Title	Lipidome analysis in brain and peripheral plasma following milk fat globule membrane supplementation in rodents
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Publication date	2022-09-06
Original Citation	Davies, R., van Diepen, J.A., Brink, L.R., Bijlsma, S., Neufeld, K.M., Cryan, J.F., O'Mahony, S.M., Bobeldijk, I. and Gross, G. (2022) 'Lipidome analysis in brain and peripheral plasma following milk fat globule membrane supplementation in rodents', Molecular Nutrition & Food Research, 66(22), 2200177 (8pp). https://doi.org/10.1002/mnfr.202200177
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://doi.org/10.1002/mnfr.202200177 - 10.1002/mnfr.202200177
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Lipidome analysis in brain and peripheral plasma following milk fat globule membrane supplementation in rodents

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Received: MONTH DD, YYY; Revised: MONTH DD, YYY; Accepted: MONTH DD, YYY
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.202200177.

ABBREVIATIONS

Abbreviation	Definition
ARA	Arachidonic acid
ApoE3L	Apolipoprotein E*3.Leiden
bMFGM	Bovine milk fat globule membrane
DHA	Docosahexaenoic acid
FC	Fold change
HM	Human milk
lysoPC	Lysophosphatidylcholine
MFGM	Milk fat globule membrane
NIST	National Institute of Standards and Technology
OPLS-DA	Orthogonal Partial Least-Squares Discriminant Analysis
PC	Phosphatidylcholine
PCA	Principal component analysis
SM	Sphingomyelin
TBHQ	Tertiary butylhydroquinone
TNO	The Netherlands Organization for Applied Scientific Research
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
VIP	Variable importance in projection

ABSTRACT

Scope

Milk fat globule membrane (MFGM) is an essential component of milk. Bovine MFGM (bMFGM) has been shown to support cognitive development and increase relative concentrations of serum phospholipids. This study investigated bioavailability of bMFGM components after oral administration in two preclinical models to explore whether dietary bMFGM induced parallel changes to plasma and brain lipidomes.

Methods and results

Transgenic APOE*3.Leiden mice (n=18/group) and Sprague-Dawley rats (n=12/group) were fed bMFGM-enriched (MFGM+) or Control diet, after which phospholipid profiles were determined in peripheral plasma, hippocampus and prefrontal cortex tissue by targeted mass spectrometry. Multivariate analysis of lipidomic profiles demonstrated a clear separation between MFGM+ and Control plasma samples across rodents. In plasma, sphingomyelins contributed the most to the separation of lipid patterns among both models, where three sphingomyelins (d18:1/14:0, d18:1/23:0, d18:1/23:1[9Z]) were significantly and consistently higher in the circulation of MFGM+ vs Control groups. A similar trend was observed in rat prefrontal cortex, although no significant separation of the brain lipidome was demonstrated.

Conclusion

bMFGM-enriched diet alters plasma phospholipid composition in rodents, predominantly increasing sphingomyelin levels in the systemic circulation with some similar, but non-significant, trends in central regions of the brain. These changes may contribute to the beneficial effects of dietary bMFGM on neurodevelopment during early life.

Key words: milk fat globule membrane, lipidomics, blood plasma, brain, rodents

INTRODUCTION

Optimal nutrition during the rapid growth and development of infancy plays a vital role in shaping a child's lifelong health trajectory. Breastfeeding provides short- and long-term health benefits for both the mother and child, and the World Health Organization recommends exclusive breastfeeding at least for the first 6 months of the child's life.[1] Unfortunately, breastfeeding is not always accessible for young infants; therefore, optimal nutrition in the form of human milk (HM) substitutes is essential. As HM is the gold standard for understanding pediatric nutrition, current research aims to identify its key bioactive components, and understand mechanisms of action underlying their benefits in infant growth and development. One component of interest present in all mammalian milk is milk fat globule membrane (MFGM), a triple-layer membrane complex that surrounds the secreted fat droplets and its composition includes, but is not limited to, sphingolipids, phospholipids, cholesterol, proteins, fatty acids and glycoproteins.[2, 3, 4]

Evidence on beneficial health effects of MFGM is accumulating, particularly with respect to brain development and function.[5, 6] In particular, SM, gangliosides, sialic acid, cholesterol and choline are components of MFGM that could contribute to effects on cognitive function in human and animal models.[7] MFGM was historically omitted from infant formula manufacturing as vegetable oils were used as the lipid source to produce formulations. However, recent technological advances have successfully provided the possibility to incorporate sources of bMFGM in infant formula, which better approximates the composition of HM lipids.[8]

During the past decade, studies have shown multiple benefits of infant formulas with added bMFGM, including improved brain development, support against pathogens, as well as preferable metabolic outcomes in infants.[9, 10, 11] Although, the exact underlying mechanisms of action are yet to be fully understood, it has been demonstrated in both clinical and

preclinical settings that added bMFGM in infant formula supports brain development.[9, 12] However, the bulk of preclinical evidence is based on studies of individual components, such as polar sphingolipids and phospholipids as well as gangliosides.[13] Phosphatidylcholines (PC) are phospholipid sources of choline, which is a vital nutrient for early brain development.[14] Sphingomyelins (SM), sphingolipids containing sialic acid, are involved in critical aspects of brain neuronal development, such as myelination and neuroplasticity.[15] Lysophosphatidylcholine (lysoPC) is an endogenous phospholipid found in micromolar concentrations in blood and has been associated with improved birth weight and infant development.[16] Phospholipids including SMs and gangliosides are two of the most important types of polar milk lipids present in MFGM. In infants, significant differences in the serum lipidome of infants that received bMFGM was largely due to the proportions of PCs and SMs. Also, phospholipids are functionally important and calculation of MFGM doses for other research studies has been based on phospholipid levels in breast milk.[17] Therefore, this was selected as the major polar lipid class to focus on.

Preclinical data have demonstrated that dietary bMFGM impacted plasma and serum lipid profiles.[13] In a double-blind, randomized, controlled trial, infants receiving bMFGM in infant formula, compared to a standard formula, had altered serum/plasma and erythrocyte membrane lipid profiles and increased circulating concentrations of PCs and SMs.[18] The beneficial neurodevelopment improvements seen with dietary bMFGM[9, 12] may be attributed to increased bioavailability of relevant MFGM lipid components, such as SMs, to the brain. As it is not ethically possible to access brain tissue from infants to test this hypothesis, we investigated if dietary bMFGM impacts brain lipid profiles similarly to changes in the systemic circulation in rodents. Using two rodent models (Apolipoprotein E*3.Leiden [ApoE3L] mice and Sprague-Dawley rats), rodents' diets were supplemented with bMFGM and changes in the circulating and brain lipidome were analyzed. We hypothesized that oral

intake of bMFGM impacts concentrations of MFGM lipid components within the systemic circulation and the brain.

