Neurodevelopmental outcomes of healthy Chinese term infants fed infant formula enriched in bovine milk fat globule membrane for 12 months- A randomized controlled trial

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Running title: The role of MFGM on neurodevelopment and growth

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ABSTRACT

Background and Objectives: Human milk fat globule membrane (MFGM) has multifunctional health benefits. We evaluated neurodevelopment and growth of healthy term infants fed bovine milk-derived MFGM-enriched formula (MF) over 12 months. **Methods and Study Design:** A prospective, multi-centre, double-blind, randomized trial was conducted in Fuzhou, China. Healthy term infants (n=212), aged <14 days, were assigned randomly to be fed MF or a standard formula (SF) for 6 months and then switched to stage 2 MF and SF formula until 12 months. A reference group (n=206) contained healthy breastfed infants (BFR). Neurodevelopment was assessed with Bayley-III Scales. **Results:** At 12 months, the composite social emotional (+3.5) and general adaptive behaviour (+5.62) scores were significantly higher in MF than SF (95% CIs 0.03 to 6.79 and 1.78 to 9.38; p=0.048 and 0.004, respectively). Mean cognitive (+2.85, 95% CIs -1.10 to 6.80, P = 0.08), language (+0.39, 95% CIs -2.53 to 3.30, p=0.87) and motor (+0.90, 95% CIs -2.32 to 4.13, p=0.49) scores tended to be higher in MF than SF, but the differences between the two groups were not significant. BFR scored higher on Bayley-III than either MF or SF at 6 and 12 months. Cognitive scores were significantly higher in BFR than SF (95% CI 0.05 to 7.20; p=0.045), but not MF (p=0.74) at 6 months. Short-term memory was significantly higher in MF than SF at 12 months (95% CI 1.40 to 12.33; p=0.008). At 4 months, serum gangliosides were significantly higher in MF and BFR than SF (95% CI 0.64 to 13.02; p=0.025). Milk intake, linear growth, body mass and head circumference were not significantly different between formula-fed groups. **Conclusions:** MFGM supplementation in early life supports adequate growth, increased serum gangliosides concentration and improves some measures of cognitive development in Chinese infants.

Key Words: infant feeding, neurodevelopment, MFGM, complex milk lipids, gangliosides

INTRODUCTION

Suboptimal nutrition during the first 2 years of life affects brain development, ranging from neuroanatomy, neurochemistry, neurophysiology, and neuropsychology to long-lasting influences on cognitive events well into adulthood.[1] In the first 6 months, human milk is uniquely optimized for the needs of infant growth, neurodevelopment, and protection against infections and chronic diseases.[2,3] Breastfeeding is linked to higher intelligence in later life relative to formula feeding,[4,5] which may in part be due to compositional differences. The

milk fat globule membrane (MFGM)[6,7] of mammalian milks comprises three polar lipid layers consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, plasmalogens, gangliosides, cholesterol, protein, and membrane-specific glycoproteins. Gangliosides are sialic-acid-containing glycosphingolipids in milk almost exclusively associated with the MFGM.[8] All mammalian milk contains beneficial gangliosides, including human milk. These complex lipids, gangliosides and phospholipids, are also found in high concentrations in the brain, where they influence membrane structural fluidity, synapse formation, neurogenesis, information storage, neurotransmission, and memory formation.[9-11] Sphingolipids and cholesterol are essential components of myelin,[12] which is an electrical insulator and enhances cell signal transmission and cellular communication in the brain neural network.[13] These lipids also contribute to gut maturation and protection against respiratory and gastrointestinal infections in infancy and childhood[6,14] through the main mechanism of "unintended" target receptors for bacterial adhesion in specific tissues,[15] e.g gangliosides can be putative decoys that interfere with pathogenic binding in the intestine.[15]

Human milk is the gold standard for feeding infants. However, when breastfeeding is not possible, infant formula is the only suitable alternative. Infant formulae differ from breastmilk. The MFGM is present in human milk, but not in most infant formulae, because it is typically removed during cow milk processing to infant formula. Vegetable oils, which lack the bioactive lipid components of MFGM, have been used as the only source of lipids matching the fatty acid profile of human milk.[7] The recent commercial availability of bovine MFGM has increased its availability as a functional ingredient in infant formula.[16] Animal studies have shown that MFGM may alter brain lipid composition and functional and cognitive development, possibly through early up-regulation of the genes involved in brain function.[6,17-19] Clinical studies have shown that infants fed bovine MFGM supplemented formula, low in energy (60 in the treatment vs 66 kcal/100 mL in the control) and protein (1.20 in the treatment vs 1.27 g/100 mL in the control) from 2 to 6 months performed better in cognitive tests at 12 months using the Bayley Scales of Infant and Toddler Development III (Bayley-III) test, compared to infants fed standard formula in Swedish infants.[20] Milk lactoferrin with bovine MFGM resulted in a higher neurodevelopmental profile at 12 months and improved language subcategories at 18 months compared with a control group.[21] Infant formula supplemented with phospholipids and gangliosides from the MFGM and with higher arachidonic acid improved cognitive development in healthy infants aged 0–6 months.[22]

However, the responses in previous studies[20-22] may not be due entirely to MFGM, as experimental and control formulae varied in more parameters than their MFGM concentration.

In this study, we conducted a prospective, double-blind, 4-centre, randomized controlled trial to evaluate the effect of bovine MFGM as a source of gangliosides and phospholipids in the first 12 months of age in healthy term infants in Fuzhou, China. Two study sites (Fuzhou Women and Children Health Care Hospital (FWCHCH) and 2nd Fuzhou Hospital (2FH)) were situated in an urban region, and two other sites were set in a rural context. The exclusive breastfeeding rate was about 49-60% within 6 months of age across study sites. The primary outcome was the neurodevelopmental outcome at 12 months of age measured using the Bayley-III development test. The secondary outcomes were growth, neurodevelopmental outcome (Bayley-III) at 6 months of age, attention and short-term memory at 6 and 12 months, and serum ganglioside levels at 4 months.

MATERIALS AND METHODS

Ethics statement

The study was conducted in accordance with the guidelines of the Declaration of Helsinki,[23] the International Conference on Harmonization Guidelines on Good Clinical Practice, and all other relevant regulations. The study was approved by the Human Ethics Committee of Xiamen University (Ethic No. 20150817), FWCHCH, Fuqing Women and Children Health Care Hospital, 2FH, and Changle Hospital (Ethic Nos. 20151111, 20151110FQ, 20151110FZ, and 20160307 respectively). This trial was registered at the Australian New Zealand Clinical Trials Registry (ANZCTR 12616001571460). Parents or caregivers had been fully informed orally and in writing about the study. Written informed consent was obtained from the parents or caregivers of all infants before enrolment.

Participants

The study population consisted of healthy full-term infants aged <14 days who were born at the obstetric units and child healthcare units of four hospitals in the Fuzhou region, Fujian Province, China, between January 2016 and October 2016. The inclusion criteria were: (I) healthy newborn male and female infants regardless of mode of delivery (caesarean section or vaginal delivery); (II) gestational age between 37 and 41 weeks; (III) birth weight between 2500 and 4000 g; (IV) intention to predominantly breast feed (breast fed >90%) or formula feed (formula fed >60%) during the first 6 months after giving birth; (V) intention to remain in Fuzhou for approximately 15–18 months. The exclusion criteria were: (I) 5-min Apgar

score <7; (II) obvious cerebral and/or other major birth defects or evidence of genetic disease at birth; (III) mothers who were not expected to comply with exclusive breastfeeding or formula feeding. Mothers who were intending to breastfeed or were breastfeeding were invited to take part in the study in the breastfed reference (BFR) group.

The sample size calculation was based on Timby et al,[20] who reported an effect-size of four points (increase) and a standard deviation of nine points on the Bayley-III test between Swedish infants fed MFGM-enriched infant formula and those fed a standard infant formula (n=80 per group, p=0.008). Assuming a similar effect size and standard deviation, 88 infants per group were required for a power of 90% and a significance of 5% (based on a two-sided t-test). To allow for 25% dropout, 120 infants per formula-fed group were required. To allow for a greater dropout rate, 200 breastfed infants were included.

