Supplementation with milk fat globule membrane from early life reduces maternal separation-induced visceral pain independent of enteric nervous system or intestinal permeability changes in the rat

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ABSTRACT

Nutritional approaches have emerged over the past number of years as suitable interventions to ameliorate the enduring effects of early life stress. Maternal separation (MS) is a rodent model of early life stress which induces widespread changes across the microbiota-gut-brain axis. Milk fat globule membrane (MFGM) is a neuroactive membrane structure that surrounds milk fat globules in breast milk and has been shown to have positive health effects in infants, yet mechanisms behind this are not fully known. Here, we investigated the effects of MFGM supplementation from birth on a variety of gut-brain signalling pathways in MS and non-separated control animals across the lifespan. Specifically, visceral sensitivity as well as spatial and recognition memory were assessed in adulthood, while gut barrier permeability, enteric nervous system (ENS) and glial network structure were evaluated in both early life and adulthood. MS resulted in visceral hypersensitivity, which was ameliorated to a greater extent by supplementation with MFGM from birth. Modest effects of both MS and dietary supplementation were noted on spatial memory. No effects of MS were observed on enteric neuronal or glial networks in early life or adulthood, however an increase in the immunoreactivity of III-tubulin in adult colonic myenteric ganglia was noted in the MFGM intervention non-separated group. In conclusion, dietary supplementation with MFGM from birth is sufficient to block MS-induced visceral hypersensitivity, highlighting its potential value in visceral pain-associated disorders, but future studies are required to fully elucidate the mechanistic role of this supplementation on MS-induced visceral pain.

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1. Introduction

Stressful events during the early postnatal period have been shown to have particularly detrimental effects on host development and physiology by leading to long-lasting perturbations in several systems including the gastrointestinal (GI), endocrine (Heim et al., 2002; Osadchiy et al., 2019), peripheral and central nervous systems (Juruaena et al., 2020; O’Mahony et al., 2008; Osachiy et al., 2019). Early life stress (ELS) has been previously shown to result in heightened visceral sensitivity (Coutinho et al., 2002; O’Mahony et al., 2009; O’Mahony et al., 2020; Videlock et al., 2009) characterised by a diffuse sensation of pain centred around the midline of the body and upper abdomen (Sikandar and Dickenson, 2012). The diffuse nature of the sensation of visceral pain results in poor localisation to the site of pain and is due to a paucity of visceral sensory innervation (Sikandar and Dickenson, 2012). ELS is also a known risk factor for the...
development of both stress-related psychiatric disorders such as depression and anxiety in humans (Gougle et al., 2016; Scott et al., 2010) as well as functional GI disorders such as irritable bowel syndrome (Bradford et al., 2012). Dysfunctional communication between the gut and the brain via the microbiota-gut-brain axis is critical in the manifestation of these disorders, with studies showing a causal relationship between ELS, mood disorders, and gut dysfunction (Bradford et al., 2012) (for review see Cryan et al., 2019; O’Mahony et al., 2011; Sánchez et al., 2001; Wilmes et al., 2021). Maternal separation (MS) is a well-established rat model of ELS and gut-brain axis dysfunction (O’Mahony et al., 2011). In rats, MS has been shown to induce depressive and anxiety-like behaviours, increase gut epithelial barrier permeability and visceral sensitivity, and lead to stress hyper-responsivity (De Palma et al., 2015; Holochneider et al., 2016; McVey Neufeld et al., 2019; Moussaoui et al., 2017; O’Mahony et al., 2009; O’Mahony et al., 2011).

The enteric nervous system (ENS) is a network of neurons and glial cells embedded in the gut wall that is crucial for normal physiological GI function (Nezami and Srinivasan, 2010) and plays a key role in gut-brain axis signaling (Carabotti et al., 2015). MS has been shown to negatively impact on the ENS by increasing colonic cholinergic activity resulting in alterations in cholinergic regulation of epithelial permeability (Gareau et al., 2007a). Epithelial barrier integrity has also been shown to be compromised following MS, leading to bacterial translocation (Moussaoui et al., 2014). It has also been suggested that the ENS may play a role in the pathophysiology of visceral pain (Vergnolle, 2003). The ENS, spinal sensory afferent nerves, and enteric mast cells have been posited to play a role in visceral pain, however, mechanisms behind this are unclear (Wood, 2011).

Dietary interventions as strategies to ameliorate MS-induced psychopathology have proven effective with studies in rats using probiotics reporting a reversal of MS-induced depressive (Desbonnet et al., 2010) and anxiety-like behaviours (McVey Neufeld et al., 2019), as well as MS-induced gut barrier dysfunction (Gareau et al., 2007b). Modulation of diet for the symptomatic relief of GI disorders has also proven effective with probiotics having been shown to reduce abdominal pain in children with irritable bowel syndrome (Guandalini et al., 2010), as well as reduce the frequency and intensity of abdominal pain occurrences in school-aged children with functional GI disorders (Newlove-Delgado et al., 2017). Other studies have also reported reduced abdominal pain following administration of different probiotics (Ducrotté et al., 2012; Gawrońska et al., 2007; Whorwell et al., 2006).