EXPERIMENTAL SECTION

Animals and study design

Mice

Thirty-six male ApoE3L transgenic mice were bred at the animal facility at The Netherlands Organization for Applied Scientific Research (TNO)/InnoSer-Leiden. These transgenic mice, expressing APOE3Leiden, develop hyperlipoproteinemia because of defective binding of E3Leiden-containing remnant lipoproteins to the LDL receptor.[19] The ApoE3L transgenic mice thereby display a more human-like plasma lipoprotein profile and respond more comparably to humans, with respect to diet-induced hyperlipidemia and experimental manipulations. [20]

During the study, the mice were housed in clean conventional animal rooms in Makrolon cages (3–6 mice per cage) with a room temperature of 20–24°C, a relative humidity of 40–70%, and a light cycle of 7 am to 7 pm. Mice pups were fed by the dams during the first few weeks of life, and MFGM supplementation only started after weaning. Animals were randomized at 4 weeks of age into two groups, based on body weight, plasma lipids and blood glucose concentrations after a 5-hour fasting period.

From Week 4 to Week 12 of age, one group of mice (Control, n=18) received an AIN93G isocaloric control diet,[21] and the second group (MFGM+, n=18) received an AIN93G diet enriched with 2.3% (w/w) bMFGM (MFGM-10 Lacprodan; Arla Foods Ingredients) balanced on casein only. Through supplementation with MFGM-10, some whey protein was

also added to the diet of the treatment group (see **Error! Reference source not found.** for the full diet composition). Diet and sterilized tap water were available *ad libitum*. The mice in the current study were not sacrificed, only blood was collected. At 12 weeks of age, blood from the mice was taken after a 5-hour fasting period. The tail was fixed and the mice were allowed to move freely during blood collection so to avoid additional stress. An incision was made in the tail vein to collect tail blood using microvette tubes containing EDTA-dipotassium salt (CB 300 K2E microvettes; Sarstedt, Nürnbrecht, Germany). Tubes containing blood were placed on ice immediately. Plasma was obtained after centrifugation (10 minutes at 9000 x g) of the samples in a lab centrifuge at 4°C. Plasma (supernatant after centrifugation) was separated and stored in an Eppendorf vial and stored at <-70°C for further use.

The study was conducted in accordance with the general principles governing the use of animals in experiments of the European Communities (2010/63/EU) and Dutch legislation. The study was approved by the Animal Ethical Committee (DEC-3682) and the TNO animal welfare body (TNO-126).

Rats

Twenty-four male Sprague-Dawley rats were housed in the Preclinical Research Facility, Biosciences Institute, University College Cork, under a temperature of 21±1°C, with a relative humidity of 40–70% and a 12-hour light cycle of 7am to 7pm and fed *ad libitum*. Rat pups were fed by the dams during the first few weeks of life, and MFGM supplementation only started after weaning. The rats were randomized into two groups.

From Week 3 to Week 13 of age, one group of rats (Control, n=12) received an AIN93G control diet,[21] and the second group (MFGM+, n=12) received an AIN93G diet enriched with 1.5% (w/w) MFGM (MFGM-10 Lacprodan; Arla Foods Ingredients) balanced on

casein, corn starch, soyabean oil and calcium. Through supplementation with MFGM-10, some whey protein was also added to the diet of the treatment group (see **Error! Reference source not found.** for the full diet composition). Both diets had a blend of docosahexaenoic acid (DHA) and arachidonic acid (ARA) (5.3g/kg). Measurements for body weight were made weekly, water intake was measured daily, and the feed intake was measured three times per week (see **Error! Reference source not found.** for the full diet composition). Blood and brain samples were collected when rats were 13 weeks of age and processed as previously described.[22]. Blood sampling for rats was similar as to mice via tail incision, however, rats were not fasted before blood collection. Therefore, rats were restrained and the end of the tails was held with two fingers. Using a single edge razor blade a diagonal incision of 2 mm long was made at 15 mm from the end of the tail. Blood was collected in a collecting tube containing EDTA to avoid blood coagulation by increasing the pressure of the fingers on the tail above the incision. Blood was mixed with EDTA by gently inverting the tube and centrifuged at 3500 × g at room temperature for 15 minutes. Plasma was carefully aspirated and stored at -80°C. The whole brain was extracted, and both the hippocampus and the prefrontal cortex were dissected out on an ice-cold plate, snap frozen and stored at -80°C until further processing All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC, and approved by the Ethics Committee of the University College Cork and the Health Department, Dublin.

Lipidomic profiling of plasma and brain samples

Ultra-performance LC-MS/MS (UPLC-MS/MS) technology was used to identify phospholipids present in plasma and rat brain tissues (hippocampus and prefrontal cortex). Lipids were homogenized from the plasma samples (5 µl mouse and 10 µl rat plasma) using isopropanol; a polar extraction solvent. Brain lipids (15–30 mg) were extracted with a modified Bligh and Dyer extraction. The extraction solvents contained several internal

standards (C17:0 lysoPC, di-C12:0 PC and C17:0 ceramide). The extracted lipids were separated on a Waters Acquity Ethylene Bridged Hybrid C8 column [150 x 2.1 mm, 1.7 µm, temperature (T) = 50°C] using a mobile phase gradient from 98% mobile phase A [0.1% formic acid in water] to 100% mobile phase B (0.1% formic acid in acetonitrile: isopropyl alcohol 6:4) in 25.5 min with a flow of 0.5 ml/min. Mass detection was carried out using electrospray ionization in the positive mode (heater capillary temperature 300°C, spray voltage 4 kV, scan range m/z 202-1000), using the linear trap quadrupole Orbitrap MS System (Thermo Scientific, Breda, Netherlands). Injection volume was 2 µl. All measurements were performed in randomized order and a pooled sample (plasma) or pooled extract (brain) were used as quality control samples and were reviewed for instrument accuracy by principal component analysis (PCA). The National Institute of Standards and Technology (NIST) human plasma reference sample was measured between every 10 samples during the analysis procedure as a 1-point calibration. Identification of the targeted lipids was based on (relative) retention times and accurate mass of the lipids. The data was normalized relative to internal standards of the respective compound classes, i.e., for lysoPC, PC. For SM, the lysoPC internal standard was used. The results were displayed semi-quantitatively by including the analysis of commercially available (NIST) human plasma as a single calibration point.[23] A panel of 19 lysoPCs, 25 PCs and 14 SMs were reported (**Error! Reference source not found.**).