Randomization and blinding

A random permuted block design stratified by site (Fuqing, Fuzhou, and Changle) and by sex was used to generate the allocation schedule. The 2 study formula cans were labelled either in pink or blue. Parents/caregivers, investigators, and the research nurses were aware of the group assignment of breast feeding versus formula feeding. However, they remained blind to the MFGM-enriched formula (MF) until all infants had completed the intervention and analysis of the primary outcome had been conducted.

Formula composition and dietary intake

Infant formulae 0–6 months and follow-on formulae 6–12 months (Fonterra Co-operative Group Limited, New Zealand) were used for the MF and control formula (SF). Supplemented formulae contained minimum ganglioside concentrations of 17.9 mg/100 g (infant formula) and 16.9 mg/100 g (follow-on formula) and were manufactured using a bovine MFGM-rich ingredient as the source of gangliosides and milk phospholipids (SureStart™ MFGM Lipid 100; NZMP, Fonterra) from anhydrous milk fat production. The control formula was manufactured with the same macro- and micronutrient composition but without fortification with the MFGM-rich ingredient (Table 1). Both infant formulae met standard nutritional requirements for infants from 0-6 months and 6-12 months.[24]

At each visit, parents/caregivers were asked to complete a 24-h recall of breastfeeding, formula feeding and intake of complementary foods (Supplementary data). We reminded every parent/caregiver to record 24 hours of feeding via phone call or text message 1-2 days before each visit. They were also asked to report any medication or treatment initiated

throughout the trial. For product accountability, parents/caregivers in the formula-fed groups were asked to return empty formula cans at each clinical visit.

Assessment of cognitive function

The Bayley-III test for global cognitive ability assessment in infants at 6 and 12 months was performed by one trained pediatric psychologist for all participants throughout the trial. The Chinese attention and short-term memory test results from the Bayley-III cognitive assessment were also analyzed using a test method validated subsequently and standardized for young Chinese children.[25,26] These tests were based on 91 subtests of the cognition domain of Bayley III that were separated into "attention (28 subtests)" and "shorten memory (48 subtests)" by a Chinese pediatric specialist in psychiatry and psychology (supplementary data).

Assessment of growth

Physical examinations and growth measurements were made at baseline, 42±5 days and 4, 6, 8, and 12 months±5 days of age (Figure 1). Weight (HW-B70, Xiamen Zhonghenkang Technology Co. Ltd, China), recumbent length (HW-B70, Xiamen Zhonghenkang Technology Co. Ltd, China), and head circumference were recorded to the nearest 10 g, 0.1 cm, and 0.1 cm respectively at each visit. The WHO Child Growth Standards were used to convert weight, length, and head circumference into weight-for-age, length-for-age, and head-circumference-for-age Z-scores.[27,28]

Analysis of serum gangliosides

One mL of venous blood was randomly collected from 4-month-old infants in the BFR (n= 41), MF (n=26), and SF groups (n=24), with written consent from parents/caregivers. Extraction, isolation and identification of serum gangliosides were conducted using our published method[29] (Supplementary data). Briefly, the ganglioside species were separated on a Dionex UltiMate 3000 high performance liquid chromatography (HPLC) system (Thermo Scientific, MA, USA) equipped with an APS-2 Hypersil column (150 mm x 2.1 mm, particle size 3 μm; Thermo Electron Corporation, Waltham, MA, USA).[29] The sample injection volume was 10 μL. The eluate from the HPLC system was introduced into a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) using a heated electrospray ionization source.

The ganglioside species were confirmed using the HPLC retention time and the precursor ion or the daughter ion of mass spectrum from six ganglioside standards during fragmentation. The list of precursor and daughter ions was created by analyzing chromatograms of ganglioside standards and by referring to the literature.[30] Total gangliosides were estimated as the sum of the measured major molecular species, i.e. GD1a, GD1b, GD3, GM1, GM2, and GM3. The peak area for each ganglioside species was generated by the Thermo Xcalibur software using accurate mass extract. Quantification of each class of ganglioside was obtained by a specific linear equation generated from external standards. The peaks for GD1a (m/z 917 and 931), GD1b (m/z 917 and 931), GD3 (m/z 720 and 775), GM1 (m/z 1544), GM2 (m/z 1382), and GM3 (m/z 1151 and 1235) were used to quantify each class of ganglioside. The molecular species used for the quantification covered all gangliosides found in all serum samples.

Analysis of blood trace elements

The blood iron, zinc, magnesium, and calcium tests are a routine examination for 12 month-old infants in China according to "0~6 Years Old Children's Health Management Service Standards". These trace elements are essential for the growth and function of the brain.[31] Iron deficiency in the early stages of life results not only in acute brain dysfunction, but also in long-lasting abnormalities even after iron repletion.[32] Zinc influences the concentration of neurotransmitters in the synaptic cleft, probably via their interaction with neurotransmitter receptors, transporters and ion channels.[31] Magnesium plays an essential role in nerve transmission and neuromuscular conduction and protects against neuronal cell death.[33] Calcium regulates several neuronal functions, including neurotransmitter synthesis and release, neuronal excitability, and phosphorylation. Calcium is also involved in long-term processes, including memory.[34] Therefore, these blood trace elements were included as an important measurement for our study aim and as co-variates for Bayley III 12 months, as well as group comparisons. Capillary blood (40μL) was collected from the finger of infants. The samples were analyzed within 30 min using an automatic multi-element analyzer (Flame Atomic Absorption Spectrometry; BOHUI, H5300S, China). The concentrations of each trace element were calculated using a standard curve.

Statistical analysis

Comparison of individual Bayley III outcomes (cognitive, motor, and verbal scores etc), attention and short-term memory scores between MF and SF groups, and secondary pairwise

comparisons between 3 groups were analysed using a general linear model with univariate analysis adjusted for socioeconomic factors (maternal age, parental education, and family income), with group and sex as fixed effects, group and sex interaction, and site as a random factor, following the published method.[20]

Longitudinal analysis of the main effect of milk intake from 0-12 months, and weight-for-age, recumbent-length-for-age, head-circumference-for -age, and body mass index (BMI)-for-age Z-scores were carried out using general linear mixed model repeated measures analysis of variance (ANOVA) with six time points, adjusted for group, sex as a fixed factor, group and sex interaction, and site as a random factor (covariates), which is used to present infant growth from the enrolled date (0) to 12 months of age. To investigate potential different time trends within each group, we included the interaction between time and group in the model. Significant interaction terms indicate that the groups were changing in significantly different ways across time. Comparisons between means (with covariates sex and site) of growth outcome between the groups at six individual time points (14 and 42 days, then 4, 6, 8, and 12 months) and of blood ganglioside analyses were performed with a general linear model ANOVA using Bonferroni's adjustment for post hoc paired comparisons. All analyses were conducted on an intention-to-treat basis, where cases were included in the analysis irrespective of compliance with the intervention. All statistical analyses were completed with the use of SPSS for Windows 22.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Background characteristics, compliance, tolerance, and dropouts

There were no significant differences in delivery mode, birth weight, birth length, head circumference, and family income at birth between the three groups (Table 2), nor any differences at enrolment between the SF and MF groups, except that the mean maternal weight at pre-delivery of the SF group was 3 and 2 kg heavier than that of the MF and BFR groups respectively (p=0.045). BFR mothers were significantly younger, with a high rate of first-time births, and the parents were more educated than those of the formula-fed groups ($p \leq 0.001$, Table 2).

Infants (103 girls and 103 boys) were recruited into the BFR group, 108 infants (55 girls and 53 boys) were recruited into the MF group, and 104 infants (55 girls and 49 boys) into the SF group (Figure 1). From initiation, the dropout rates were 14.8% (MF), 20.2% (SF), and 12.1% (BFR). There was no significant difference in dropout rate among the formula-fed groups. The most common reason for discontinuation was related to formula intolerance, as

evidenced by constipation (MF, n=3; SF, n=5), vomiting (SF, n=1), and allergic reaction (SF, n=1). The most common reason for withdrawing from the BFR group was the perception of insufficient milk production. Other reasons were loss of contact, voluntary withdrawal, and inability to follow protocols.

