was mixed with 40 µL of DMB reagent (7 mM DMB, 40 mM TFA, 18 mM sodium hydrosulfite, 1 M 2-mercaptoethanol) and kept at 4 °C for 48 h. The derivatized sample was diluted with Milli-Q water (150 µL). An UHPLC system (Waters Chromatography B.V., Etten-Leur, The Netherlands) was used to separate Sia. DMB derivatives of Sia were eluted under isocratic conditions using 5% (v/v) methanol, 9% (v/v) acetonitrile in Milli-Q water for 7 min at a flow rate of 0.18 mL/min.

2.6. Corticosteroid quantification

Corticosteroids in infant formulas were quantified by UHPLC-MS/MS, as previously described (van der Voorn et al., 2015). Briefly, 200 µL infant formula was mixed with 75 µL internal standard solution (consisting of 820.9 nmol/L cortisol-[13]C3, 13.8 nmol/L cortison-[13]C3, 2.9 nmol/L corticosterone-d4, 1.4 nmol/L 11-deoxycortisol-[13]C3, 0.9 nmol/L 11-deoxycorticosteron-[13]C3) and 100 µL Milli-Q water. Then, 1.5 mL hexane was added to the mixture to extract lipids twice. Afterwards, 1.2 mL of 0.17 M Zinc sulphate ($ZnSO_4$) solution in 45% methanol was mixed with the aqueous phase, centrifuge the samples at 4000 rpm for 10 min after fully vortexed. Supernatant was transferred to Solid Phase Extraction (SPE) column (Oasis, Waters, The Netherlands) for further purification of the samples. The 1 mL analyte was eluted from the cartridge and was transferred to 96-wells plate. Then dried 96-wells plate under nitrogen (N_2) at 40 °C and dissolved in 200 µL 40% methanol. Parameters LC-MS/MS corticosteroids were separated with a Kinetex Biphenyl Column (100 × 2.1 mm, 2.6 µm) (Phenomenex, The Netherlands). Mobile phase A was 10% methanol and mobile phase B was methanol. The mobile phase was starting at 80:20 (A: B), peaking at 10:90 before returning to 80:20. The chromatography was performed at 40 °C. The flow rate was 0.4 mL/min and total run time was 7.5 min.

Ionization was achieved by ESI in positive ion mode at capillary voltage of 0.80 kV and cone voltage of 30 V at a source temperature of 150 °C and desolvation at 600 °C with gas flow at 800 L/h. Quantitation was achieved by Selective Reaction Monitoring against stable isotope labeled internal standards (S-Table 1).

3. Results and discussion

3.1. Quantitation of milk oligosaccharides in infant formulas

Milk oligosaccharide content in infant formula was assessed by established HPLC-FLD chromatography (Fig. 1, S-Fig. 1) (Austin & Bénet, 2018). Identities of hMOS observerd in the infant formulas, as well as unknown peaks were evaluated with LC-MS to verify the structures or propose a structure known to occur in bovine or goat milk. Peaks for known hMOS 2′FL, 3FL, 3′GL, 6′GL LNnT, LNFP III and LSTc were observed at the expected elution times in HPLC-FLD analysis in several formulae and confirmed by LC-MS (Table 2). Furthermore, LC-MS analysis (S-Figures 2–12) showed base-peak chromatograms fitting HexNAc$_1$Lac (NAL or Lacto-N-triose; LN*t*), 3′NGL, 6′NGL, DSL, and S-GL (Table 2), with elution times potentially fitting the proposed structures. Particularly 3′NGL (overlapping with 6′SL) and 6′NGL eluting significantly later than 6′SL) observed in KAB fit with previous observations in goat milk (Chatziioannou et al., 2021). The chromatograms also show peaks supporting the presence of GOS DP3-6 and maltodextrins DP2-8. Using the same methods, 24 hMOS could be quantified in human milk (Austin & Bénet, 2018) (Fig. 1), while only 9 hMOS (neutral structures: 2′FL, 3FL, 3′GL, 6′GL, LNnT and LNFP III; acidic structures: 3′SL, 6′SL, and LSTc) were detected in infant formulas. Moreover, NAL 3′NGL, 6′NGL, DSL and S-GL are not found in human milk. The oligosaccharides analytical approach and limits of detection (LoD) and limits of quantification (LoQ) were based on the study from Austin et al. (Austin & Bénet, 2018), and reconfirmed on our own system, showing satisfactory hMOS separation, recoveries and reproducibility. With the exception of 2′FL, 3′GL, 6′GL and 3′SL, most detected MOS could not be quantified due to low concentrations or overlapping peaks. It should be noted that bovine milk contains dozens of identified MOS, but most at very low concentrations (Albrecht et al., 2014). Also goat milk contains only several structures in quantifyable concentrations (Albrecht et al., 2014; Chatziioannou et al., 2021; Van Leeuwen et al., 2020). Goat and bovine milk are not directly used to produce infant formula, but are processed into different ingredient streams, that are mixed in relevant ratio's to produce infant formula fitting the nutritional values of human milk. Potential lossed of MOS during this process may lead to reduced concentrations of MOS in the infant formula.