Statistical analysis

Raw data was converted using auto-scaling where each variable was scaled to mean of 0 and standard deviation of 1. Unsupervised PCA was used to identify outliers and provide an overview of multivariate data and quality control. Any outliers were identified using Hotelling's T^2, where observations outside the 95% confidence interval were removed from further analysis. Orthogonal Partial Least-Squares Discriminant Analysis (OPLS-DA) was

completed using the ropls package (version 1.26.0)[24] in Bioconductor, performed in R (version 4.0.2), where the number of orthogonal components was set to not applicable and the number of predictive components was set to 1.[24] The predictive performance of the model estimated by cross-validation was assessed by the cumulative Q2Y metric, where more predictive models are determined by Q2Y closer to 1. Raw data was inputted into OPLS-DA analysis as the ropls package automatically applied autoscaling as default. The number of orthogonal components was automatically computed using cross validation. The OPLS-DA analysis used a 'leave-one-out' cross-validation procedure using 7 cross-validation segments to rank the variables according to their importance. The number of random permutations of response labels to estimate R2Y and Q2Y significance by permutation testing was set to 20. Variable importance in projection (VIP) scores were used to estimate the importance of each variable in the model. Differences in relative concentrations of specific lipids between the MGFM+ and Control groups were conducted using Wilcoxon (Mann-Whitney) test and the correction for multiple comparisons was carried out using the Benjamini-Hochberg False Discovery Rate method. All statistical analyses were completed in R (version 4.0.2) and visualized using ggplot2 package. $q<0.05$ and $VIP>1$ were used to indicate statistical significance.

RESULTS AND DISCUSSION

Overall lipid composition across rodent models and sample type

Using UPLC-MS/MS targeted lipidomic technology, we investigated if oral intake of bMFGM induced changes in levels of systemic and brain MFGM lipid components, particularly lysoPCs, PCs and SMs, in rodents. Lipidomic analyses and statistical investigation were completed on a total of 58 lipids across rodents and sample types following identification and exclusion of outliers. One sample of each of the following was excluded: mouse plasma (Control), hippocampus (MFGM+), and prefrontal cortex (MFGM+). Two rat plasma (MFGM+) samples were removed. All outliers were from different animals, indicating that they were likely flagged due to technical variations rather than biological variability. Overall, food intake and animal body weight did not differ significantly between animals fed the bMFGM-enriched or control diets (data not shown).

Overall lipid composition across animal species and tissue types is depicted in Figure 1, illustrating that the distribution of plasma lipid classes (including SMs, lysoPCs and PCs) was similar between mice and rats. In rats, the relative proportion of lysoPCs was considerably greater in plasma compared with the brain tissues, whereas the relative proportion of PCs was higher in the brain. No significant MFGM+ vs Control difference was detected at the lipidome level for combined SMs, lysoPCs or PCs, or for total lipid concentrations (data not shown).

Effects of dietary MFGM on circulating lipid profiles

Dietary lipid intake is known to affect plasma lipid concentrations. Correspondingly, significant differences by univariate analysis were identified in plasma lipidome profiles of mice and rats that received the MFGM+ vs Control diets. Furthermore, multivariate testing was completed on the lipid profiles by OPLS-DA modelling, where significant plasma

responses were found for both the mouse and rat models. The predictive ability of the models, indicated by the Q2Y metric, was high for both models (mice: Q2Y=0.750; rats: Q2Y=0.882). Generated OPLS-DA of plasma lipidomic profiles demonstrated a clear separation between MFGM+ and Control samples across both mice and rats (Figure 2a and 2b). Separation between rodents assigned to MFGM+ vs Control was mainly attributed to higher concentrations of SM and PC species in mouse and SM species in rats (Table 1). VIP scores from the OPLS-DA where VIP>1 and corresponding significance values with $q<0.05$ across rodent plasma lipidomic analyses is shown in Table 1. Notably, 22 lipids in mice were significantly different for MFGM+ vs Control mice, with 10 SMs and 7 PCs increased in the MFGM+ group. Three circulating SMs were significantly higher for MFGM+ vs Control rats, which were also reflected among the highest, significant VIP values recorded in mice; namely SM (d18:1/14:0), SM (d18:1/23:0) and SM (d18:1/23:1[9Z]) (Table 1).

SM (d18:1/23:0) represented the most significant alteration in mice and the second strongest finding in rats (mice: VIP=1.92, log fold change [FC]=1.57, $q=8.01\text{x}10^{-5}$; rat: VIP=2.99, logFC=1.63, $q=5.65\text{x}10^{-3}$). In contrast, SM (d18:1/14:0) was the most changed in rats and was also among the top significantly changed lipids in the circulation of mice (mice: VIP=1.76, logFC=1.78, $q=2.80\text{x}10^{-5}$; rat: VIP=3.09, logFC=1.72, $q=5.07\text{x}10^{-3}$). Four plasma lysoPCs and one PC were significantly decreased in MFGM+ vs Control mice, specifically two structural isomers in *sn*-1 and *sn*-2 configurations for both lysoPC(20:4[5Z,8Z,11Z,14Z]) and lysoPC(22:6[4Z,7Z,10Z,13Z,16Z,19Z]), and PC(18:1[11Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]); Table 1).

While individual lipid species were listed, the relevance of changes in individual lipid species upon MFGM supplementation may be questionable. When focusing on overall changes in type of lipids, these data show that the number of SM species, as well as their fold change, were highest compared to the other phospholipid species that were analyzed. Changes in the

circulating lipidome were more apparent in the mouse vs rat rodent model. The choice of rodent models and background diet may have impacted these results. Also, the fasting in the mouse experiment may have minimized variation in the lipidome compared to the non-fasted rat samples, making the bMFGM-induced changes become more apparent. While the mouse experiment presented here was relatively simplistic with the addition of MFGM in animal diets, the rat experiment aimed to include more complex considerations into diet composition (balancing MFGM not only on protein, but also on fat; with DHA/ARA reflecting the routine addition of DHA to infant formula). Importantly, our data show that the impact of bMFGM on plasma lipid profiles from mice and rats was very similar, indicating that the addition of DHA did not majorly affect the lipid profiles in rats (with DHA) compared to mice (without DHA). Also, DHA/ARA was added to both the control and the MFGM+ group in rats, demonstrating that any changes induced by the addition of MFGM, in comparison to control animals, are due to MFGM+ supplementation rather than presence of DHA.

The effects of dietary bMFGM on lipidomic profiles were assessed in two different models for the purpose of adding strength and validity to the data. The overlap in effects of dietary bMFGM in both models highlights the changes that occur independent of experimental conditions or background diet composition. Hence, our data suggest that bMFGM most strongly impacts on circulating SMs compared to other phospholipids across preclinical models.