In recent years, dietary interventions aimed at restoration of proper gut to brain communication have come to the fore as strategies for the management of symptoms of many mood disorders. For example, the Mediterranean diet has been shown to have positive effects by reducing the symptoms of depression in humans (Dinan et al., 2019; McMillan et al., 2011; Opie et al., 2018), while a diet high in polyunsaturated fatty acids (PUFAs) has been shown to reduce the symptoms of anxiety in a cohort of undergraduate college students (Yehuda et al., 2005). A combination of eicosapentaenoic acid and docosahexaenoic acid (DHA), two PUFAs, has also been shown to reduce anxiety-like behaviour in rats (Pusceddu et al., 2015a) and reverse selective effects of ELS (Pusceddu et al., 2015b).

Milk fat globule membrane (MFGM) is a triple layer membrane structure that surrounds milk fat globules secreted by mammary epithelial cells during lactation and has been shown to potentially confer health benefits (Boels et al., 2019). MFGM contains various bioactive factors including lactoferrin and probiotics, which are beneficial for the health of infants (Rourieu and Michalski, 2015; Brink and Lonnerdal, 2020). Breastfeeding has been shown to confer several beneficial effects on health including on cognitive scores (Quigley et al., 2012) and lowering the risk of obesity later in life (Owen et al., 2005). Not only this, but exclusive breastfeeding for a longer duration has been shown to reduce the incidence of GI tract infections in infants (Kramer et al., 2001). It has been proposed that MFGM exerts effects on neurodevelopment and cognitive function with preclinical evidence showing changes in brain development in piglets following dietary supplementation with probiotics, MFGM, and lactoferrin (Mudd et al., 2016).

Furthermore, studies in humans have reported higher cognitive scores in the Bayley scales of infant development at 12 months of age in those whose diets were supplemented with MFGM (Timby et al., 2014). MFGM has also been shown to have beneficial effects on gut physiology whereby MFGM altered intestinal epithelial architecture. Specifically, it was observed that MFGM increased ileal and jejunal villus length, indicators of intestinal health, in a dose-dependent manner (Bhinder et al., 2017).

We have previously shown that post-weaning administration of MFGM ameliorated MS-induced visceral hypersensitivity and lead to an improvement in spatial learning and memory (O’Mahony et al., 2020). We also noted that administration of MFGM from weaning facilitated a faster return of stress-induced corticosterone levels to baseline, whilst also exerting effects on the gut microbiota at the family and genus level. However, it is unclear if interventions would induce greater effects if they began at an earlier timeframe. Thus, based on existing evidence as mentioned above, this current study aimed to lead a novel investigation into the effect of supplementation with MFGM from birth on MS-induced behavioural and GI physiological changes in early life and adulthood, with a particular focus on visceral sensitivity.

2. Methods

2.1. Animals and housing

Male and female Sprague Dawley rats (approximately 8 weeks of age) were purchased from Envigo, UK and were mated in the Biological Services Unit, Western Gateway Building, University College Cork, and subsequent offspring were used in this study. The day of birth was designated as postnatal day 0 (PND0). Dams and littersmates were housed in large plastic breeding cages (15 × 9cm) in a humidity- and temperature-controlled room set to 21°C ± 1°C. The light/dark cycle was set to 12 hours (light phase 7am-7pm). All experiments were conducted in accordance with European Directive 2010/63/EUC, the requirements of S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork.

2.2. Maternal separation model

Maternal separation was carried out as described previously (O’Mahony et al., 2009). Briefly, at PND0 litters were randomly assigned to maternally separated (MS) or non-separated (NS) groups. At PND2, the litters assigned to MS were moved from the main colony room to an adjacent room maintained at the same temperature (21 ± 2°C) and lighting conditions. The dam was first removed from the home cage and placed into a smaller holding cage, following which, the pups (entire litters) were gently transferred together into a small cage where they remained for 3 hours. Cages containing the pups were placed on heating pads set to 30–33°C and were filled with 3cm of bedding so pups could thermoregulate as needed. The dam was returned to the home cage and transferred back to the main colony room without her pups for this time period to avoid communication between the dam and her pups. After the 3 hour separation, dams were again brought into the adjacent room and pups were returned to their original home cages. NS litters were also transported to the same room as the MS groups to avoid the confound of transportation stress but were otherwise left undisturbed in their home cages with their dams with the exception of weekly cage cleaning. This procedure was repeated daily from PND2 to PND12 inclusive. The period of separation was carried out at the same time each day (9am–12pm). At PND21, offspring were sexed and weaned, and male offspring were used for the remainder of the study.
2.3. Dietary interventions and experimental design

2.3.1. Diets

Two custom rodent diets (control diet and MFGM-enriched diet) were used in this study and were formulated by Mead Johnson Nutrition based on AIN-93G specifications. The composition of the two diets is listed in Supplementary Table 1. Both the control and the MFGM-containing diets contained DHA/ARA oil 5.3g/kg, however, the MFGM diet differs from the control diet by the inclusion of whey protein concentrate MFGM-10 15.9g/kg. Food pellets containing both the control diet and MFGM-enriched diet were provided to pregnant dams from two days prior to the birth of the pups. Dietary supplementation continued throughout the lifespan of all experimental animals including during behavioural testing. All experimenters were blinded as to the type of diet administered. Upon completion of the experiments and subsequent data analysis, experimenters were informed of the contents of the diets.