As illustrated in Table 3, most formulas, beside NOS, BIO and RIC, show clear peaks of GOS, fitting with the added GOS according to the label (S-Table 2). We cannot detect and quantify all GOS peaks, first of all, the non-lactose DP2 peaks of GOS are lost in the on-line cleanup and particularly in formula with high levels of maltodextrins some GOS peaks cannot be integrated accurately and are left out. Moreover, the method does not detect non-reducing GOS containing Gal(β1-1α/β)Glc elements, that have been described for GOS (Van Leeuwen et al., 2014). Therefore, the rough quantitation of GOS is not accurate and likely to underestimate actual GOS concentrations.

Two bovine milk-based formulas (NOS and PFU) had particularly high 2′FL content with concentrations of 1233 and 1138 mg/L, respectively. As shown in S-Table 12, NOS and PFU contained added 2′FL (1000 mg/L) according to the nutritional label. The concentrations of 2′FL in our study were slightly higher than indicated the nutritional label, which could in part be explained by the natural presence of low levels 2′FL in the bovine milk ingredients as shown in formulas without 2′FL added (23–41 mg/L). Furthermore, there was variation in scoop-sizes when preparing the formula according to the instructions. In our experience the scoops tended to be slightly larger (6–9%, based on 10 scoops weighed) than according to the package, which may explain why

the NOS containing added 2′FL shows a higher concentration than according to the label. Moreover, the formula was prepared in non-precision glassware, to simulate home-preparation of a bottle with ~ 90 mL of warm water. 2′FL is a major structure in human milk with around 2457 mg/L at 1 month postpartum (Austin et al., 2019). Among the rest of the formulas, the levels of 2′FL in BIO and HST (bovine milk-based formulas) were around 40 mg/L. Other bovine milk-based and one goat milk-based formula (KAB) contained a comparable level of 2′FL (23–29 mg/L). We also found trace amounts of 3FL in all formulas, confirmed by LC-MS.

3′GL and 6′GL are two major MOS with variable concentrations between formulas (Table 3). Beside rice-based formula (RIC), all the remaining formulas contained 3′GL (60–895 mg/L) and 6′GL (17–262 mg/L) above LoD. The highest level of 3′GL was observed in RDY with 895 mg/L, second in NST with the concentration of 559 mg/L and third in PFU containing 376 mg/L. All three formulas have added 3′GL according to the label (S-Table 12). It should be noted that 3′GL and 6′GL are naturally present in bovine milk (Albrecht et al., 2014) as also observed in the two-bovine milk-based formulas without GOS and/or 3′GL added (NOS and BIO). Moreover, the added GOS naturally contains 3′GL and 6′GL already (Leeuwen et al., 2016). Therefore, formulas with added GOS and 150 mg/L added 3′GL will naturally contain more than 150 mg/L 3′GL. Besides the infant formulas with added MOS (NOS and PFU), the remaining products showed significantly higher concentrations of 3′GL than 2′FL, which was in good agreement with previous studies that 3′GL was more abundant than 2′FL in bovine and goat milk (Albrecht et al., 2014; Chatziioannou et al., 2021; Van Leeuwen et al., 2020). Besides three Nutricia formulas, goat-based formula (KAB) had 231 mg/L of 3′GL, which was higher than the other formulas. This observation was in accordance with the study of Albrecht et al., who reported that the relative abundance of 3′GL was higher in goat milk than in bovine milk (Albrecht et al., 2014).