The ApoE3L mouse model was selected for its established translational characteristics of human lipid metabolism.[20] The predominant circulating lipoproteins in both humans and the ApoE3L mouse model are ApoB-containing lipoproteins (i.e., VLDL/LDL), whereas HDL particles prevail in the circulation of wild-type mice and Sprague-Dawley rat model, which was additionally used for the current study. The association of 63–75% of SMs with VLDL/LDL compared to ~25% associated with HDL[25] may explain why dietary bMFGM-

induced changes in the circulating lipidome were more apparent in the humanized ApoE3L mouse model than the Sprague-Dawley rat model. Lipoprotein profiles depicting the differences in lipid composition for the conditions of each rodent model (rats, mice and +/- bMFGM) could further clarify whether the dietary supplementation affects VLDL/LDL-C and HDL-cholesterol in rats or ApoE3L mice. Unfortunately, time constraints, along with repeated freeze thaw cycles, that are likely to have compromised the stability of the samples, explain why no lipoprotein profiles are presented in the current study.

These results are in line with a human study, in which Grip *et al.* described significant differences in the serum/plasma lipidome, mostly due to the proportions of SMs, PCs and ceramides, at 4 and 6 months of age in infants receiving an infant formula with or without added bMFGM.[18] Although ceramides were not measured in the current rodent analysis, these observations demonstrate that major bMFGM lipid components become available to the systemic circulation following dietary administration.

In an untargeted analysis of the lipidomic composition of bMFGM where 393 lipid species were identified, PCs (12.4%) and SMs (7.6%) were the second and third most abundant lipid classes.[7] This is in line with our finding that, in particular, SM and PC concentrations increased in the circulation of rodents after receiving dietary bMFGM. Because the current analysis cannot discriminate between endogenously or exogenously influenced metabolism changes, the reduction of some lipids in response to dietary bMFGM, including 2 lysoPC isoforms, is difficult to interpret. It is possible that dietary bMFGM may influence other metabolomic processes, which leads to this decrease in certain lipid species.

Milk fat globule membrane supplementation to the animals was not directly post-natal, rather, it was initiated relatively early in life (comparable to human toddler age) and relevant for pediatric nutrition. Preclinical models enabling nutritional interventions during the weaning period ("pup-in-a-cup") have been used in previous studies.[13] However, while this model

simulates intake during the "breastfeeding" period of a pup's life, there are several drawbacks to these models, such as the impact of the feeding procedure through gastric cannulas, influence of rodent milk substitutes and artificial rearing of the pups, making it difficult to compare with maternally reared reference animals. Furthermore, although there are parallels between this study and the research described by Moukarzel *et al*., other major differences allow this study to provide a novel investigatory angle on the infant situation. We analyzed MFGM exposure for a different duration and age of animal; Moukarzel *et al*. investigated pups fed every 10 minute in a 30-minute cycle from day 5 to day 18, in comparison to the 3/4-week-old pups fed *ad libitum* here. Lipid classes rather than specific lipid species are measured by Moukarzel *et al*. with particular emphasis on phosphatidylethanolamine and phosphatidylserine. Lastly, lipidomics in the plasma is analyzed in this investigation. Whereas, blood was not collected in the study by Moukarzel *et al*. and therefore it does not enable translation from circulation to brain levels.

Effect of dietary MFGM on rat brain lipid profiles

Lipidomic analyses were completed to determine whether lipid patterns observed in rat plasma after receiving dietary bMFGM were reflected in tissues from the central brain, hippocampus and prefrontal cortex. OPLS-DA analyses detected no significant differences in rat brain samples from MFGM+ and Control groups, thus VIP data was not obtainable. Rodent brain lipidome profiles were not clearly separated between MFGM+ and Control groups, although similar trends identified in plasma samples were observed for some individual SMs in the prefrontal cortex. Two lipids, PC (16:1[9Z]/22:2[13Z, 16Z]) and SM (d18:1/16:0), were modestly higher in the rat prefrontal cortex tissue for MFGM+ vs Control groups; however, no significant differences remained for lipids across brain tissues following Benjamini-Hochberg correction for multiple testing. Various factors could have contributed to the lack of significance in the differences between the brain data such as the

methodology chosen, or the numbers of animals included. For instance, compared to a more comprehensive lipid analysis, in this study a targeted phospholipid panel, including lysoPC, PC and SM, was applied which limits the assessment of the range of lipids or their metabolites that may be impacted by dietary bMFGM. Unlike levels of plasma SMs and PCs, their low abundance within the brain of rats may have resulted in any subtle changes that occurred to fall below the sensitivity level of detection by MS.

Similarities between the plasma and brain lipid profiles were observed in plasma after receiving dietary bMFGM, despite the detection of no significant differences in the brain samples. The similarities in the patterns of the three lipids, SM (d18:1/14:0), SM (d18:1/23:0) and SM (d18:1/23:1[9Z]), that were increased in rodent plasma and the two top lipids in the rat prefrontal cortex (PC [16:1 [9Z]/22:2[13Z, 16Z] and SM [d18:1/16:0]) across samples are visualized in Figure 3a and Figure 3b, respectively. In the brain, these lipid types were not significantly different between MFGM+ and Control groups, in either the hippocampus or the prefrontal cortex tissues. However, lipid profiles in the prefrontal cortex, particularly lipids PC (16:1 [9Z]/22:2[13Z, 16Z]) and SM (d18:1/16:0) (Figure 3b), did show similar trends to those observed in plasma. These two lipids also displayed the greatest difference between MFGM+ and Control groups, observed in the prefrontal cortex, and were significantly different prior to Benjamini-Hochberg correction for multiple comparisons. These lipids were not significantly increased in rodent hippocampus or plasma, with the exception of SM (d18:1/16:0), which was significantly increased in the plasma of mice in the MFGM+ group ($q<0.05$), but did not meet the VIP>1 threshold for inclusion.

Results in the current study illustrated that dietary bMFGM impacted plasma lipid profiles and these trends were similar for specific SMs in the prefrontal cortex. Collectively, our data demonstrates that dietary bMFGM components become bioavailable to the systemic circulation and may increase concentrations of lipid species in areas of the brain that can play

a role in the beneficial effects of dietary bMFGM. In particular, SMs are of key interest for their role in brain development. In a previous study, SM was quantified in infant nutrition products that participants received during the first three months of life.[26] Higher SMs were significantly associated with higher rates of change in verbal development in the first two years of life.[26] Potential mechanisms of action explored in preclinical models might include increased proliferation, maturation and differentiation of oligodendrocyte precursor cells, as well as increased axon myelination.