2.3.2. Experimental design

Male offspring were divided into four experimental groups: NS-Control, MS-Control, NS-MFGM and MS-MFGM (see Fig. 1) and were tested at two different life stages; PND21 and PND100. At PND21, offspring from each experimental group were culled by decapitation (n=8–9 per group) and ileum and colon segments were gently excised and used for ex vivo intestinal permeability and immunohistochemistry experiments. At weaning, the remaining offspring were randomly group-housed (2–3 per cage) and underwent behavioural tests and visceral sensitivity assessment in adulthood (see Fig. 1). Following the assessment of visceral sensitivity, adult offspring (PND100) were culled by decapitation (n=11–12 per group) and ileum and colon segments were removed and used for ex vivo intestinal permeability and immunohistochemistry experiments. From both PND21 and PND100 animals, the entire GI tract (from stomach to anus) was removed, and the length of the small intestine and colon was measured. The weight of the caecum including its contents was also measured. Body weight was recorded at PND21 (prior to culls in early life), at PND60 (prior to behavioural assessment) and at PND100 (prior to culls in adulthood). Experimental design and timeline are shown in Fig. 1.

2.4. Behavioural procedures

Behavioural assessment began at 10 weeks of age (PND70) in male offspring from each of the experimental groups. Between each test, animals were given a minimum of 1 week of a washout period to reduce the impact of the behavioural battery on subsequent behavioural tests as much as possible.

2.4.1. Novel object recognition

The novel object recognition test was carried out on PND70 and provides a readout of recognition memory and exploratory behaviour. The protocol used was adapted from Bevins and Besheer (2006). The test was carried out over 2 days. On day 1, animals were allowed to freely explore the testing apparatus, a rectangular arena equipped with an overhead camera, for 10 minutes (habituation phase). No bedding was used, and the container was wiped with 70% ethanol in between each animal to remove odour cues. On day 2, two identical objects were placed in adjacent corners approximately 5cm from the wall of the arena, and animals were allowed a 10-minute exploration period (acquisition training period). Following the acquisition training period, animals were removed from the arena for a period of 1 hour. After this time, animals were returned to the arena, which this time contained one “familiar” object (the same used in the acquisition training period) and one “novel” object that the animals had not encountered previously. Interactions between the animal and the two objects were recorded by the overhead camera for 5 minutes for later analysis. The objects were cleaned before each trial with 70% ethanol to remove odour cues. The time spent exploring each of the objects was scored blinded using EthoVision (Noldus, UK). Exploratory behaviour was defined as orienting the nose towards the object at a distance of less than 2cm, or direct contact with the object. A discrimination index was calculated according to the following formula: (t[novel] − t[familiar])/(t[novel] + t [familiar]). As rats are naturally explorative, a decrease in the discrimination index compared with controls indicates a deficit in recognition memory.

2.4.2. Morris water maze

The Morris Water Maze (MWM) was performed at PND77, using a protocol adapted from (O’Mahony et al., 2014). The MWM is used to assess spatial learning and reference memory of rodents, and it relies on distal cues to navigate from different start locations around the perimeter of an open swimming arena to find a submerged escape platform. Spatial learning is assessed across repeated trials, and spatial and reference memory is determined by preference for the platform area when the platform is absent (O’Mahony et al., 2014). The maze used was a circular pool 180cm in diameter and was filled with water (21°C ± 1°C) to a depth of 31cm. A transparent platform with a diameter of 10cm was placed in the middle of one of the quadrants so that it was submerged up to 3cm below the level of the water and was not visible from the surface. Four spatial cues were arranged around the maze to provide landmarks that would aid navigation to the platform. The test was conducted over 5 days. The animals received 4 days of training that consisted of 4 trials per day (acquisition training). At the beginning of each trial, animals were placed in 1 of 4 starting positions facing the wall of the tank and allowed to swim for 120 seconds or until it located the
escape platform. A different starting position selected in a semi-randomised pattern was used for each of the 4 trials on a given day. If the animal was unable to locate the platform within the allocated 120 seconds, the researcher gently guided the animal to the platform and detained the animal there for 30 seconds. On the fifth day of the test (probe trial), the platform was removed, and the animals were placed in a novel starting position and allowed to explore the arena for 60 seconds. The amount of time spent in the quadrant where the platform was situated previously was recorded using EthoVision (Noldus, UK) and was scored by an observer blinded to the experimental groups. Increased latency to find the platform and a decrease in the time spent in the quadrant where the platform was previously with respect to control animals is indicative of spatial memory deficits.

2.4.3. Visceral sensitivity assessment through colorectal distension

The colorectal distension protocol was carried out as previously described (O’Mahony et al., 2009) on PND91. Animals were fasted for 16 hours prior to the start of the procedure. Animals were lightly anaesthetised with isoflurane and a 6-cm long polyethylene balloon with a connecting catheter was inserted into the colon, 1 cm proximal to the anus. The catheter was secured to the tail of the animal with surgical tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 minutes prior to the start of the procedure. The colorectal distension paradigm used was an ascending phasic distension from 0 to 80mmHg over an 8-minute period. Air inflation and pressure were monitored during the procedure using a customised barostat tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 minutes prior to the start of the procedure. The colorectal distension paradigm used was an ascending phasic distension from 0 to 80mmHg over an 8-minute period. Air inflation and pressure were monitored during the procedure using a customised barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). Pain behaviours were identified as abdominal retraction, withdrawal and stretching (O’Mahony et al., 2012). A trained observer, blinded to the experimental groups, scored each animal for the threshold pressure, when the first pain behaviour was observed, as well as the total number of pain behaviours displayed across all pressure ranges by each animal.