6′GL was the second major oligosaccharides in infant formulas, which is not specifically reflected in the nutritional label. 6′GL levels were more abundant in Nutricia (RDY, NST, PFU, PRE, OMN) and goat milk-based formula (KAB) (>100 mg/L of 6′GL), compared with other formulas (<100 mg/L). Since 6′GL is present in most GOS formulations (Leeuwen et al., 2016) as well as naturally in bovine and goat milk (Albrecht et al., 2014), it is not possible to determine the source of the 6′GL observed in the formulas, nor assess whether the source (regular bovine, organic bovine or goat milk) has any significant influence on the presence of 3′GL and 6′GL. To note, the concentrations of 3′GL and 6′GL were significantly higher in infant formulas than human milk at 1 month postpartum (mean concentrations are 6 and 29 mg/L, respectively) (Austin et al., 2019; Lefebvre et al., 2020).

We detected evidence for presence of 3′SL and 6′SL in all infant formulas by LC-MS, except in rice based-formula. The amounts of 3′SL ranged from 33 mg/L (OMN) to 72 mg/L (BIO). The 6′SL concentration was not possible to assess, due to overlap with a GOS DP4 peak. In case of infant formulas without added GOS, the 6′SL concentration could be separately integrated and was in both cases < LoQ. Therefore the 6′SL concentration is considered to be a trace amount in all bovine milk-based infant formulas. The amounts of 3′SL and 6′SL in formulas were significantly lower than in human milk (Austin et al., 2019; Samuel et al., 2019). Low levels of LNnT, LNFP-III and LSTc were detected by LC-MS in some milk formulas as well, but all < LoD of the UPLC analysis and very low ion-intensity in the mass spectrometry.

To our surprise, traces of 2′FL, 3FL, LNFP-III and LSTc were identified in rice based-infant formula by LC-MS, but were not visible in the UPLC-FLD chromatogram. Since these MOS only occur in animal milk, there might be limited animal source in rice protein-based formulas, or possibly the formula is produced in a facility where also animal milk-based formulas being produced, resulting in traces of these oligosaccharides present in this formula.

The chromatograms (Fig. 1) also show peaks for maltodextrins (starch-derived α1,4-glucose linked oligosaccharides, DP2-8). The
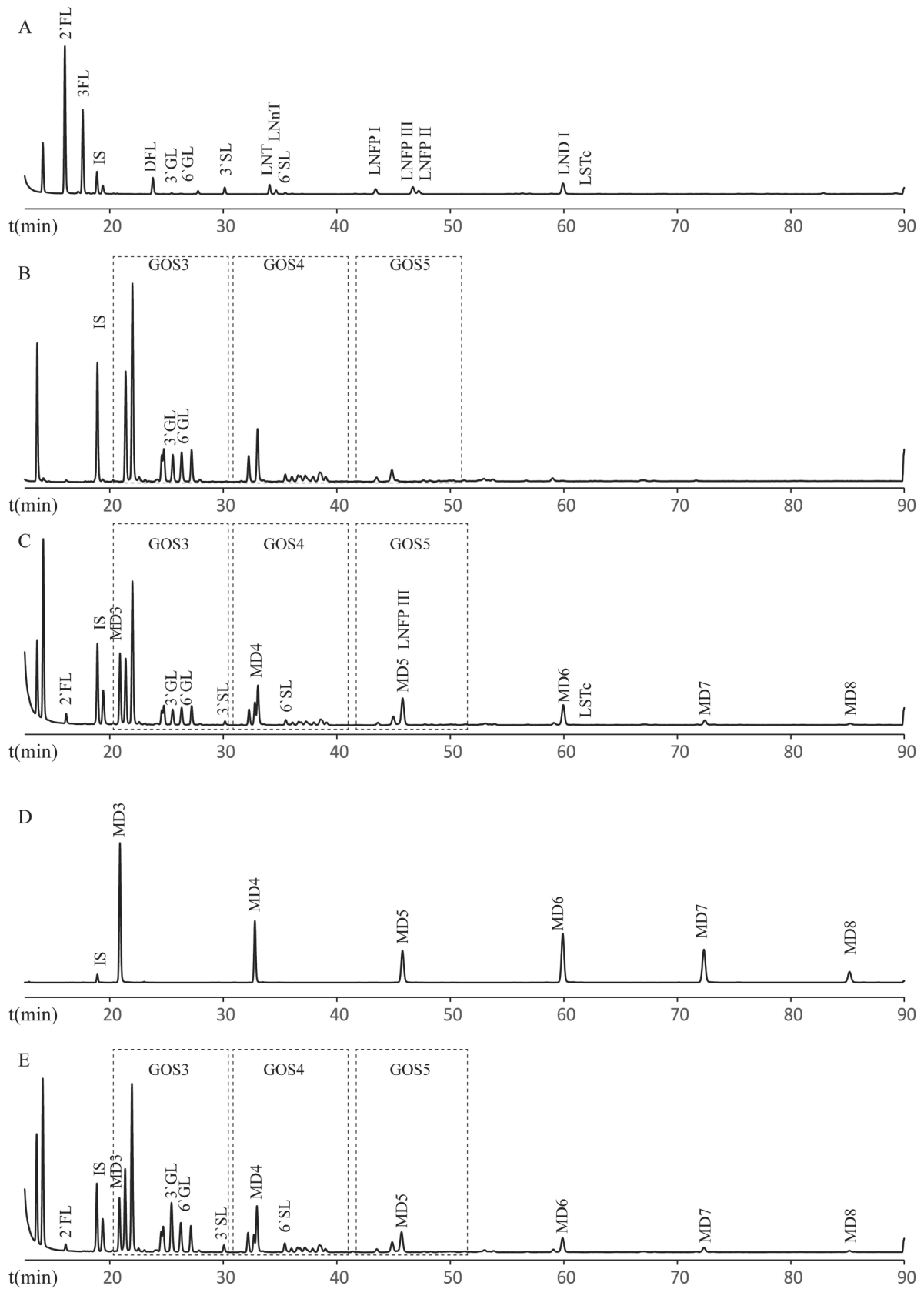
Fig. 1. UPLC-FLD profiles of milk oligosaccharides in: (A) human milk (milk group 1), (B) GOS, (C) HAC, (D) RIC, (E) KAB.