Concluding remarks

In conclusion, we demonstrated mice and rat pups receiving dietary bMFGM significantly impacted plasma lipid profiles with some similar, but non-significant, trends for specific SMs in the prefrontal cortex. In particular, higher concentrations of SM and altered concentrations of PC were detected in groups receiving dietary bMFGM. Changes observed in this study may contribute to the observed beneficial effects of dietary bMFGM previously reported on cognitive development in infants. However, further research is needed to confirm the observed effects of added bMFGM in infant formula on infant lipid metabolism. Larger sample sizes may additionally be needed to detect subtle changes in low abundant lipid species and improve the statistical parameters for the multivariate analysis. Future studies with advanced methodologies using a comprehensive lipid profiling that includes a wider panel of metabolites would provide further insight into the subtle changes that occur in the lipidome after receiving dietary bMFGM.

ACKNOWLEDGEMENTS

We would like to thank Brian M. Berg, Timothy G. Dinan, and Eric A.F. van Tol for their contributions to the studies underlying the sample collection and analyses described in this manuscript. Thank you to Shahzadgai Khan and Niche Science and Technology for their medical writing support with this manuscript.

FUNDING SOURCES

This study was financially supported by Reckitt|Mead Johnson Nutrition.

CONFLICT OF INTEREST

Rosalind Davies, Janna A. van Diepen, Lauren Brink and Gabriele Gross are all employees of Reckitt.

REFERENCES

1. Breastfeeding. World Health Organization 2018. https://www.who.int/news-room/facts-in-pictures/detail/breastfeeding [Accessed 09/11/21]
2. I.H. Mather, T.W. Keenan, J Membr Biol. **1975**, *21*, 65.
3. S. Patton, T.W. Keenan, Biochim Biophys Acta Rev Biomembr. **1975**, *415*, 273.
4. F.B.P. Wooding, J Ultrasruct Res. **1971**, *37*, 388.
5. T.A. Mudd, S.L. Alexander, K. Berding, R.V. Waworuntu, B.M. Berg, S.M. Donovan, R.N. Dilger, Dietary Prebiotics. **2016**, *4*, 2296.
6. V.L. Spitsberg, J Dairy Sci. **2005**, *88*, 2289.
7. L.R. Brink, B. Lönnerdal, J Nutri Biochem. **2020**, *85*, 108465.
8. J.H.J. Hageman, M. Danielsen, A.G. Nieuwenhuizen, A.L. Feitsma, T.K. Dalsgaard, Int Dairy J. **2019**, *92*, 37.
9. N. Timby, E. Domellof, O. Hernell, B. Lonnerdal, M. Domellof, Am J Clin Nutr. **2014**, *99*, 860.
10. O. Hernell, N. Timby, M. Domellof, B. Lonnerdal, J Pediatr. **2016**, *173S*: S60.
11. Y. Xia, B. Jiang, L. Zhou, J. Ma, L. Yang, F. Wang, H. Liu, N. Zhang, X. Li, P. Petocz, B. Wang, Asia Pac J Clin Nutr. **2021**, *30*, 401.
12. F. Li, S.S. Wu, C.L. Berseth, C.L. Harris, J.D. Richards, J.L. Wampler, W. Zhuang, G. Cleghorn, C.D. Rudolph, B. Liu, D.J. Shaddy, J. Colombo, J Pediatr. **2019**, *215*, 24.
13. S. Moukarzel, R. Dyer, C. Garcia, A. Wiedeman, G. Boyce, J. Weinburg, B.O. Keller, R. Eleango, S.M. Innis, Sci Rep. **2018**, *8*, 15277.
14. S.H. Zeisel, J Am Coll Nutr. **2013**, *23*, 621.
15. S. Sonnino, A. Prinetti, J Neurochem. **2016**, *137*, 485.

16. J. LaBarre, M. Puttabyatappa, P.X.K. Song, J.M. Goodrich, L. Zhou, T.M. Rajendiran, T. Soni, S.E. Domino, M.C. Treadwell, D.C. Dolinoy, V. Padmanabhan, C.F. Burant, Sci Rep. **2020**, *10*, 14209.
17. G. Bhinder, J.M. Allaire, C. Garcia, J.T. Lau, J.M. Chain, N.R. Ryz, E.S. Bosman, F.A. Graef, S.M. Crowley, L.S. Celiberto, J.C. Berkmann, R.A. Dyer, K. Jacobson, M.G. Surette, S.M. Innis, B.A. Vallance, Sci Rep. 2017, 7, 45274.
18. T. Grip, T.S. Dyrlund, L. Ahonen, M. Domellof, O. Hernell, Hyötyäinen, M. Knip, Lönnerdal, Oresšič, N. Timby, Pediatr Res. **2018**, *84*, 726.
19. A.M. van den Maagdenberg, M.H. Hofker, P.J. Krimpenfort, I. de Bruijn, B. van Vlijmen, H. van der Boom, L.M. Havekes, R.R. Frants, J Biol Chem. 1993, 268, 10540.
20. S. Zadelaar, R. Kleemann, L. Verschuren, J. de Vries-Van der Weij, J. van der Hoorn, H.M. Princen, T. Kooistra, Arterioscler Thromb Vasc Biol. **2007**, *27*, 1706.
21. P.G. Reeves, F.H. Nielsen, G.C. Fahey Jr, J Nutr. **1993**, *123*, 1939.
22. S.M. O'Mahony, K.A. Neufeld, R.V. Waworuntu, M.M. Pusceddu, S. Manurung, K. Murphy, Eur J Neurosci. **2019**, *51*, 1042.
23. A.E. Kozijn, M.T. Tartjiono, S. Ravipati, I. Bobeldijk, H. Weinans, R. Stoop, Osteoarthritis and Cartilage. **2018**, *27*, 118.
24. E.A. Thevenot, A. Roux, Y. Xu, E. Ezan, C. Junot, J Proteome Res. **2015**, *14*, 3322.
25. V.O. Oninla, B. Breiden, J.O. Babalola, K. Sandhoff, J Lipid Res. **2014**, *55***,** 2606.
26. N. Schneider, J. Hauser, M. Oliveira, E. Cazaubon, S.C. Mottaz, B.V. O'Neill, P. Steiner, S.C.L. Deoni, eNeuro. **2019**, *6*.