2.5. Ex vivo permeability assessment

Intestinal permeability was assessed ex vivo. Distal ileum (a 1.5 cm segment taken 2 cm proximally to the caecum) and middle colon (a 1.5 cm segment) specimens of PND21 and PND100 animals were mounted into vertical NaviCyte diffusion chambers with 4 mm round aperture (0.126 cm² exposed tissue area). No seromuscular stripping was performed. 4.0ml of Krebs buffer (1.2mM NaH₂PO₄, 116mM NaCl, 4.8mM KCl, 1.2mM MgCl₂, 25mM NaHCO₃, 2.5mM CaCl₂ and 10mM D-glucose) was added to both the mucosal and serosal chambers for colonic specimens. For ileal specimens, Krebs buffer was added to the serosal chamber and 10mM mannitol was added to the mucosal chamber to avoid activation of sodium/glucose co-transporters in the epithelial cells of the small intestine and the associated increase in tight junction permeability (Turner et al., 1997). Chambers were continuously supplied with carbogen (95% O₂ and 5% CO₂). Tissues were not clamped, and electrophysiological measures were not recorded. Transepithelial permeability was investigated by measuring mucosal-to-serosal flux of 4kDa fluorescein isothiocyanate (FITC)-dextran (FD4, Sigma-Aldrich, Ireland) using a sampling method as described previously (Golubeva et al., 2017). Briefly, FITC was added to the mucosal chamber to a final concentration of 2.5mg/ml and 200μl samples were taken from the serosal chamber every 30 minutes for the following 180 minutes. Samples were measured at 485nm excitation/535nm emission wavelengths. FITC mucosal-to-serosal flux was presented in μg/h/cm².

2.6. Immunohistochemistry of colonic frozen sections

Colonic segments (1.5 cm specimens adjacent to those used in the ex vivo permeability assessment) were collected from PND21 and PND100 animals from each of the experimental groups. Colonic segments were flushed with a 10mM Phosphate buffered saline (PBS) and 10mM glucose solution and fixed in 4% paraformaldehyde (PFA) at 4°C for 4 hours. Following the fixation period, segments were washed in 10mM PBS and any excess PFA was dabbed off. Colonic segments were then placed in a cryoprotective solution comprised of 30% sucrose in 10mM PBS and 0.02% Na₃ for 72 hours at 4°C to prevent microbial growth. Colonic samples were then washed in 10mM PBS and embedded in optimal cutting temperature (OCT) medium (VWR chemicals, Dublin, Ireland). Colonic samples were frozen at –20°C and then placed at –80°C until sectioning. Colonic samples were sectioned at 16μm thickness using a cryostat (Leica CM1900, Germany) and sections were mounted on SuperFrost Plus slides for immunohistochemistry as previously described (Caputi et al., 2017). Briefly, colonic frozen sections were thawed at room temperature for 15 minutes and then rinsed with three washes of 5 minutes with Tris buffered saline (TBS). Sections were dried and incubated with 0.05M NH₄Cl for 20 minutes at room temperature. After three 5-minute washes with TBS, colonic sections were blocked and permeabilised with blocking solution A (4% goat serum and 0.3% Triton X-100 in TBS) or blocking solution B (3% horse Serum and 0.3% Triton X-100 in TBS) for 90 minutes at room temperature. Colonic sections were incubated with chicken anti-JJL-tubulin (1:100, ab41499, abcam, UK) and mouse biotin-conjugated HuC/D (1:50, Thermo Fisher Scientific, cat no A-21272, UK) diluted in 0.5% goat serum in TBS, or with rabbit monoclonal (EP1576V) anti-S100 (1:200, ab52642 abcam, UK) diluted in 0.5% horse serum in TBS overnight in a humidity chamber at 4°C. After three 10-minute washes with TBS, sections were incubated with the following secondary antibodies: goat anti-chicken IgG Alexa Fluor 568-conjugated (1:500, Thermo Fisher Scientific, cat no A-11041) and streptavidin Alexa Fluor 488-conjugated (1:500, Thermo Fisher Scientific, cat no S32354) diluted in TBS and 0.5% goat serum, or donkey anti-rabbit IgG Alexa Fluor 488-conjugate (1:500, Thermo Fisher Scientific, cat no A-21206) diluted in TBS and 0.5% horse serum. Sections were incubated with secondary antibodies for 2 hours at room temperature. Following the 10-minute washes with TBS, colonic sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000, Thermo Fisher Scientific) diluted in TBS and 0.5% goat serum or 0.5% horse serum for 30 minutes at room temperature. After three 10-minute washes with TBS, sections were dried and mounted with Polyvinyl Alcohol DABCO mounting medium and stored in the dark at –20°C until analysis.