Table 2
Milk oligosaccharides identified in infant formulas by LC-MS.

Name	Abbreviation	[M + H] $^+$		Composition	RT
		Found	Calculated		
Lactose	Lac	463.19	463.19	Hex2	15.9
2′-fucosyllactose	2′FL	609.25	609.25	Hex2Fuc1	20.4
3-fucosyllacose	3FL	609.25	609.25	Hex2Fuc1	22.0
3′-galactosyllactose	3′GL	625.24	625.25	Hex3	29.7
6′-galactosyllactose	6′GL	625.24	625.25	Hex3	30.7
6′-N-acetylglusosaminyllactose	NAL	666.27	666.27	Hex2HexNAc1	25.5
3′-sialyllactose	3′SL	754.29	754.29	Hex2Neu5Ac1	33.5
6′-sialyllactose	6′SL	754.29	754.29	Hex2Neu5Ac1	38.5
3′-Neu5Gc-lactose	NGL	770.28	770.28	Hex2Neu5Gc1	38.7
6′-Neu5Gc-lactose	6′NGL	770.28	770.28	Hex2Neu5Gc1	44.2
Lacto-N-neotetraose	LNnT	828.32	828.33	Hex3HexNAc1	38.3
Sialyl-galactosyllactose	S-GL	916.34	916.34	Hex3Neu5Ac1	46.2
Lacto-N-fucopentaose III	LNFP-III	974.38	974.38	Hex3HexNAc1Fuc1	48.5
Disialyllactose	DSL	1045.35	1045.38	Hex2Neu5Ac2	48.2
Sialyllacto-N-tetraose c	LSTc	1119.40	1119.42	Hex3HexNAc1Neu5Ac1	58.0
GOS DPs and MD2-8		**[M+H]** $^+$		**Composition**	
GOS3		625.24		Hex3	
MD3		625.24		Hex3	
GOS4		787.30		Hex4	
MD4		787.30		Hex4	
GOS5		949.35		Hex5	
MD5		949.35		Hex5	
GOS6		1111.40		Hex6	
MD6		1111.40		Hex6	
MD7		1273.46		Hex7	
MD8		1435.51		Hex8	

Table 3
Concentrations of oligosaccharides (mg/L) detected in infant formulas by UPLC-FLD profiling.