TABLES

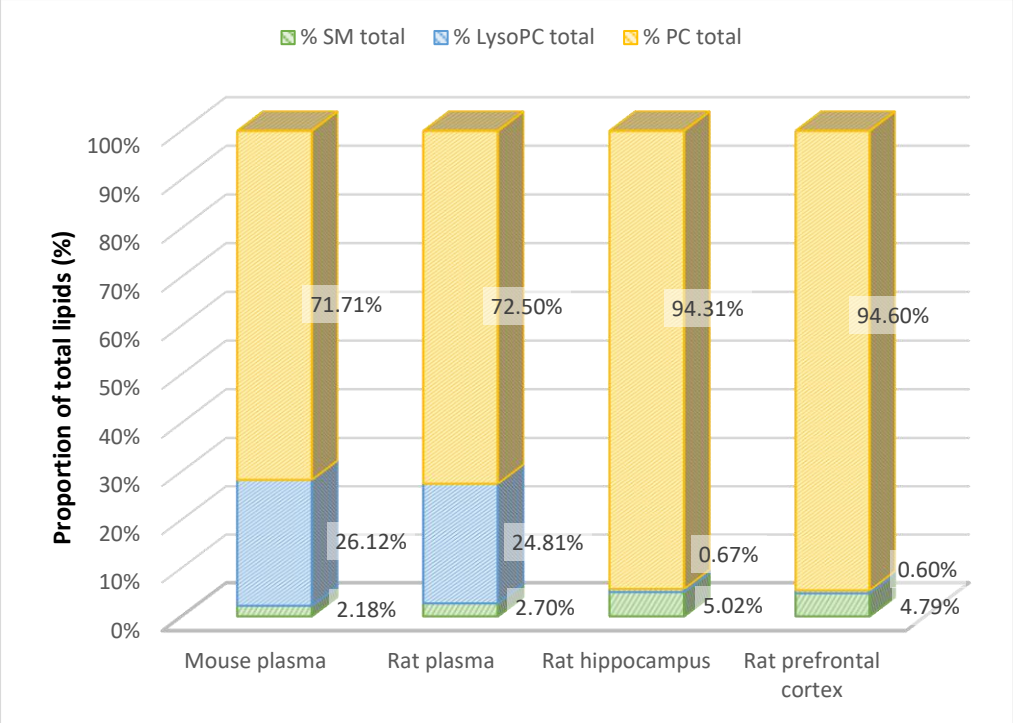
Table 1 Top differential lipids for MFGM+ versus Control in mouse plasma (top) and rat plasma (bottom). Lipids were considered significant when q<0.05 and VIP scores from OPLS-DA>1.

	Lipid name	HMDB ID	Lipid family	VIP	q-value	logFC
Mouse plasma	SM(d18:1/23:0)	HMDB12105	SM	1.92	8.01E-05	1.57
	PC(14:0/20:2(11Z,14Z))	HMDB07880	PC	1.78	1.18E-04	1.48
	SM(d18:1/22:0)	HMDB12103	SM	1.78	5.06E-04	1.34
	SM(d18:1/14:0)	HMDB12097	SM	1.76	2.80E-05	1.78
	SM(d18:1/20:0)	HMDB12102	SM	1.75	8.01E-05	1.54
	PC(14:0/18:2(9Z,12Z))	HMDB07874	PC	1.72	1.18E-04	1.47
	SM(d18:1/24:0)	HMDB11697	SM	1.65	5.16E-04	1.23
	SM(d18:1/23:1(9Z))	HMDB0240614	SM	1.52	1.18E-04	1.47
	LysoPC(20:4(5Z,8Z,11Z,14Z))-*sn*1	HMDB10395	LysoPC	1.45	2.93E-03	-1.10
	PC(14:0/20:3(5Z,8Z,11Z))-*sn*2	HMDB07881	PC	1.44	4.37E-04	1.19
	LysoPC(20:4(5Z,8Z,11Z,14Z))-*sn*2	HMDB10395	LysoPC	1.43	2.77E-03	-1.08
	PC(14:0/22:2(13Z,16Z))	HMDB07888	PC	1.36	7.99E-04	1.17
	SM(d18:1/18:0)	HMDB01348	SM	1.33	2.32E-03	1.14
	PC(14:0/20:3(5Z,8Z,11Z))	HMDB07881	PC	1.32	2.63E-03	1.14
	SM(d18:1/22:1(13Z))	HMDB12104	SM	1.22	3.71E-03	1.07
	SM(d18:0/16:1(9Z))	HMDB13464	SM	1.22	4.42E-03	0.97
	PC(14:0/20:1(11Z))	HMDB07879	PC	1.17	2.63E-03	1.09
	PC(14:1(9Z)/24:1(15Z))	HMDB07927	PC	1.16	2.63E-03	1.03
	SM(d18:1/18:1(9Z))	HMDB12101	SM	1.12	1.88E-02	0.89
	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))-*sn*2	HMDB10404	LysoPC	1.09	1.79E-02	-0.93
	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))-*sn*1	HMDB10404	LysoPC	1.07	2.02E-02	-0.90
	PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	HMDB08090	PC	1.02	3.46E-02	-0.86
Rat plasma						
	SM(d18:1/14:0)	HMDB12097	SM	3.09	5.07E-03	1.72
	SM(d18:1/23:0)	HMDB12105	SM	2.99	5.65E-03	1.63
	SM(d18:1/23:1(9Z))	HMDB0240614	SM	2.62	1.68E-02	1.45

FC=fold change; HMDB=Human Metabolome Database; ID=identification; PC=phosphatidylcholine; SM=sphingomyelin; VIP=variable importance in projection;