2.7. Immunohistochemistry of colonic whole mount preparations

Freshly isolated middle-proximal colon segments (a 3 cm long segment from PND21 animals and a 5 cm long segment from PND100 animals of each of the experimental groups) were gently flushed with a 10mM PBS and 10mM glucose solution to remove any luminal content. Colonic segments were then tied with string at one end and filled with 4% PFA (in 10mM PBS) before being tied off at the other end. Segments were then placed in fixative solution (4% PFA in 10mM PBS) for 2 hours (PND21) or 4 hours (PND100) at room temperature to ensure fixation from the inside and outside of the tissue. After two 30-minute washes in 10mM PBS, colonic segments were placed in a solution of 10mM PBS and 0.02% Na₃ to prevent microbial growth and were stored at 4°C until analysis. For immunohistochemistry experiments, colonic segments were divided into 0.5cm segments, opened along the mesenteric border, and placed as a flat sheet onto Sylgard-coated dishes with the mucosal side down. Using a dissecting microscope, tissues were separated into two layers: the outer musculature with adhering serosa, and the submucosa/mucosa. The circular muscle was removed to yield whole mount sections of longitudinal muscle with the myenteric plexus attached (LMMP) as previously performed (Brun et al., 2013). LMMP preparations were gently stretched and pinned down on the bottom of a Sylgard-coated dishes and washed in PBS-T (PBS with 1% Triton X-100) for 45 minutes with gentle agitation. After blocking nonspecific binding sites with PBS-T containing 4% goat serum for 1.5 hours at room temperature, LMMPs were incubated overnight at room temperature with mouse biotin-conjugated Huc/D (1:100, Thermo Fisher Scientific, cat no 0.5% horse serum for 30 minutes at room temperature. After three 10-minute washes with TBS, sections were dried and mounted with Polyvinyl Alcohol DABCO mounting medium and stored in the dark at –20°C until analysis.
A-21272, UK) diluted in PBS-T and 4% goat serum. Three 15-minute washes with PBS-T were carried out following the incubation period and LMMPs were incubated with streptavidin Alexa Fluor 488-conjugate (1:500, Thermo Fisher Scientific, cat no S32354) diluted in PBS-T and 10-minute washes with PBS-T were carried out. LMMPs were mounted (1:500, Thermo Fisher Scientific, cat no S32354) diluted in PBS-T and LMMPs were incubated with streptavidin Alexa Fluor 488-conjugate

2.8. Confocal image acquisition and analysis

Images of colonic frozen sections and LMMP preparations were acquired using an Olympus FV1000 confocal laser scanning microscope equipped with an oil immersion 60× objective lens for colonic frozen sections and 40×, 20× and 10× objective lenses for LMMP preparations. The immunoreactivity of βIII-tubulin, HuC/D, and S100β in colonic frozen sections was determined as previously described (Sten-kamp-Strahm et al., 2013). Four full-thickness images per animal were taken using the 60× objective lens and were processed as maximum intensity projections. A tracing tool was used to define the muscularis externa or the myenteric ganglia in order to estimate tissue area. βIII-tubulin, HuC/D, and S100β staining in the muscularis externa and/or the myenteric ganglia were then thresholded in a blinded fashion to allow Fiji Image J software (version 1.52e) to estimate stained areas and/or the myenteric ganglia were then thresholded in a blinded fashion to allow Fiji Image J software (version 1.52e) to estimate stained areas within muscularis externa or myenteric ganglia. Density index calculations (stained area/area of muscularis externa or myenteric ganglia) were generated. In colonic LMMP preparations, changes in the number of HuC/D+ neurons were assessed in PND21 and PND100 animals from each of the experimental groups. For cell quantification, five to ten visual fields were blindly chosen within the areas where the myenteric plexus was intact and acquired using a 20× objective (for anti-HuC/D staining in colonic specimen at PND21; 636 × 636µm/visual field) and a 40× objective (for anti-HuC/D staining in colonic specimen at PND100; 318 × 318µm/visual field). HuC/D+ myenteric neurons were blindly quantified and expressed as neuronal count per visual field.

2.9. Statistical analysis

Data were analysed using the statistical software package SPSS 24.0 (IBM) and were expressed as mean ± SEM. Differences between the experimental groups were assessed using: a repeated-measures two-way ANOVA, a two-way ANOVA, mixed design ANOVA, and LSD Fisher post-hoc test where appropriate. A p-value of 0.05 was set as the threshold of statistical significance. “n” indicates the number of animals per experimental group.

3. Results

3.1. Perinatal coadministration of DHA and MFGM does not alter rat body weight or gastrointestinal anatomy

The coadministration of DHA and MFGM from birth was well tolerated by the animals and there was no change in body weight observed throughout the lifespan of the animals from each of the experimental groups (data not shown). Food intake was also not affected between experimental groups. No changes in either small intestine or colon length, or caecum or spleen weight were observed at PND21 or PND100 in animals exposed to MS or the dietary intervention (data not shown).

3.2. Coadministration of DHA and MFGM reduces maternal separation-induced visceral hypersensitivity

Two-way ANOVA revealed a significant main effect of early life stress (F (1,39) = 14.785, p < 0.001) and of diet (F (1,39) = 5.213, p = 0.028) on threshold pressure in response to colorectal distension. No diet × early life stress interaction was observed (F (1,39) = 0.272, p = 0.61).