Infant formula	2′FL mg/L	3FL mg/L	3′GL mg/L	6′GL mg/L	3′SL mg/L	6′SL mg/L	LNnT mg/L	LNFP-III mg/L	LSTc mg/L	GOS[a] mg/L	MD[b] mg/L
NCC	29.4	<LoQ	125.2	109.7	22.1	Det[c]	<LoD	n.a.	<LoD	2257.5	303.1
NOS	1238.9	<LoQ	198.5	49.9	60.4	<LoQ	n.a.	n.a.	n.a.	297.5	387.0
NST	24.6	<LoQ	558.9	234.5	50.6	Det	n.a.	n.a.	n.a.	4425.6	223.5
PFU	1143.8	<LoQ	377.6	199.3	53.3	Det	n.a.	n.a.	<LoD	4311.7	154.1
OMN	<LoQ	<LoD	129.5	151.3	32.5	Det	n.a.	n.a.	<LoD	3657.8	13381.5
RDY	25.2	<LoD	895.2	261.6	0.0	Det	n.a.	n.a.	< LoD	4669.0	87.5
PRE	23.6	<LoQ	161.4	165.5	0.0	Det	<LoD	n.a.	<LoD	3988.6	2772.7
HAC	27.7	<LoD	60.3	69.4	18.8	Det	<LoD	<LoQ	<LoD	1698.8	1115.1
HST	39.6	<LoQ	97.1	79.8	47.1	Det	n.a.	<LoQ	<LoD	1808.6	71.1
BIO	41.2	<LoQ	88.4	16.7	72.0	<LoQ	n.a.	n.a.	n.a.	155.3	10931.0
KAB	23.0	<LoD	232.2	138.9	39.5	Det	<LoD	n.a.	<LoD	2643.5	950.5
RIC	<LoD	<LoQ	n.a.	n.a.	n.a.	n.a.	n.a.	<LoD	<LoD	106.0	26351.4
LoD	3.9		1.9	2.4	7.5		7.1		4.2		
LoQ	13.0	4.0	6.3	8.0	25.0	20.0	24.0	6.6	14.0		
HM (1 m)[d]	2457.0	401.5	5.8	29.1	136.4	493.4	187.5	262.3	237.1		

[a] Rough quantitation of cumulative of identified GOS peaks, including 3′GL and 6′GL.
[b] Total of maltodextrin peaks identified DP2–DP8.
[c] Det. Indicates presence of 6′SL was supported by LC-MS analysis, quantification was not possible due to co-elution with a GOS DP4 peak.
[d] Averaged from 40 random human milk samples at 1 month postpartum from the LifeLines NEXT cohort.

formulas OMN, BIO, PRE, HAC and RIC show addition of maltodextrins or starch on the nutritional label, which fits the observations of high maltodextrin (>1 g/L) (Table 3). These oligosaccharides can be rapidly degraded to glucose by salivary and pancreatic amylases and used as rapid energy source. In most cases the levels of maltodextrins detected is lower than the label suggests, probably due to larger maltodextrin/starch structures outside the separation gradient of the analytical method. In case of HAC and RIC, the reported number of starch/maltodextrins is much higher than detected, which is probably explained by the use of starch and long-chain maltodextrins, instead of only maltodextrins up to DP8. Interestingly, all other formulas show presence of maltodextrins (71–951 mg/L), even when there is no mention of maltodextrins or starch on the nutritional label. Some of the maltodextrins could stem from spray-drying of ingredients, where maltodextrins are

often used as drying and stabilizing agent. However, it is hard to explain 951 mg/L identified maltodextrins from just addition as drying agent in the production of the separate ingredients.

3.2. Determination of sialic acids in infant formulas

Total and free Sia (Neu5Gc, Neu5Ac and Kdn) were determined in infant formulas. All of the infant formulas contained Sia (Table 4). There were large differences between bovine milk-based formulas. The total Sia concentration ranged from 141 mg/L (PFU) to 293 mg/L (HST), more than a factor 2 difference. Our values are consistent with previous studies that the total Sia concentration ranged from 65 to 334 mg/L in bovine milk-based infant formulas (Herrmann et al., 2021; Lacomba et al., 2011; Martín et al., 2007; SøRensen, 2010; Spichtig et al., 2010;

Table 4
Concentration of total and free sialic acids in infant formulas determined by UPLC-FLD.