The incidence of gastrointestinal events over 0–12 months was not significantly different across groups (MF, n=11; SF, n=14; BFR, n=12; p=0.055); the percentage of total adverse events were 55% (60/108) in the MF, 47% (49/104) in the SF and 47% (97/208) in the BFR respectively. Over the first 0–6 month period, skin rash [MF, n=4 (4%); SF, n=0 (0%); BFR, n=12 (6%); p=0.002] and upper respiratory infection [MF, n=7 (6.5%), ; SF, n=1 (1%); BFR, n=15 (7%); p=0.003] were the most frequent adverse events, and constipation (MF, n=3; SF, n=2; BFR, n=0; p=0.15) and diarrhea (MF, n=2; SF, n=3; BFR, n=5; p=0.35) were the most frequent gastrointestinal problems. The incidence of regurgitation and vomiting at 42 days, 4 or 6 months did not vary (p>0.05) between groups.

Dietary intake

There were no significant differences in daily intake of formula-milk volume, energy, protein, fat and carbohydrates between the MF and SF groups throughout the study (p=0.60-0.77, Table 3). The MF and SF groups consumed their study formula >90% of the time between 0-4 months and >85% between 4-6 months, the remaining percentage was breastfeeding. The milk consumption in the BFR was ~100% breast milk between 0-4 months and >85% between 4 - 6 months, the remaining percentage was other kinds of infant formula. Formula milk intake in the BFR gradually increased from 15% at 6 months to ~60% at 12 months (Table 3).

Bayley-III composite and scaled scores

At 12 months, the Bayley-III social emotional and adaptive behaviour composite scores were 3.50 (95% CI 0.03 to 6.79, p=0.048) and 5.62 (95% CI 1.78 to 9.38, p=0.004) points higher in the MF than in the SF group (Table 4). Although the cognitive score was 2.86 points higher in the MF group than in the SF group, the difference was not statistically significant (p=0.08), even when males and females were analysed separately (Supplementary Table 1A-B). No significant differences in the motor or language domains between the MF and SF groups were found, whether all infants were included in the analysis or when males and females were analysed separately (p=0.25-0.87, Table 4 and Supplementary Tables 1A-B). All composite

scores of the BFR group were higher than those for MF and the SF groups at 12 months (p=0.15-1.00, Table 4).

At 12 months of age, 173 BFR infants (95%), 83 MF infants (90%), and 77 SF infants (93%) had composite cognitive scores within the normal range, i.e. ≥85. Seven BFR, 7 MF, and 5 SF group infants presented a mild delayed performance with composite cognitive scores between 70 and 85. Two BFR, 2 MF, and 1 SF group infant were classified as expressing severely delayed development (<70). When the composite scores of the two infants with medically diagnosed severe neurodevelopmental delay (score=60) were excluded, the social emotional (+3.50) and adaptive behaviour (+5.62) scores were significantly higher in the MF than in SF at 12 months (p=0.048 and 0.004 Supplementary Table 2).

There were no differences in all Bayley-III composite scores between the MF and SF groups at 6 months (p=0.16-0.95, Table 5). The fine motor scores for BFR infants were significantly higher than for SF at 6 months (95% CIs 0.36 to 2.21, p=0.003, Table 5), but not than for MF (p=0.08, Table 5). Furthermore, when analysed separately, the cognitive (+5.24), motor domain (+7.99), and social emotional (+9.65) scores in male infants (Supplementary Table 3A) and the fine motor (+1.52) score in females (Supplementary Table 3B) of the BFR group were significantly higher than those of SF (p=0.001-0.040), but not MF (p=0.18-0.62, Supplementary Tables 3A-B). The BFR group performed significantly better in expressive language in both male and female, gross motor in males and overall motor domain in females compared with the formula-fed groups (p=0.001-0.041, Supplementary Tables 3A-B).

The composite cognitive (95% CI 3.33 to 6.95; $p < 0.001$), language (95% CI 3.71 to 5.90; p<0.001), and motor (95% CI 0.13 to 3.66; p=0.036) scores increased significantly from 6 to 12 months of age among all three groups, based on a general linear mixed model repeated measures analysis of variance (ANOVA) with two time points adjusted for group and sex as a fixed effect, group and time interaction, and site as a random effect (Supplementary Figure 1A-C). Differences in social emotional scores between 6 and 12 months were not significant among the three groups (p=0.18, Supplementary Figure 1D). The general adaptive scores did not improve from 6 to 12 months of age among the three groups (Supplementary Figure 1E). The results of the pairwise comparisons showed no significant differences in all scores between the MF and SF from 6 to 12 months of age (p=0.60-1.00), but there was a significant difference in the composite cognitive, and in the motor scores between the BFR group and the two formula-fed groups (p=0.001-0.018, data not shown).

Attention and short-term memory

The composite score of attention did not differ between MF and SF (p>0.05) at both 6 and 12 months of age, although their scores were lower than for BFR at 6 months (p=0.020), but not at 12 months (p=0.24, Figure 2). For short-term memory, the mean score for MF (102.15±1.87) was significantly higher than for SF (95.29±1.86) at 12 months (95% CI 1.40 to 12.33, p=0.002), but not at 6 months (p=0.35, Figure 2). The same trend was found when the raw scores were analyszed separately (Supplementary Figure 2A-B).

Growth assessment

All investigated growth parameters were within the normal range across groups, both when all infants were included in analyses or when male and female infants were analysed separately (Figure 3A–H). The time trends comparison for the growth parameters in the MF and SF showed that there was no significant difference in weight-for-age (p=0.60 and 0.57), length-for-age (p=0.90 and 0.98), head-circumference-for-age (p=0.30 and 0.82), and BMI-for-age (p=0.53 and 0.34) Z-scores in male and female infants. Although the weight-for-age, head-circumference-for-age, and BMI-for-age Z-scores at 42 days in both male and female infants for BFR tended to be higher than those of MF and SF, the overall differences were not significant (p=0.056-0.538, Figure 3A-H). However, the weight-for-age of female infants at 4 months of age was the highest in BFR following by SF and then MF, with the overall difference being significant (p=0.024, Figure 3B). The head-circumference-for-age of BFR male infants was slightly smaller than that of MF (p=0.013, Figure 3E) at 6 months of age and SF at 8 months of age (p=0.025, Figure 3E). The overall difference in the head-circumference-for-age between the three groups in male was statistically significant at 6 months (p=0.016) and 8 months (p=0.027), but this was not observed with females (Figure 3F). At 12 months of age, all anthropometric outcomes were not significantly different among study groups (Figure 3A-H).

Serum gangliosides

The MF group expressed significantly higher concentrations of serum GM3, GD3, GM1, GD1a, GD1b and the sum of these gangliosides than the SF group (p=0.001-0.045, Figure 4). All ganglioside concentrations were significantly higher in BFR than in SF (p=0.001-0.024, Figure 4). However, no significant differences between the BFR and MF groups (p=0.06-0.47, Figure 4) were observed for the summed and individual serum gangliosides at 4 months, with the exceptions of GM1 and GM3 (p=0.021 and 0.019, Figure 4).

Blood trace elements

MF and SF groups had similar concentrations of blood trace elements at 12 months (p=0.33-0.79, Figure 5). BFR tended to have higher blood concentrations of iron by 55.6% and 53.6%, calcium 51.6% and 49.2%, and magnesium 28.9% and 31.5% than the MF and SF groups, respectively. However, the overall differences between the groups were not significant (p=0.45 - 0.81, Figure 5).