LSD Fisher post hoc test revealed a significant decrease in threshold pressure in MS-Control animals compared to NS-Control animals (p = 0.003) and MFGM administration significantly restored threshold pressure to near control levels in MS-MFGM compared to MS-Control animals (p = 0.05) (Fig. 2A). Two-way ANOVA revealed a significant main effect of early life stress (F (1,35) = 25.124, p < 0.001) as well as of diet (F (1,35) = 11.484, p = 0.002) on the total number of pain behaviours displayed in response to colorectal distension. No diet × early life stress interaction was noted (F (1,35) = 2.553, p = 0.12). LSD Fisher post hoc test revealed a significant increase in the total number of pain behaviours displayed by MS-Control animals compared to the NS-Control group (p < 0.001) and that the total number of pain behaviours displayed by MS-MFGM animals was significantly less than that of the MS-Control group (p = 0.001) (Fig. 2B).

3.3. Perinatal coadministration of DHA and MFGM improves spatial learning without affecting reference memory in the Morris water maze

Animals successfully learned the location of the platform over the four training days as shown by a repeated-measures two-way ANOVA which revealed a significant main effect of time (F (3,105) = 104.522, p < 0.001) and of diet with respect to time (F (3,105) = 2.713, p = 0.049). However, during the acquisition training, no effect of early life stress with respect to time was found (F (3,105) = 0.563, p = 0.64), nor was there a diet × early life stress interaction with respect to time (F (3,105) = 0.229, p = 0.88) (Fig. 2C). Further investigation using a mixed design ANOVA with trial day as the repeated measures factor and diet as the independent factor revealed that diet approached the threshold of statistical significance as a main effect between NS-Control and NS-MFGM on day one only (F (1,22) = 3.866, p = 0.06) (Fig. 2D). A significant effect of diet was also noted by mixed design ANOVA with trial day as the repeated measures factor and diet as the independent factor between MS-Control and MS-MFGM on day one (F (1,21) = 4.337, p = 0.05) and day four (F (1,21) = 4.704, p = 0.04) (Fig. 2E). Mixed design ANOVA with trial day as the repeated measures factor and ELS as the independent factor also revealed an effect of early life stress between NS-Control and MS-Control on day 4 only (F (1,21) = 5.158, p = 0.034) (Fig. 2F). No differences were observed in the percentage of time spent in the platform quadrant during the probe trial between animals from each experimental group, suggesting that reference memory was not affected by MFGM administration or MS (data not shown).

3.4. Maternal separation and MFGM do not affect recognition memory in the novel object recognition test

A two-way ANOVA revealed no effect of diet (F (1,41) = 1.018, p = 0.32), nor of a diet × early life stress interaction effect (F (1,41) = 0.098, p = 0.76) on the discrimination index in the novel object recognition test (Fig. 2G).

3.5. Intestinal permeability in early life or adulthood was not affected by maternal separation or dietary interventions

A two-way ANOVA revealed no effect of early life stress on trans-epithelial permeability of the ileum to 4kDa FITC at PND21 (F (1,29) = 0.16, p = 0.69) (Fig. 3A) or PND100 (F (1,40) = 0.021, p = 0.88) (Fig. 3B). Likewise, no effect of diet on transmucosal permeability of the ileum to FITC was noted at PND21 (F (1,29) < 0.001, p = 0.99) (Fig. 3A) or PND100 (F (1,40) = 0.014, p = 0.91) (Fig. 3B). No diet × early life stress interaction on transmucosal permeability of the ileum to FITC was noted at PND21 (F (1,29) = 1.529, p = 0.29) (Fig. 3A) or PND100 (F (1,40) = 3.411, p = 0.07) (Fig. 3B). With respect to the colon, a two-way ANOVA revealed no effect of early life stress on transmucosal permeability of colonic segments to 4kDa FITC at PND21 (F (1,32) = 0.037, p = 0.85) (Fig. 3C) or PND100 (F (1,39) = 0.216, p = 0.65) (Fig. 3D). Similarly, no effect of diet on transmucosal permeability of 4kDa FITC to the colon at PND21 was noted (F (1,39) = 0.227, p = 0.61) (Fig. 3D).
permeability of the colon to FITC was noted at PND21 (F (1,32) = 0.162, p = 0.69) (Fig. 3C) or PND100 (F (1,39) = 0.032, p = 0.86) (Fig. 3D).

3.6. No effect of maternal separation or perinatal dietary intervention on neuronal and glial architecture of the enteric nervous system in early life

The immunoreactivity of βIII-Tubulin, a neuronal marker present in the nerve fibres of all subsets of neurons in the ENS, was evaluated in the muscle layer of colonic frozen section from PND21 animals. A two-way ANOVA showed no effect of early life stress (F (1,31) = 2.168, p = 0.15), nor of diet (F (1,31) = 1.777, p = 0.19), nor of a diet × early life stress interaction (F (1,31) = 0.717, p = 0.40) on βIII-Tubulin immunofluorescence in the colonic muscle layer of PND21 animals (Fig. 4A and C). The density index of βIII-Tubulin in the colonic myenteric ganglia was also assessed, and no differences were induced by early life stress (F (1,30) = 3.315, p = 0.08), nor by diet (F (1,30) = 0.045, p = 0.83), nor by a diet × early life stress interaction (F (1,30) = 0.007, p = 0.93) (Fig. 4A and D). Colonic frozen sections were also stained for HuC/D, a pan-neuronal cell body marker in the ENS, and no effect of early life stress (F (1,31) = 0.001, p = 0.98), nor of diet (F (1,31) = 0.187, p = 0.67), nor of a diet × early life stress interaction (F (1,31) = 1.952, p = 0.17) on HuC/D immunoreactivity was observed in colonic myenteric
ganglia from PND21 animals. (Fig. 4A and E). Enteric glial networks were also investigated in early life. A two-way ANOVA revealed no effect of early life stress (F(1,29)<0.001, p = 0.99), nor of diet (F(1,29) = 0.001 p = 0.98), nor of a diet × early life stress interaction (F(1,29) = 2.071, p = 0.16) on density index of the enteric glial marker S100β in myenteric ganglia of PND21 animals. (Fig. 4B and F).