Infant formula	Total Sia mg/L	Neu5Ac mg/L	Neu5Gc mg/L	Kdn mg/L	Free Sia mg/L	Neu5Ac mg/L	Neu5Gc mg/L	Kdn mg/L	Total Neu5Ac/ Neu5Gc ratio
NCC	205.7	171.8	32.1	1.9	30.5	3.1	27.3	<LoQ	5.4:1
NOS	176.7	143.0	31.3	2.4	38.9	22.4	16.1	<LoQ	4.6:1
NST	179.6	151.6	25.7	2.3	29.8	12.7	16.8	<LoQ	5.9:1
PFU	140.9	118.6	20.8	1.5	29.3	11.2	17.7	<LoQ	5.7:1
OMN	256.7	223.3	30.9	2.5	25.8	8.2	17.3	<LoQ	7.2:1
RDY	159.2	123.1	32.7	3.5	25.0	10.7	13.9	<LoQ	3.8:1
PRE	187.7	130.9	51.8	5.0	31.2	11.8	18.8	<LoQ	2.5:1
HAC	244.0	215.7	26.2	2.1	21.6	5.7	15.9	<LoQ	8.2:1
HST	292.9	248.7	41.2	3.0	31.6	9.1	22.2	<LoQ	6.0:1
BIO	256.8	210.2	41.6	4.9	46.6	21.6	23.3	1.7	5.1:1
KAB	261.5	91.1	170.4	<LoQ	30.1	2.8	26.0	1.3	0.5:1
RIC	27.3	2.7	24.3	<LoQ	16.1	<LoQ	14.9	<LoQ	0.1:1
HM (n = 40)[a]	1126.5	1117.7	8.8	–	33.5	23.6	9.9	–	127:1

[a] Averaged from 40 random month 1 milk samples from the LifeLines NEXT cohort.

Wang et al., 2001; Wylie & Zandberg, 2018). OMN and BIO contained comparable amounts of total Sia (around 257 mg/L). RDY contained less total Sia than Nutricia powder formulas. Moreover, the concentration of total Sia in Nutricia preterm formula (ready to feed) (188 mg/L) was higher than in Nutricia normal formula (ready to feed) (159 mg/L). The formulas all contain significantly less Sia than human milk (1126.5 mg/L; Table 4). Neu5Ac was the major Sia in the bovine milk-based formulas. Total Nue5Ac ranged from 119 to 249 mg/L and total Neu5Gc from 21 to 52 mg/L among bovine milk-based formulas (Table 4). Regarding the individual forms, Neu5Ac is the dominant Sia in bovine milk-based formulas which is in line with all previous studies (Claumarchirant et al., 2016; Lacomba et al., 2011; Martín et al., 2007; Salcedo et al., 2011; SøRensen, 2010; Spichtig et al., 2010; Wylie & Zandberg, 2018)(Claumarchirant et al., 2016; Lacomba et al., 2011; Martín et al., 2007; Salcedo et al., 2011; SøRensen, 2010; Spichtig et al., 2010; Wylie & Zandberg, 2018). The total levels of Neu5Ac in our study were slightly higher than the 115–157 mg/L range reported by some studies (Lacomba et al., 2011; Martín et al., 2007; Salcedo et al., 2011), but were in line with other reports (Claumarchirant et al., 2016; SøRensen, 2010; Spichtig et al., 2010; Wylie & Zandberg, 2018).

Total amounts of Neu5Gc in our study were slightly higher than in previous studies (Claumarchirant et al., 2016; Lacomba et al., 2011; SøRensen, 2010; Spichtig et al., 2010; Wylie & Zandberg, 2018). A large variation of Sia concentration in bovine milk has been observed (Chen et al., 2014; Martín et al., 2007), which could partially explain the differences between studies. Furthermore, the Neu5Ac/Neu5Gc ratio was also different among bovine milk-based formulas. Powder formulas had a higher ratio than ready to feed formulas, term formulas contained a higher ratio than preterm formula. For powder bovine milk-based formulas, HAC had the highest ratio, which was two times higher than NOS. There was a higher Neu5Ac/Neu5Gc ratio in OMN than the remaining formulas which contained a comparable ratio. Interestingly, both HAC and OMN are hypoallergenic infant formulas, using hydrolysed bovine milk protein. In addition, infant formulas also contained limited Kdn, up to 5 mg/L (PRE). Small amounts of Sia are found in the free form in our study. The majority Sia is present in bound form, only 9 to 22% Sia in free form in bovine or goat milk-based infant formulas (Table 4). Interestingly, the ratio Neu5Ac/Neu5Gc is more strongly towards Neu5Gc in the free form Sia than in the bound fraction. In some cases, most of the Neu5Gc detected is in the free form (e.g. NCC 27 mg/L Neu5Gc free, 32 mg/L total). The amount of free Sia is comparable with that found in human milk (10 mg/L Neu5Gc free, 33 mg/L total).