DISCUSSION

Human milk is species-specific and uniquely optimized for the needs of the developing infant. Infant formula based on animal milk cannot mimic the complex nutritional composition of human breast milk, although some infant formulas supplemented with bovine MFGM have already been launched in several markets.[16] There is, however, not sufficient evidence for the health benefits of bovine MFGM enriched infant formula on neurodevelopment and growth in the early life of humans. The current study assessed the impact of an MFGM-supplemented infant formula, which supported normal growth, was well tolerated, and gave no indication of adverse effects. The Bayley-III test validated for use in Chinese infants[35-37] provided the cornerstone for the measurement of cognitive development in this study. The MF group had significantly higher scores for social emotional and general adaptive behaviour at 12 months than the SF group. Although the cognitive score for the MF group was higher than that of the SF group by 2.86 points, the difference between MF and SF groups were not significant (p>0.05, Table 4). There were no differences between the groups for language and motor skills at 12 months, and no differences between the two formula-fed groups for the composite scores at 6 months. Our findings were slightly different from recent reports by Timby et al[20] using the same Bayley-III version and Li et al[21] using an adapted Chinese Bayley-III version, which reported that MFGM supplementation of infant formula improved the mean cognitive scores at 12 months of age by 4.0 (95% CI 1.1 to 7.0) and 8.7 points respectively. Timby et al,[20] however, reported the MFGM formula contained lower energy and lower protein than the control formula. The formula used in the Li et al[21] study was supplemented with both bovine MFGM and lactoferrin. In our study, the two formulae differed only by the addition of the MFGM ingredient. Therefore, the nutrient intakes of infants in the current study were different from those in previously published trials.

In a further study, formula supplemented with a concentrated MFGM complex lipid improved cognitive development using the Griffith Scales at 6 month of age.[22] The present study utilized infant formulae containing similar ganglioside levels as the formula used by

Gurnida et al,[22] when analyzed using the same techniques (Rowan A., personal communication). The two formulae used by Gurnida et al also differed in arachidonic acid content[22]. Thus, neither the nutritional composition of infant formula nor the test method for cognitive function were the same between the current and previous studies. In the current study, other key nutrients for gut and brain development including DHA, prebiotics and probiotics were the same in the two formulae. Their presence may have improved cognitive outcomes in both groups, making it more challenging to observe a large difference due to the supplementation with MFGM.

Our results have shown that BFR infants generally had better performance in the Bayley-III test compared with the two formula-fed groups at 6 and 12 months (Table 4 and 5). At 6 months, the cognitive score of the BFR group was significantly higher than the SF group, but not the MF group (Table 5). MFGM supplemented formula-fed infants were more similar to the BFR group for cognitive scores than the SF group. These findings are consistent with previous studies demonstrating significantly higher mean Bayley-III cognitive scores for breast-fed infants compared to standard formula-fed infants.[20,21,38] A recent study using supplements of MFGM together with FOS, inulin, probiotics, and LCPUFAs, did not stimulate neurodevelopment measured as general movement at 2, 3 or 4 months of age, but some differences were reported for visual function at 12 months of age.[39] In the present study, there was progression in neurodevelopment in the two formula fed groups from 6 to 12 months, which approached the scores for the BFR. To our knowledge, this is the first randomized, controlled, and double-blinded trial to evaluate the effects of supplementation on neurodevelopment in healthy term infants with bovine MFGM alone for 12 months as measured by Bayley-III in Chinese infants.

To further assess the impact of infant feeding on cognitive ability, we analyzed the scores of the Chinese attention and short-term memory test generated from the Bayley-III cognitive score (Supplementary Table 4). Our key finding was that short-term memory with MFGM supplementation was 6.86 points higher than for the SF at 12 months (p=0.008), but not at 6 months (p=0.94). Importantly MFGM supplementation allowed attention and short-term memory to approach the level with breast feeding.

Gangliosides are important for the growth of neurons, synaptic connections, and memory formation.[11,40] High concentrations of gangliosides have been detected in both brain[41] and human breast milk[30] at different stages of lactation. The most abundant types of ganglioside in human milk are GM3 and GD3, expressed early and late in lactation, 30. We found the most abundant serum gangliosides in infants at 4 months of age were GM3, followed by GD3,

GD1b, GM1, GM2 and GD1a, a finding similar to previous reports.[22,42] We observed that the BFR group had the highest concentration of the serum gangliosides, followed by the MF group and then the SF group. This suggests that MFGM enrichment of infant formula may increase serum ganglioside concentrations to be more similar to that of the breast-fed infant, potentially supporting improved cognitive development.[10,22]

Human breast milk is the optimal source of nutrition for the early stage of human life, as it provides all the necessary nutrients for normal growth and development. The finding that all growth outcomes measured in this study at 42 days tended to be higher for BFR than those of MF and SF suggests that human breast milk is a species-specific food and composed of the exact nutrition for a baby's needs at this stage. We have investigated in this study the need for infant formulae to be designed to resemble breast milk as closely as possible in terms of composition and function. The growth results in the current study were within the WHO standards across the three groups. Therefore, the nutrients provided by the MF or SF formulae were sufficient to meet infant needs and were well tolerated.

The current study has some limitations. Although social emotional (+3.5) and general adaptive scores (+5.63) in the MF group were significantly higher than the SF group, this significance was achieved after we adjusted for all confounding variables. In addition, the cognitive score for MF was +2.85 points higher than that for the SF group, while ~100 subjects are required in each formula-fed group to detect differences of +5 between the groups. Further, the current study did not evaluate complementary food and its potential effect on neurodevelopment. The unexpected findings of a bit higher incidence of adverse respiratory infection in the BFR compared to the SF group at 0-6 months need further investigation. However, overall incidences of adverse event in the study groups were relatively low. The loss of individuals at consecutive follow-up in MF, SF, and BFR over 1 year may cause bias in the usual statistical analysis and the study outcomes. However, there are no universally applicable methods for handling missing data. We have used our best knowledge to conduct sensitivity analyses to encompass different scenarios of assumptions and to discuss the consistency or discrepancy among them. Overall key findings from this study were summarised in Figure 6.

Conclusion

MFGM-enriched formula in early life supports adequate growth and increases serum gangliosides levels, which may improve social emotional, general adaptive, and short-term memory measures of cognitive development in infancy using the validated Bayley-III

assessment. MFGM reduced the gap in cognitive development between breast-fed and formula-fed infants for all sub-scores of the Bayley-III test, suggesting that the MF group might resemble more closely the BFR group. However, the unique properties of human milk cannot be replaced by animal milk based or plant based infant formula for full and optimal development of human infants. Further studies with different MFGM levels in the formula and long-term follow-up studies are warranted to evaluate infant growth and the metabolic fate of MFGM gangliosides and phospholipids. This will assist in unravelling the true potential of MFGM in early life intervention on neurocognitive function and health in later life.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURE

All authors declare no competing interests.

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Table 1. Macronutrient and micronutrient compositions of the infant and follow-on formulae enriched in MFGM (MF) and the control infant and follow-on formulae (SF)

Nutrient per 100 mL	MF IF	SF IF	MF FO	SF FO
Energy (kcal)	67	67	68	68
Proteins (g)	1.7	1.7	2.2	2.2
Carbohydrate (lactose; g)	7.4	7.4	7.8	7.8
Fructooligosaccharides (mg)	58	58	77	77
Lipids (g)	3.4	3.4	3.2	3.2
Linoleic acid (mg)	458	497	408	459
α-linolenic acid (mg)	62	53	N.D.	N.D.
Arachidonic acid (mg)	8.9	8.5	7.8	7.0
DHA (mg)[†]	8.4	8.2	11.9	10.4
MFGM components				
Phospholipids (mg)	71.5	39.4	75.5	36.5
Phosphatidylcholine (mg)	20.6	11.0	21.3	10.3
Phosphatidylethanolamine (mg)	19.1	9.6	20.4	9.1
Phosphatidylinositol (mg)	6.9	4.5	6.9	3.9
Phosphatidylserine (mg)	6.7	3.3	7.2	3.2
Sphingomyelin (mg)	12.8	6.3	13.3	5.9
Gangliosides (mg)[‡]	2.5	1.4	2.7	1.5
Probiotic (*Bifidobacterium lactis* HN019; cfu)	1.0×10^8	1.0×10^8	1.0×10^8	1.0×10^8

N.D.: not determined; IF: infant formula for 0–6 months; FO: follow-on formula for 6–12 months; cfu: Colony-forming unit.
[†]Because of batch-to-batch variation and the necessity to produce two batches during the study, 35% of the infants received an MF or SF FO with 7.7 mg DHA/100 mL for the 6–12 months intervention period, and 24, 18, and 23% of the infants received, for the last month, 2 months, and 3 months of the intervention period respectively, an MF or SF FO with 9.6 mg DHA/100 mL. All other nutrients were present at the same levels across batches.
[‡]Measured as GD3 as described in Fong et al.[43]