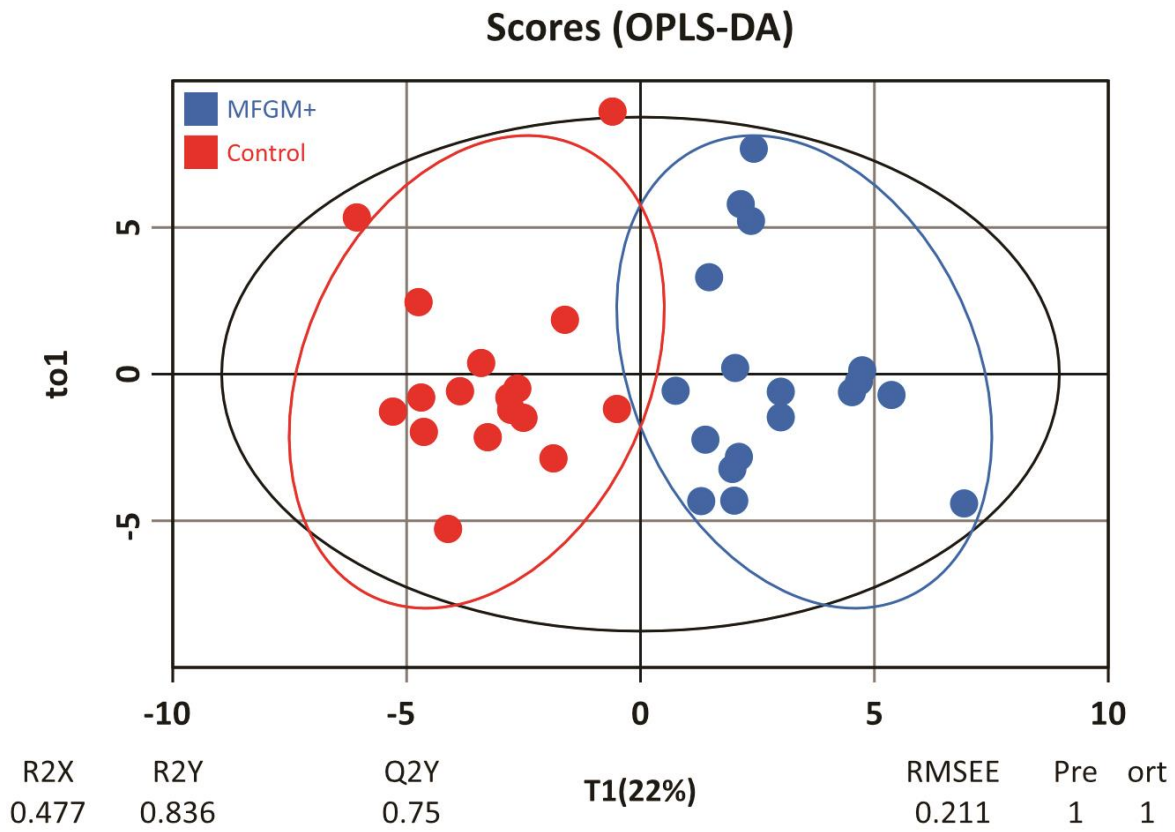
FIGURES

Figure 1: Proportional total lipid profile for each lipid family across tissue types. MFGM+ and Control samples are combined to observe differential profiles between plasma and brain tissues. No significant differences in lipid families were demonstrated between MFGM+ and Control groups (not shown).



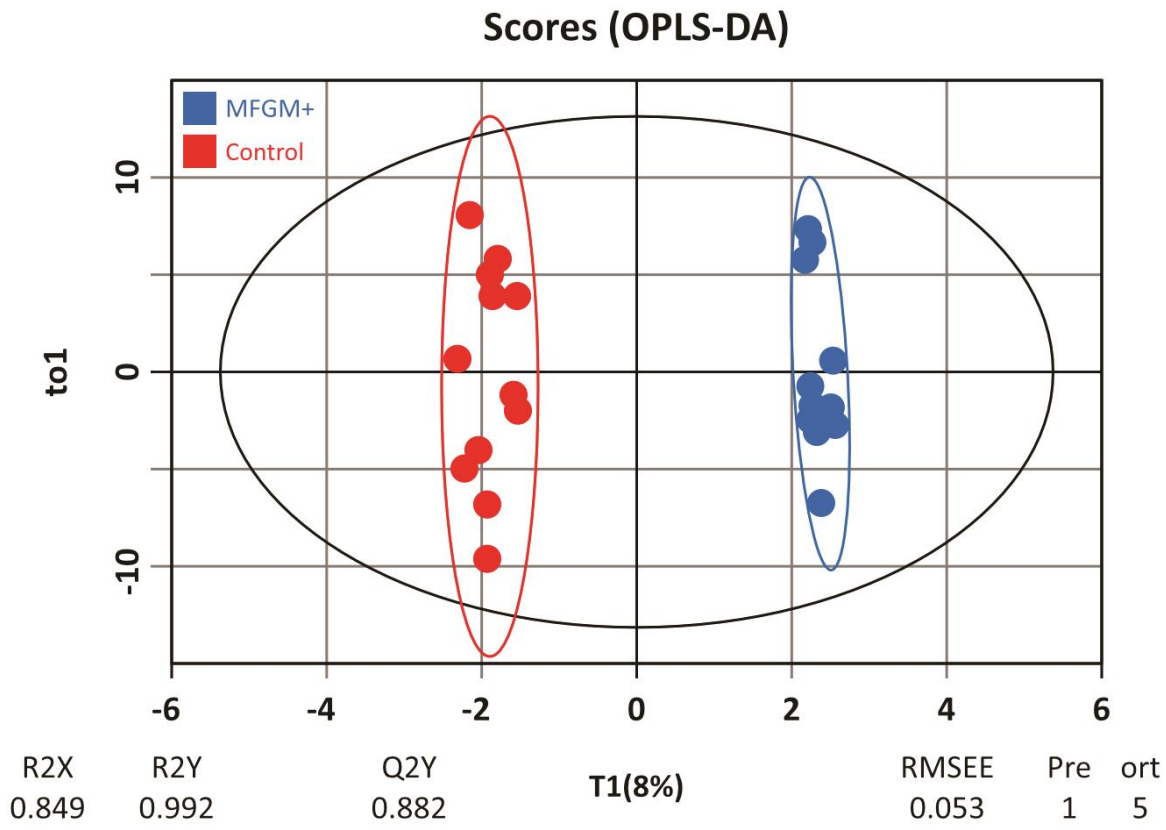
LysoPC=lysophosphatidylcholine; PC=phosphatidylcholines; SM=sphingomyelin

Figure 2A: Supervised OPLS-DA score plot. A 2-component model comparing the profile of 58 plasma lipids in mice from the MFGM+ (blue, n=18) and Control (red, n=17) group. A significant separation in the data estimated using OPLS-DA metrics (1 + 1, R2X (cum) 0.477, R2Y (cum) 0.836, Q2Y (cum) 0.750) is shown.