3.7. Dietary intervention but not maternal separation affects enteric neuronal processes in adulthood in colonic myenteric ganglia

In colonic frozen sections of PND100 animals, a two-way ANOVA showed no effect of early life stress (F(1,26) = 0.099, p = 0.76), nor of diet (F(1,27) = 1.749, p = 0.2), nor of a diet × early life stress interaction (F(1,27) = 2.634, p = 0.12) on the immunoreactivity of βIII-Tubulin in the colonic muscle layer (Fig. 4G and I). The immunofluorescence of βIII-Tubulin in the colonic myenteric ganglia was also assessed, and no differences were induced by early life stress (F(1,26) = 0.03, p = 0.86), however, there was a significant effect of diet (F(1,26) = 6.772, p = 0.015), and a diet × early life stress interaction (F(1,26) = 5.98, p = 0.022). LSD Fisher post hoc test revealed a significant increase in βIII-Tubulin immunoreactivity in the myenteric ganglia of NS-MFGM animals when compared to the NS-Control group (p = 0.001) (Fig. 4G and J). Colonic frozen sections were also stained for HuC/D, however no effect of early life stress (F(1,27) = 0.066, p = 0.8), nor of diet (F(1,27) = 3.314, p = 0.08), nor of a diet × early life stress interaction (F(1,27) = 0.381, p = 0.54) on HuC/D immunoreactivity was noted (Fig. 4G and K). The immunoreactivity of the enteric glial marker S100β was also investigated at PND100. A two-way ANOVA revealed no effect of early life stress (F(1,27) = 0.199, p = 0.66), nor of diet (F(1,27) = 1.216, p = 0.28), nor of a diet × early life stress interaction (F(1,27) = 1.079, p = 0.31) on density index of S100β in myenteric ganglia of PND100 animals (Fig. 4H and I).

3.8. No effect of maternal separation or diet on enteric nervous system in early life: whole-mount colonic preparations

Two-way ANOVA showed no effect of early life stress (F(1,26) = 2.754, p = 0.11), nor of diet (F(1,26) = 0.003, p = 0.95), nor of a diet × early life stress interaction (F(1,26) = 0.008, p = 0.93) on the number of HuC/D-stained cells in colonic whole mount preparations in early life (Supplementary Figs. 1A and 1C). Similarly, no differences in HuC/D-stained cells in colonic whole mount preparations at PND100 were induced by early life stress (F(1,29) = 1.087, p = 0.31), nor by diet (F(1,29) = 1.228, p = 0.28), nor by a diet × early life stress interaction (F(1,29) = 0.93, p = 0.76) (Supplementary Figs. 1B and 1D).

4. Discussion

We have previously shown that post-weaning administration of MFGM attenuated the effects of ELS in rats (O’Mahony et al., 2020). The mechanisms underpinning such effects were unknown but may involve changes in ENS function and intestinal permeability (Bhinder et al., 2017; Ortega-Anaya and Jimenez-Flores, 2019; Yu et al., 2021). In this study, the diet of the dams was supplemented with MFGM from two days prior to birth of the pups and continued throughout the lifespan of the offspring to assess its potential in reversing the effects of ELS. MFGM was selected as the candidate intervention as it has been shown to have beneficial health effects both preclinically (Mudd et al., 2016) and clinically on neurodevelopment (Hernell et al., 2016), and narrow the gap in cognitive performance between formula-fed and breastfed infants. MFGM has also been shown to reduce the incidence of infections (Hernell et al., 2016). However, the mechanistic potential of MFGM in reduction of ELS-induced deficits has not yet been extensively explored.

This study is to our knowledge the first to investigate the effect of dietary supplementation with MFGM from birth on rat ENS and behaviour following exposure to ELS. The MS model is a robust model of ELS and gut-brain axis dysfunction in rats (Botschuijver et al., 2019;
Cowan et al., 2019; O’Mahony et al., 2009; O’Mahony et al., 2011; Rincel et al., 2019b).

Visceral sensation from the gut has been shown previously to be heavily influenced by ELS with exposure to stress during the stress hyporesponsive period in early life resulting in heightened sensitivity of the GI tract (Felice et al., 2015; O’Mahony et al., 2012). In line with this, we found that MS resulted in visceral hypersensitivity in response to the noxious stimulus of colorectal distension. This was noted both by an increased total number of pain behaviours observed in response to distension of the colorectal region and by a decreased threshold to this stimulus. Supplementation with MFGM reduced this, normalising the number of pain behaviours and threshold pressure to near control levels. This is in agreement with our previous study showing that supplementation with MFGM after weaning ameliorated MS-induced visceral hypersensitivity (O’Mahony et al., 2020), supporting the potential value of MFGM against visceral pain-associated disorders.