Table 2. Demographic information for breast-fed reference (BFR), MFGM-enriched formula (MF), and standard formula (SF) groups

	MF (n=108) M: 53, F: 55	SF (n=104) M: 49, F: 55	BFR (n=206) M: 103, F: 103	*p* value† Overall
Caesarean section (%)‡	45 (41.7%)	36 (34.6%)	67 (32.5%)	0.17
Birth weight (kg)§	3.21±0.36	3.24±0.39	3.28±0.33	0.18
Birth length (cm)	49.51±1.72	49.63±1.92	49.54±1.40	0.84
Birth height (cm)	33.88±1.24	33.89±1.38	34.15±1.20	0.10
Maternal age (y)	29.38±5.00	29.69±5.00	27.70±4.56	0.001
Maternal weight (kg)	65.83±8.37	68.68±10.35	66.73±7.25	0.045
Maternal education (%)¶				<0.001
Higher education	25 (23.1%)	25 (24.0%)	84 (40.8%)	0.001
High school	34 (31.5%)	27 (26.0%)	73 (35.4%)	0.24
Middle school	41 (38.0%)	46 (44.2%)	48 (23.3%)	<0.001
Primary school	5 (4.6%)	5 (4.8%)	1 (0.5%)	0.026
Illiterate	3 (2.8%)	1 (1.0%)	0 (0.0%)	0.06
Paternal education (%)				<0.001
Higher education	23 (21.3%)	25 (24.0%)	81 (39.3%)	0.001
High school	32 (29.6%)	28 (26.9%)	70 (34.0%)	0.42
Middle school	46 (42.6%)	44 (42.3%)	51 (24.8%)	0.001
Primary school	7 (6.5%)	5 (4.8%)	3 (1.5%)	0.06
Illiterate	0 (0.0%)	2 (1.9%)	1 (0.5%)	0.22
Parity (%)				<0.001
1	34 (31.5%)	32 (30.8%)	99 (48.1%)	0.002
2	59 (54.6%)	50 (48.1%)	94 (45.6%)	0.32
3	11 (10.2%)	18 (17.3%)	13 (6.3%)	0.01
4+	4 (3.7%)	4 (3.8%)	0 (0.0%)	0.019
Family income†† (%)				0.19
>10,000 RMB	17 (15.7%)	10 (9.6%)	33 (16.0%)	0.28
5,000–10,000 RMB	47 (43.5%)	43 (41.3%)	90 (43.7%)	0.92
3,000–5,000 RMB	38 (35.2%)	34 (32.7%)	69 (33.5%)	0.93
2,000–3,000 RMB	3 (2.8%)	8 (7.7%)	4 (1.9%)	0.03
<2,000 RMB	1 (0.9%)	2 (1.9%)	0 (0.0%)	0.16
Unknown	2 (1.9%)	7 (6.7%)	10 (4.9%)	0.23

RMB: Ren Min Bi; M: Male; F: Female.
†A general linear model, group and sex as fixed effects and site as a random factor.
‡The data in parentheses shows the results in percentage within the group
§Mean±SE (same for all such values)
¶Maternal education at delivery
††Monthly family income

Table 3. Means (± SD) daily intake of formula-milk volume, energy, protein, fat and carbohydrates between MFGM formula and standard formula groups during first year of life[†]

Daily intake	MF			SF			MF vs SF	
	No.	Mean	SD	No.	Mean	SD	*p* value[†]	Adjusted *p* value[‡]
FF volume intake (ml/d)								
42d	96	823.17	286.91	82	890.67	268.01		
4M	92	966.74	271.09	82	937.13	351.08		
6M	91	959.51	294.17	81	958.46	275.55	0.55	0.65
8M	90	893.39	276.59	80	908.34	276.38		
12M	91	757.76	307.20	83	829.94	280.74		
Energy (kcal/d)								
42d	96	551.53	192.23	82	596.75	179.57		
4M	92	647.72	181.62	82	627.88	235.22		
6M	91	642.87	197.09	81	642.17	184.62	0.55	0.65
8M	90	607.50	188.08	80	617.67	187.94		
12M	91	515.29	208.89	83	564.36	190.90		
Protein (g/d)								
42d	96	13.99	4.88	82	15.14	4.56		
4M	92	16.44	4.61	82	15.93	5.97		
6M	91	16.31	5.00	81	16.29	4.68	0.50	0.60
8M	90	19.66	6.9	80	19.98	6.08		
12M	91	16.67	6.76	83	18.26	6.18		
Fat (g/d)								
42d	96	27.99	9.76	82	30.28	9.11		
4M	92	32.87	9.22	82	31.86	11.94		
6M	91	32.62	10.00	81	32.59	9.37	0.56	0.66
8M	90	28.59	8.85	80	29.07	8.84		
12M	91	24.25	9.83	83	26.56	8.98		
Carbohydrate (lactose g/d)								
42d	96	60.92	21.23	82	65.91	19.83		
4M	92	71.54	20.06	82	69.35	25.98		
6M	91	71.00	21.77	81	70.93	20.39	0.54	0.64
8M	90	69.68	21.57	80	70.85	21.56		
12M	91	59.11	23.96	83	64.74	21.90		
Meal size (ml/time)								
42d	96	97.92	19.86	82	99.39	19.99		
4M	92	135.00	26.61	82	132.44	31.84		
6M	91	151.98	36.33	81	149.51	34.40	0.64	0.70
8M	90	162.33	39.29	80	166.19	32.21		
12M	91	171.15	39.58	83	180.00	36.92		
Number of daily meals (Time/day)								
42d	96	8.38	2.24	82	8.96	2.04		
4M	92	7.21	1.56	82	7.05	1.96		
6M	91	6.37	1.50	81	6.49	1.67	0.70	0.77
8M	90	5.61	1.56	80	5.46	1.49		
12M	91	4.46	1.49	83	4.62	1.31		

[†]The main effects between groups were analysed using a general linear mixed model repeated measures analysis of variance (ANOVA) with six time points without adjustment.
[‡]adjusted for group and sex as a fixed effect, group and time interaction and site as a random effect.

Table 4. Bayley-III composite scores (mean±SE) at 12 months for the MFGM-enriched formula (MF), vs standard formula (SF) and breast-fed reference (BFR) group

	MF (n=92)		SF (n=83)		$p^{\dagger}$ (adjusted $p^{\ddagger}$)	BFR (n=182)		$p^{\dagger}$ (adjusted $p^{\ddagger}$)		
	Mean	SE	Mean	SE	MF vs SF	Mean	SE	Overall	BFR vs MF	BFR vs SF
Cognitive	97.61	1.35	94.75	1.34	0.77 (0.08)	97.48	1.18	0.08 (0.14)	-	-
Language	95.20	1.00	94.81	0.99	0.27 (0.87)	96.32	0.87	0.06 (0.38)	-	-
Receptive[§]	9.00	0.24	8.80	0.24	0.71 (0.51)	9.13	0.21	0.35 (0.49)	-	-
Expressive[§]	9.33	0.17	9.39	0.17	0.08 (0.61)	9.57	0.15	0.012 (0.45)	0.009 (-)	-
Motor	92.37	1.10	91.46	1.10	0.80 (0.49)	92.29	0.97	0.06 (0.75)	-	-
Fine motor[§]	8.81	0.13	8.74	0.13	0.89 (0.58)	8.91	0.12	0.43 (0.53)	-	-
Gross motor[§]	8.63	0.31	8.41	0.31	0.79 (0.56)	8.50	0.28	0.08 (0.84)	-	-
Social emotional	94.18	1.48	90.68	1.48	0.82 (0.048)	93.74	1.30	0.15 (0.10)	-	-
General adaptive	95.73	1.56	90.11	1.55	0.06 (0.004)	92.19	1.37	0.1 (0.01)	-	-

[†]*p* value without adjustment.
[‡]*p* value adjusted for maternal age, maternal and paternal education, family income and blood trace elements (iron, zinc, magnesium and calcium) at 12 months (the blood trace elements and Bayley III were measured at same day) using general linear model with univariate analysis group and sex as fixed effects, group and sex interaction, and site as a random factor. A randomized MF and SF comparison and a non-randomized BFR vs EF and SF comparison were analysed separately.
[§]Subscale with scaled score.