In bovine milk-based formulas, one full whey-based bovine formula and five formulas with a whey: casein ratio of 60:40 were higher in total Sia and Neu5Ac content than those with a whey: casein ratio of 50:50 (S-Table 3). This trend has been previously reported: whey protein concentrates are a better source of Sia than casein (Lacomba et al., 2011;

Wylie & Zandberg, 2018). However, it is not the case for NOS, which contains a higher whey percentage but lower Sia levels. The same observation was found in a previous study, which could not be explained by whey/casein ratio differences (Claumarchirant et al., 2016; Wang et al., 2001). Moreover, compared to bovine formula in powder, there was a lower concentration of total Sia and Neu5Ac content in ready to feed bovine formula, both have the same whey and casein ratio as well as brand, suggesting that the dehydrating procedure and storage conditions do not significantly influence Sia concentration (Wylie & Zandberg, 2018). There is one ready to feed bovine formula for preterm infants in the present study, containing a higher Sia concentration than the normal ready to feed bovine formula. A previous study indicated that human milk of mothers of preterm infants contained more Sia than milk of mothers who delivered on term (Wang et al., 2001). However, preterm formula had a whey: casein ratio of 60:40, while term formula of the same brand had a whey: casein ratio of 50:50 in our study.

Neu5Gc was dominant in the goat milk-based formula. Goat milk-based formula (KAB) contained the second highest amount of total Sia (262 mg/L). In line with previous studies, formula produced from goat milk contains a high level of Sia and the levels of Neu5Gc exceeded those in all bovine milk-based formulas by more than 3 fold (de Sousa et al., 2015; Röhrig et al., 2017; Tolenaars et al., 2021; Wylie & Zandberg, 2018). It has been reported that Neu5Gc concentrations are higher than those of Neu5Ac in goat milk (de Sousa et al., 2015), fitting our observations on goat milk based infant formula. The role of Neu5Gc in human health is still unclear. It has been reported that Neu5Gc associated with chronic inflammation, cancer and ischemic heart disease(Pundir et al., 2020). Tangvoranuntakul et al. showed that exogenous Neu5Gc can be absorbed by human cells and incorporated it into newly synthesized glycoproteins in an *in vitro* study(Tangvoranuntakul et al., 2003) (Tangvoranuntakul et al., 2003). However, the metabolic fate of Neu5Gc in formula-fed infants remains uncertain.

The rice-based formula also contained limited amounts of Sia with higher levels of Neu5Gc than of Neu5Ac. The presence of Sia in a rice protein-based product is surprising, since plants do not produce sialic acid, while Neu5Ac and Neu5Gc only occur in animal-derived glycans. This would support presence of either an animal-based ingredient, or production in a facility where animal products are processed as well, resulting in traces of Sia-containing elements in this product.

As shown in Table 4, the total Sia and Neu5Ac levels in infant formulas are significantly lower than in human milk, as has been observed previously (Liu et al., 2022; Wang et al., 2001). Considering the suggested relevance of Sia in infant nutrition for infant cognition and brain development (Liu et al., 2022), adding a source of Neu5Ac Sia to infant formulas might be a step toward mimicking the molecular composition and functionality of human milk.

3.3. Measurement of corticosteroids in infant formulas

Five corticosteroids, i.e., cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-deoxycorticosteron, have been determined and quantified in infant formulas. The concentrations (nmol/L) observed for the steroid hormones included in this study are summarized in Table 5. To date, there are limited studies focused on infant formula hormone content (Barreiro et al., 2015). This is the first time to report five corticosteroids in infant formulas.

As shown in Table 5, each hormone varied in concentration among the different infant formulas. Cortisol was the dominant corticosteroid in infant formulas. BIO had the highest cortisol concentration (1.786 nmol/L), following is PFU (1.407 nmol/L), which is twice as much as in KAB (goat milk-based formula, 0.715 nmol/L). NCC only contained limited amounts of cortisol (0.096 nmol/L). While no cortisol was detected in OMN, HAC and RIC. Cortisone was the second most abundant corticosteroid in infant formulas after cortisol. Similar to cortisol, BIO had the highest concentration (0.360 nmol/L), followed by PFU (0.213 nmol/L), which is twice as high as KAB (0.151 nmol/L) and NST (0.133 nmol/L). However, no cortisone was detected in NCC, OMN, HAC and RIC. The concentration of corticosterone was highest in BIO (0.158 nmol/L), second in PFU (0.137 nmol/L). However, no corticosterone was detected in NCC, OMN, HAC and RIC. 11-deoxycortisol can be detected in all infant formulas, ranging from 0.002 to 0.031 nmol/L. Small amounts of 11-deoxycortisol were detected in the rice protein-based formulas, which is surprising. Besides goat milk based and rice-based formula, infant formulas also contained limited amounts of 11-deoxycorticosteron, with concentrations from 0.002 to 0.035 nmol/L.