We noted that animals exposed to ELS displayed a deficit in spatial memory in the Morris water maze on the final day of the acquisition training, suggesting that initially the MS group learned the location of the platform as fast as the NS group, with this effect having tapered off by day 4. This impaired cognitive performance has been previously noted following a longer MS paradigm where the authors suggest that the effects of MS on spatial memory are related to changes in the postnatal development of the hippocampus (Cao et al., 2014). Interestingly, we noted that MS MFGM-treated animals displayed significantly better cognitive performance on day 1 and day 4 of the acquisition training compared to the MS control diet animals, highlighting the potential benefit of MFGM on brain function, particularly on learning. Interestingly, a subtle deficit in visuospatial memory was noted in a cohort of patients with irritable bowel syndrome (Kennedy et al., 2014), strengthening the link between impaired cognition and visceral pain-associated disorders such as irritable bowel syndrome. Interestingly, the MS-induced deficits in our current study were much more modest than what we have previously shown (Felice et al., 2014; McVey Neufeld et al., 2019; O’Mahony et al., 2009; O’Mahony et al., 2020).

This may be due to the fact that the control diet used in the present study was enriched with DHA, a polyunsaturated fatty acid that has been shown to have pro-cognitive effects (Lauritzen et al., 2016; Mulder et al., 2018).

MS has previously been shown to result in deficits in recognition
function in vitro by decreasing cytokine production following spleno
mount preparations where no effect of ELS or MFGM supplementation
either PND21 or PND100. The same may be said for the colonic whole
the density index of 

muscle layer at PND21 was unaffected by ELS. However, an increase in
β
III-Tubulin in both the colonic myenteric ganglia and the colonic

of intestinal barrier function following MS ( García-Roderas et al., 2004; Oines et al., 2012; S

It has been shown that the gut microbiota plays an important role in
vesicular sensation, with male germ-free mice showing baseline visceral
hypersensitivity ( Laczynski et al., 2017), while female germ-free mice
do not, but instead display microbiota-dependent modulation of visceral
pain across the oestrous cycle ( Tramullas et al., 2021). Similarly, early
life antibiotic-induced depletion of the gut microbiota results in altered
sensation of visceral pain in adulthood ( O’ Mahony et al., 2014). MS has
been shown previously to affect transepithelial barrier permeability as
noted by an increased flux of Horseradish peroxidase in maternally
separated rats ( Gareau et al., 2006, 2007a ). Other studies have also
reported MS-induced increases in transepithelial permeability ( Barreau
et al., 2004; Oines et al., 2012; Soderholm et al., 2002). However, a
study using a combination of pre- and probiotics reported normalisation
of intestinal barrier function following MS ( García-Roderas et al., 2006 )
while a probiotic, VSL#3, was successful in preventing MS-induced
vesicular pain, and also tightened the gut epithelial barrier ( Dai et al.,
2012). This increase in transepithelial permeability may lead to bacte-
rial translocation from the gut, feeding into the manifestation of visceral
pain. Therefore, we hypothesised that epithelial barrier permeability
plays a role in MS-induced visceral hypersensitivity. However, no effect
of either MS or dietary intervention was noted at either life stage. This is
in agreement with a previous study, which found no effect of MFGM on
transepithelial barrier permeability to FITC-dextran ( Bhinder et al.,
2017). Possible reasons behind the protection of epithelial barrier
permeability against the effects of MS include the presence of DHA in the
control diet. DHA has been shown to be effective against increased
permeability as a result of the addition of interleukin-4 to a monolayer of
human colon-derived carcinoma cells ( Willemsen et al., 2008).

Previous studies have shown that ELS affects the morphology and
functionality of the ENS. Piglets exposed to early life adversity (early
weaning stress) displayed a higher veratridine (activates voltage-gated
sodium channels resulting in neuron depolarisation)-induced short-circuit
current in the ileum than late weaning controls ( Medland et al.,
2016). Moreover, early weaning increased the number of ileal submu-
coal neurons in adulthood compared to late weaning controls. How-
never, how ELS impacts upon development of the ENS is not yet known.
Here we show that both the immunoreactivity of HuC/D and βIII-Tubulin in both the
colonic myenteric ganglia and the colonic muscle layer at PND21 was unaffected by
ELS. However, an increase in the density index of βIII-Tubulin in colonic myenteric
ganglia was noted at PND100 in the NS MFGM-treated group only. Similarly, no effect
of either ELS or dietary intervention on enteric glial structure was noted at
either PND21 or PND100. The same may be said for the colonic whole
mount preparations where no effect of ELS or MFGM supplementation
was noted at PND21 or PND100 ( Supplementary Fig. 1 ).

Despite a clear mechanism of action, MFGM supports immune
function in vitro by decreasing cytokine production following spleno-
cyte stimulation ( Zanabria et al., 2014 ). It was observed that the effects
of MFGM did not occur under non-stimulatory conditions, therefore
potentially suggesting MFGM may act in conditions that, in part,
simulate a stressed condition. Immune changes may be one of the
driving factors of ELS-induced visceral hypersensitivity as MS can cause
immune dysregulation including upregulation of cytokines and growth
factors which can sensitise peripheral nociceptors and thus amplify
vesicular pain perception ( Fuentes and Christianson, 2018 ). It is therefore
reasonable to suggest that the effects on the immune system induced by
MFGM are likely to reduce the impact of ELS on visceral pain perception.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2022.109026.

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