Table 5. Bayley-III composite scores (mean ± SE) at 6 months for the MFGM-enriched formula (MF) vs standard formula (SF) and breast-fed reference (BFR) groups

	MF (n=91)		SF (n=82)		$p^{\dagger}$ (adjusted $p^{\ddagger}$)	BFR (n=182)		$p^{\dagger}$ (adjusted $p^{\ddagger}$)		
	Mean	SE	Mean	SE	MF vs SF	Mean	SE	Overall	BFR vs MF	BFR vs SF
Cognitive	91.64	1.17	89.68	1.23	0.26 (0.21)	93.30	0.95	0.010 (0.05)	-	0.009 (-)
Language	90.53	0.60	89.31	0.63	0.18 (0.16)	90.49	0.49	0.07 (0.24)	-	-
Receptive[§]	7.12	0.17	6.83	0.18	0.24 (0.20)	6.65	0.14	0.22 (0.08)	-	-
Expressive[§]	9.57	0.10	9.42	0.10	0.33 (0.37)	10.06	0.08	<0.001 (<0.001)	<0.001 (<0.001)	<0.001 (<0.001)
Motor	86.24	1.45	85.08	1.53	0.56 (0.53)	92.55	1.18	<0.001 (<0.001)	<0.001 (0.001)	<0.001 (<0.001)
Fine motor[§]	7.41	0.30	6.96	0.32	0.32 (0.28)	8.24	0.25	<0.001 (0.003)	0.026 (-)	0.001 (0.003)
Gross motor[§]	7.99	0.23	8.04	0.24	0.96 (0.95)	9.24	0.19	<0.001 (<0.001)	<0.001 (<0.001)	<0.001 (<0.001)
Social emotional	90.03	1.63	89.61	1.71	0.89 (0.79)	93.62	1.32	0.023 (0.08)	-	-
General adaptive	97.12	1.10	96.93	1.15	0.97 (0.83)	98.57	0.89	0.17 (0.40)	-	-

[†]*p* value without adjustment.
[‡]*p* value adjusted for maternal age, maternal and paternal education, and family income using general linear model with univariate analysis group and sex as fixed effects, group and sex interaction, and site as a random factor. A randomized MF and SF comparison and a non-randomized BFR vs EF and SF comparison were analysed separately.
[§]Subscale with scaled score.

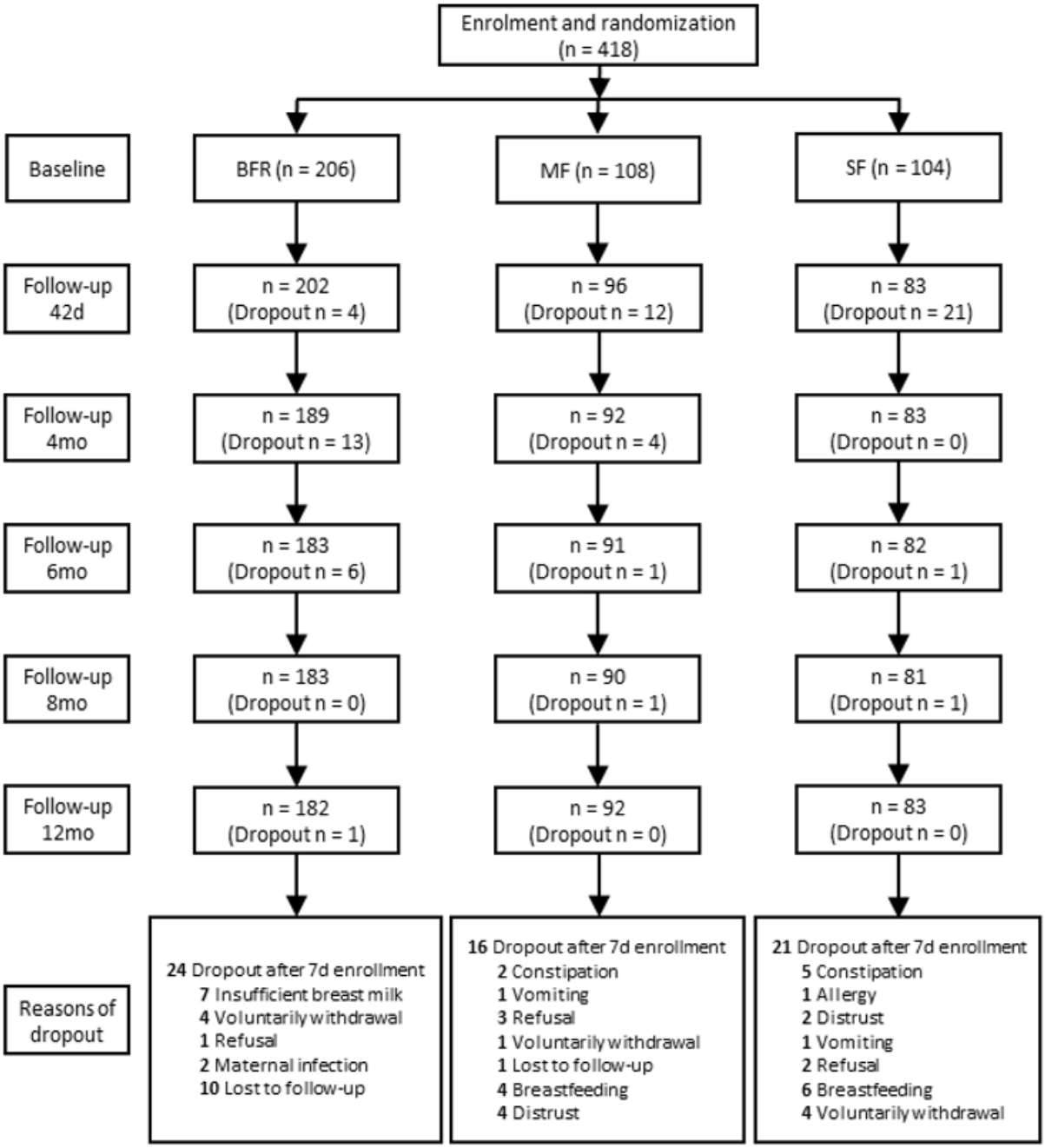
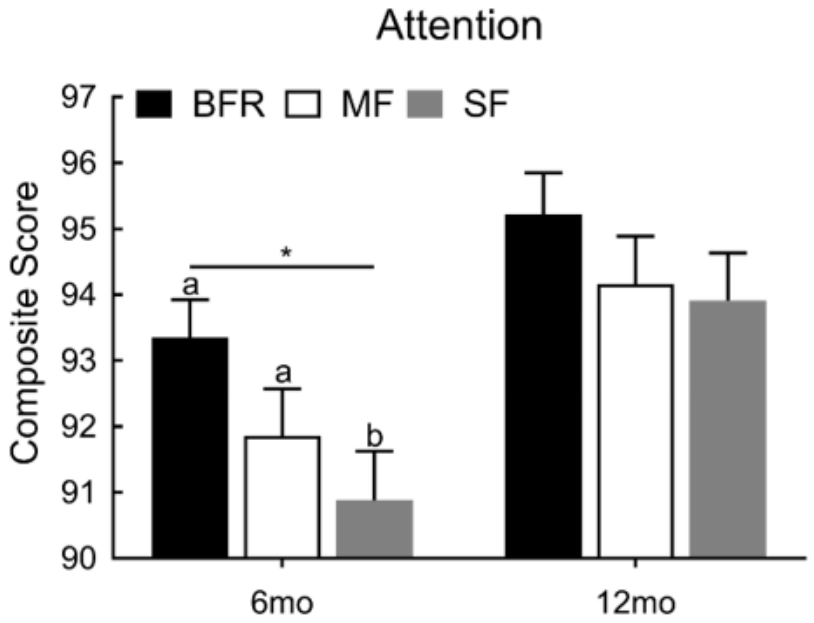


Figure 1. Flow chart of change in infant numbers in the MF, SF, and BFR groups during the study period and dropout reasons. MF: MFGM-enriched formula; SF: standard formula; BFR: breast-fed reference.