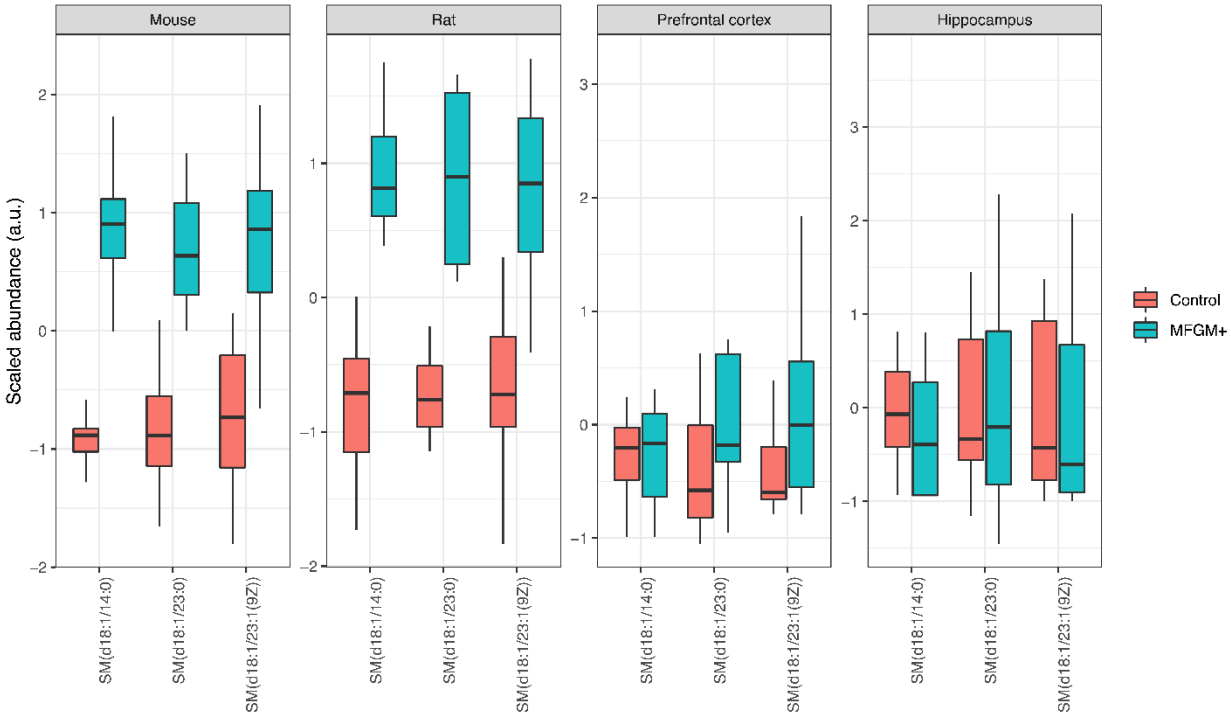
Con=control; MFGM=milk fat globular membrane; OPLSA-DA analysis=orthogonal partial least squares discriminant analysis

Figure 2B: Supervised OPLS-DA score plot of a 2-component model comparing the profile of 58 plasma lipids from rats in the MFGM+ (blue, n=10) and Control (red, n=12) group. A significant separation in the data estimated by OPLS-DA metrics (1 + 5, R2X (cum) 0.849, R2Y (cum) 0.992, Q2Y (cum) 0.882) is shown.



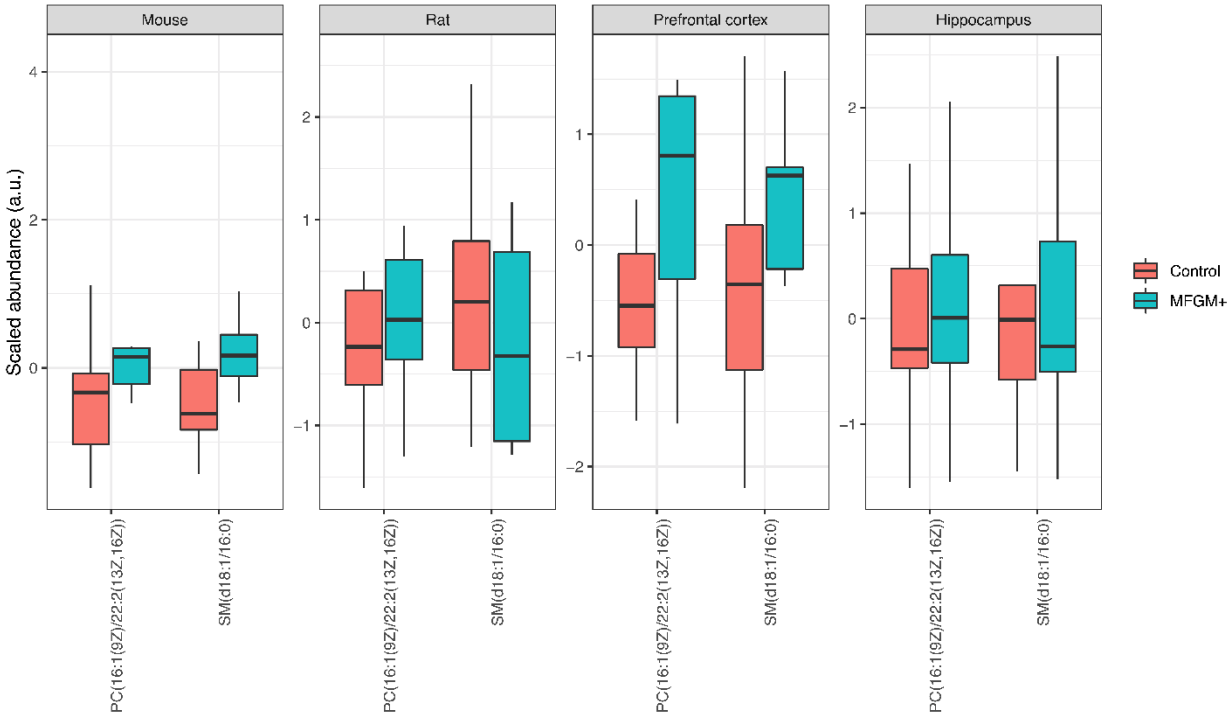
Con=control; MFGM=milk fat globular membrane; OPLSA-DA analysis=orthogonal partial least squares discriminant analysis

Figure 3A: Brain and plasma profile comparison for top 3 SM lipids, SMd18:1/23:1[9Z], SMd18:1/14:0 and SMd18:1/23:0, for MFGM+ (blue) versus Control (red) groups.



MFGM=milk-fat globular membrane; PC=phosphatidylcholine; SM=sphingomyelin

Figure 3B: Brain and plasma profile comparison for top 2 lipids in prefrontal cortex, PC(16:1[9Z]/22:2[13Z,16Z]) and SM(d18:1/16:0), for MFGM+ (blue) versus Control (red) groups.



MFGM=milk-fat globular membrane; PC=phosphatidylcholine; SM=sphingomyelin

Graphical abstract text

Milk fat globule membrane (MFGM) is a complex component of milk that contributes to neurodevelopment in infants. This study aimed to investigate the bioavailability of lipids in the blood and brain of rodents fed MFGM-enriched vs Control diets. The levels of lipids were analysed by mass spectrometry. A clear difference was observed in the lipid profiles from the blood of animals fed MFGM-enriched vs Control diets, with similar patterns also seen in the brain. These changes may contribute to the beneficial effects of dietary MFGM on cognitive development in early life.

Influence of milk fat globule membrane on plasma and brain lipidomes