According to corticosteroid concentrations from literature, the levels of cortisol and cortisone in infant formulas were obviously lower than those in human milk, i.e., 4–23 nmol/L cortisol and 11–33 nmol/L cortisone (van der Voorn et al., 2015). Moreover, the dominant corticosteroid in bovine and goat milk-based infant formulas was cortisol, while cortisone is dominant in human milk (Pundir et al., 2020; Toorop et al., 2020; van der Voorn et al., 2015, 2016; Xu et al., 2011). It has been noted previously that cortisol levels are higher than those of cortisone in bovine milk (Xu et al., 2011). Cortisol concentrations in bovine and goat milk-based infant formulas in our study were lower than those reported in native bovine and goat milk, i.e., 3.53 nmol/L in raw bovine milk (Xu et al., 2011) and 1.38 nmol/L in raw goat milk (Díaz et al., 2013). There are several factors that might influence cortisol levels in bovine and goat milk, like lactation stage (Gellrich et al., 2015), breed (Sgorlon et al., 2015), parity, mammary gland health status (Díaz et al., 2013), and time of the day (Romero et al., 2015). Moreover, processing conditions of the milk may influence the corticosteroid concentrations. Corticosteroids are hydrophobic compounds, partially associated with the fat fraction of the milk. In infant formulas most fat is derived from plant and fungal sources, with some added fish oil and very little milk derived fat to result in a composition more similar to that of human milk.

Corticosteroids are steroid hormones; inactive cortisone can be converted to active cortisol in the body. Corticosteroids play an important role in infant physiological, behavioral, metabolic and cognitive development (Hollanders et al., 2017). However, the concentration of corticosteroids in infant formulas is significantly lower than in human milk, especially cortisone. Moreover, it has been reported that exogenous factors might affect endogenous hormone balance (Hollanders et al., 2017). Further studies should determine what levels of corticosteroids in milk are functionally desirable and whether addition to infant formula would be recommended.

4. Conclusions

All infant formulas showed concentrations of maltodextrins, also when they were not mentioned on the nutritional label and are thus not incorporated into the nutritional values. Also, natural levels of MOS are present the infant formulas, although at limited concentrations compared with human milk. We found all the infant formulas contained Sia and corticosteroids, however, their concentrations are lower than in human milk. Moreover, the bioactive components are different among different infant formulas. Since the nutritional labels only reflect ingredients specifically added, there are some components with a significant presence, that is not reflected in the nutritional label. Most of the bioactive components were present in lower concentrations than in human milk collected at 1-month lactation. We would recommend further studies into extra addition of these functional bioactive factors in the early stage infant formula.

CRediT authorship contribution statement

Fan Liu: Conceptualization, Data curation, Methodology, Writing – original draft. **Jan van der Molen:** Data curation, Methodology. **Folkert Kuipers:** Supervision, Writing – review & editing. **Sander S. van Leeuwen:** Conceptualization, Data curation, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 5
Concentrations of corticosteroids in infant formulas determined by LC-MS/MS.

Infant formula	Cortisol nM	Cortisone nM	Corticosterone nM	11-deoxycortisol nM	11-deoxycorticosteron nM
NCC	0.096	n.a.	n.a.	0.003	0.015
NOS	0.348	0.099	0.060	0.004	0.005
NST	0.570	0.133	0.063	0.008	0.009
PFU	1.407	0.213	0.137	0.031	0.035
OMN	n.a.	n.a.	n.a.	0.007	0.008
RDY	0.393	0.073	0.074	0.005	0.007
PRE	0.556	0.064	0.082	0.005	0.011
HAC	n.a.	n.a.	n.a.	0.002	0.002
HST	0.288	0.067	0.038	0.005	0.012
BIO	1.786	0.360	0.158	0.027	0.021
KAB	0.715	0.151	0.057	0.006	n.a.
RIC	n.a.	n.a.	n.a.	0.003	n.a.
HM[a]	4–23	11–33	–	–	–

[a] Data derived from van der Voorn et al. (van der Voorn et al., 2015).

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113589.

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