A



B

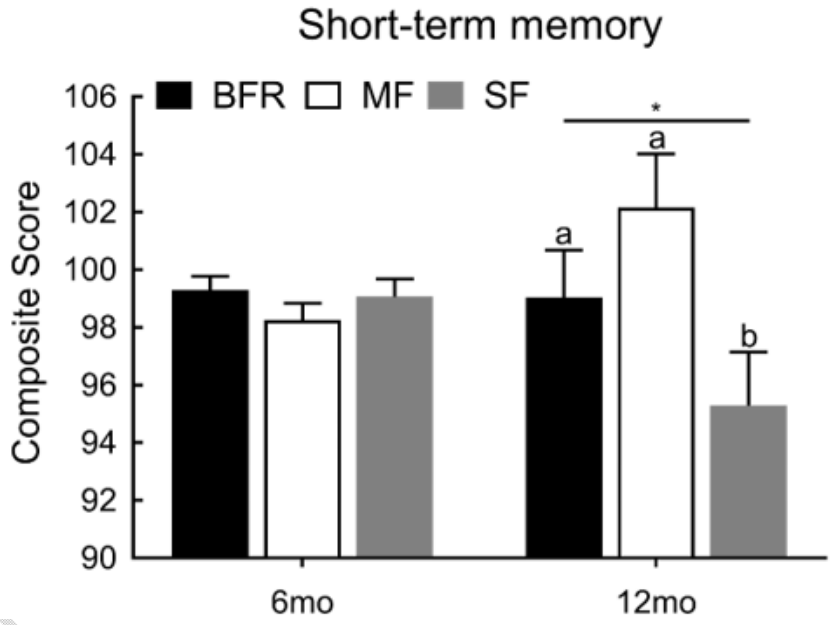


Figure 2. Comparison of composite attention and short-term memory scores, presented as mean ± SE, in the MF group (white column), SF group (grey column), and BFR group (black column) at 6 and 12 months of age. A. Composite attention score among the three groups. B. Composite short-term memory score among the three groups. MF, MFGM-enriched- formula; SF, standard formula; BFR, breast-fed reference. Data were analyzed using a general linear model with univariate analysis adjusted for socioeconomic factors (maternal age, parental education, and family income) with group and sex as fixed effects, group by sex interaction, group and time interaction and site as a random factor. $^{*}p<0.05$ (overall comparison). Columns bearing different letters within same timepoint are statistically significantly ($p<0.05$)

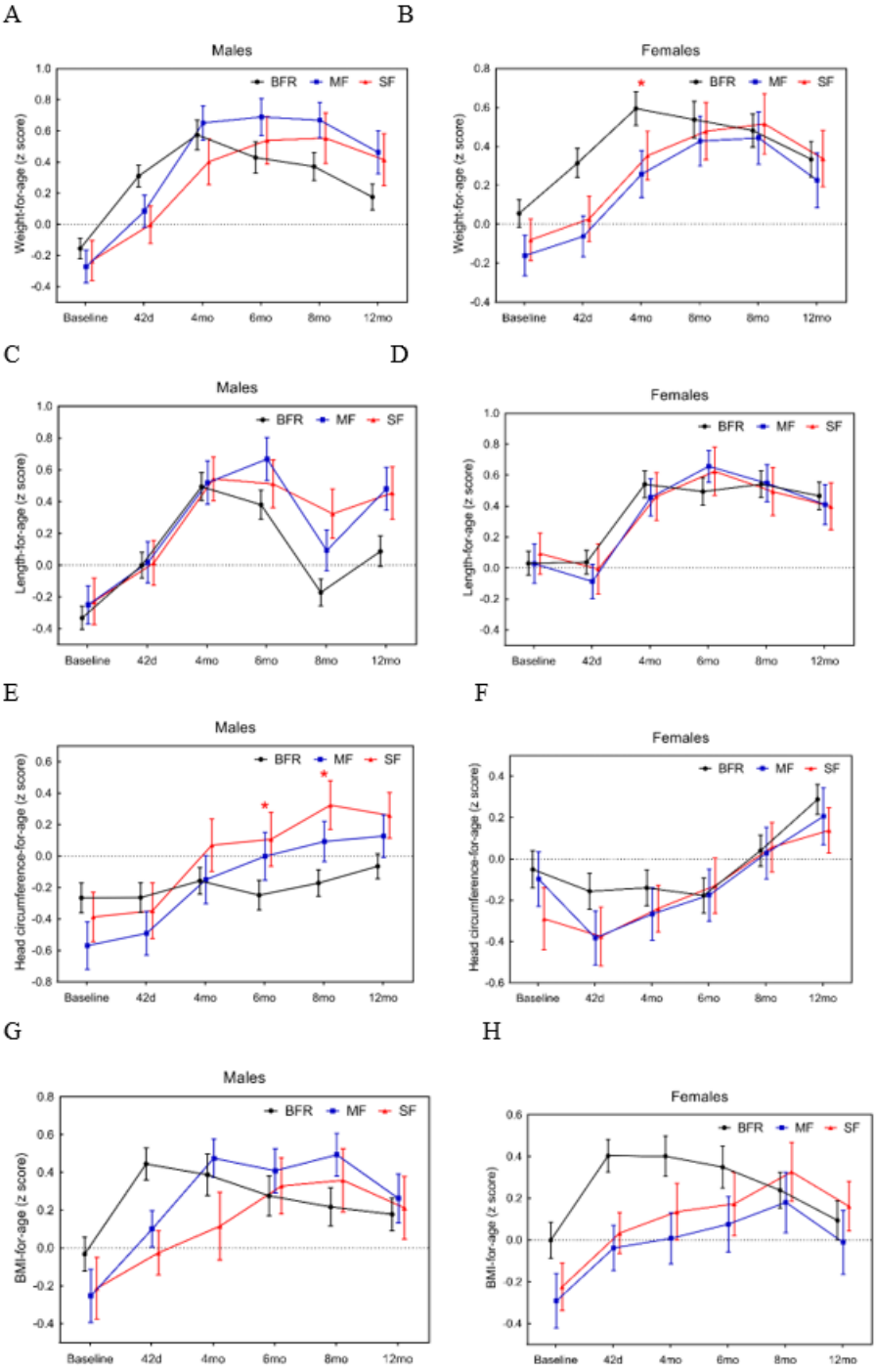


Figure 3. Dynamic variation of Z-scores (mean ± SD, 95% CI) of infants from 0 to 12 months in MF (blue line, n=108), SF (red line, n=104), and BFR (black line, n=206) groups. Weight-for-age Z-score in males (A) and females (B); length-for-age Z-score in males (C) and females (D); head-circumference-for-age Z-score in males (E) and females (F); BMI-for-age Z-score in males (G) and female (H). Data were analyzed using a general linear mixed model repeated measures with six time points adjusted for group and sex as fixed effects, group by sex interaction and site as a random effect with post hoc Bonferroni adjusted comparisons when the overall comparison was significant. To investigate potential different time trends within each group, we included the interaction between time and group in the model.

$^{*}p<0.05$ (overall comparison). MF: MFGM-enriched formula; SF: standard formula; BFR: breast-fed reference; BMI: body mass index.

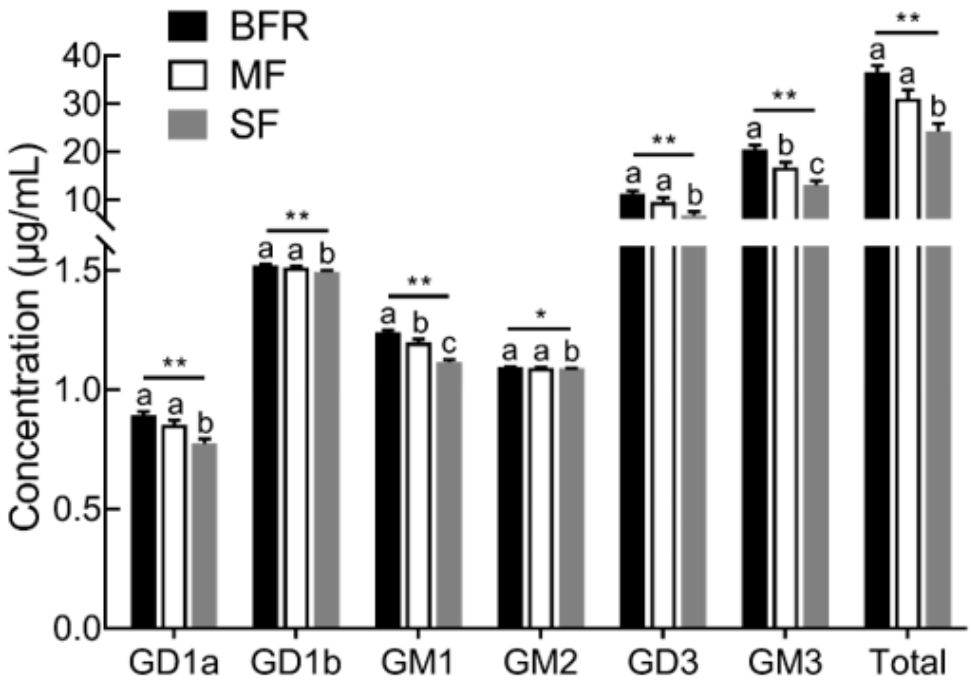


Figure 4. Comparison of the serum ganglioside concentrations (μg/mL; presented as mean ± SE) in the MF (white column, n=26), SF (grey column, n=24), and BFR (black column, n=41) groups at 4 months of age. Data were analyzed using a univariate general linear model. Group and sex were fixed effects, group by sex interaction and site was used as a random factor. Significant difference is indicated by asterisks $^{*}p<0.05$, $^{**}p<0.01$ (overall comparison). Columns bearing different letters within a group are statistically significantly (mean±SEM, $p<0.05$). MF: MFGM-enriched formula; SF: standard formula; BFR: breast-fed reference.

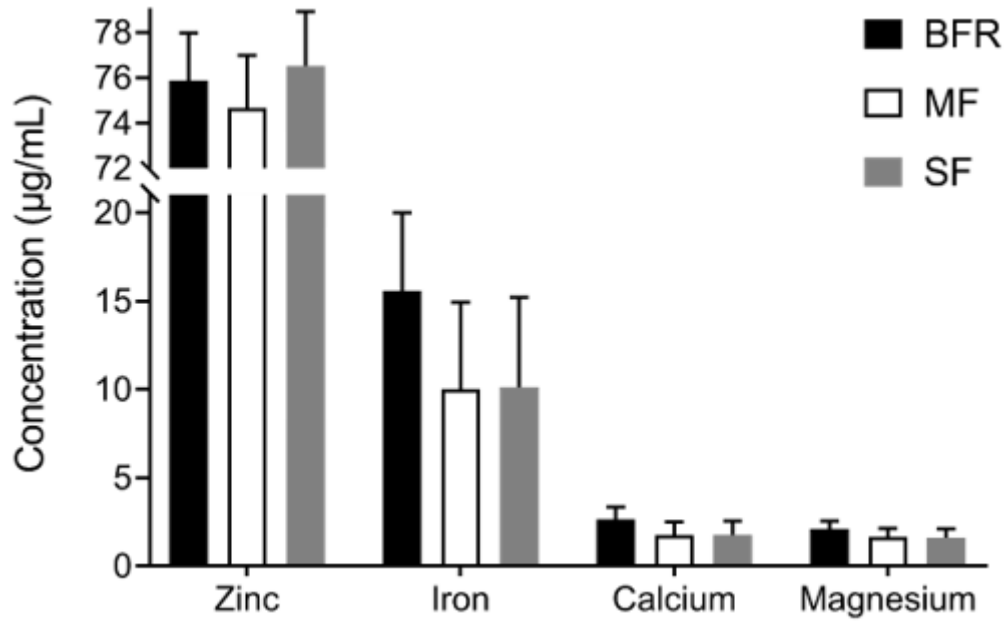


Figure 5. Comparison of the blood concentrations of zinc, iron, calcium, and magnesium (presented as mean ± SE) in the MF (white column, n=66), SF (grey column, n=60), and BFR (black column, n=122) groups at 12 months of age. Data were analyzed using a univariate general linear model. Group and sex were fixed effects, group by sex interaction and site was used as a random factor. MF: MFGM-enriched formula; SF: standard formula; BFR: breast-fed reference. The number at the top each column shows mean concentration of each blood trace elements.

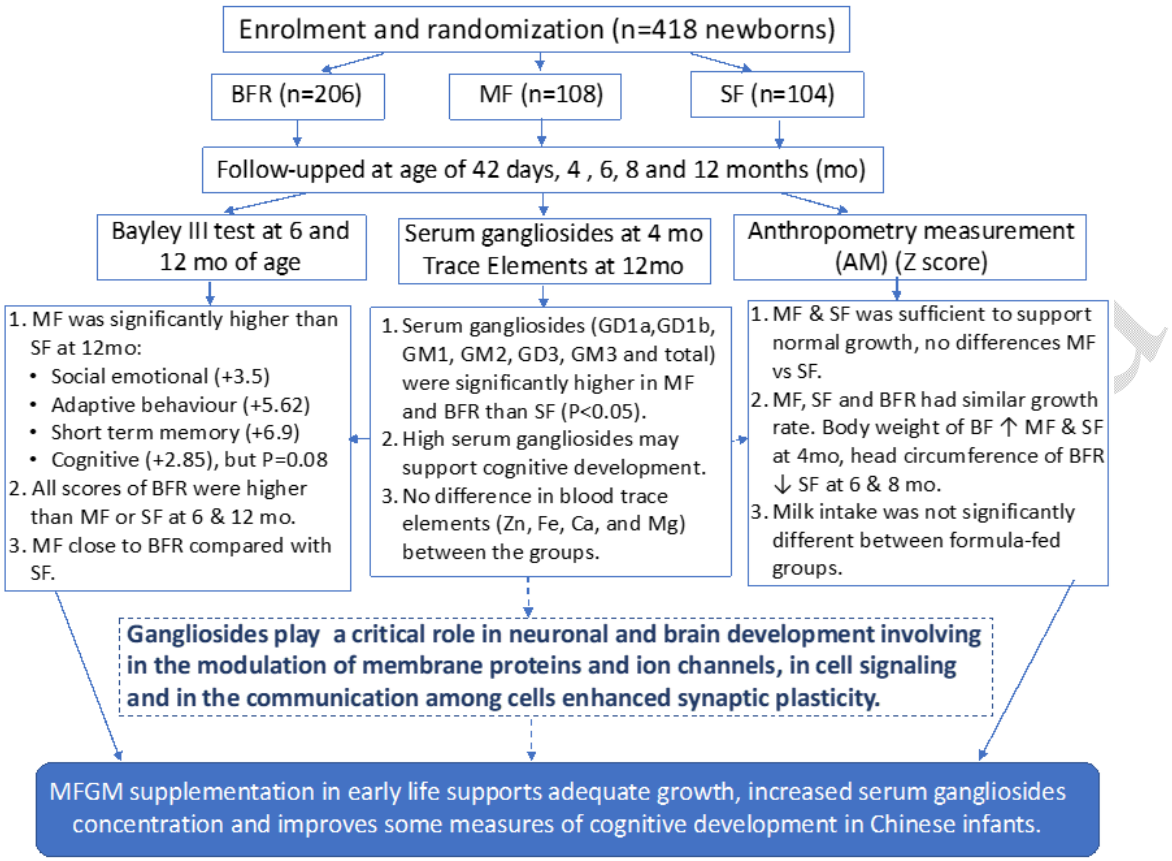


Figure 6. Summary of key findings. The mechanisms that might explain our findings are shown in bold in the figure. Zn: zinc; Fe: iron; Ca: calcium; Mg: magnesium. MF: MFGM-enriched formula; SF: standard formula; BFR: breast-fed reference.