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# The Enduring Effects of Early Life Stress on the Microbiota-Gut-Brain Axis are Buffered by Dietary Supplementation with Milk Fat Globule Membrane and a Prebiotic Blend

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**Running title:** MFGM reduces effects of early life stress

**Keywords:** maternal separation, visceral hypersensitivity, neurodevelopment, Postnatal

**Abstract**

Nutritional interventions targeting the microbiota-gut-brain axis are proposed to modulate stress-induced dysfunction of physiological processes and brain development. Maternal separation (MS) in rats induces long-term alterations to behavior, pain responses, gut microbiome and brain neurochemistry. In this study, the effects of dietary interventions (milk fat globule membrane (MFGM) and a polydextrose/galactooligosaccharide prebiotic blend (PDX/GOS)) were evaluated. Diets were provided from postnatal day 21 to both non-separated (NS) and MS offspring. Spatial memory, visceral sensitivity and stress reactivity were assessed in adulthood. Gene transcripts associated with cognition and stress and the caecal microbiota composition were analysed.

MS-induced visceral hypersensitivity was ameliorated by MFGM and to greater extent with the combination of MFGM and prebiotic blend. Furthermore, spatial learning and memory were improved by prebiotics and MFGM alone and with the combination. The prebiotic blend and the combination of the prebiotics and MFGM appeared to facilitate return to baseline with regard to HPA axis response to the restraint stress which can be beneficial in times where coping mechanisms to

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stressful events are required. Interestingly, the combination of MFGM and prebiotic reduced the long-term impact of MS on a marker of myelination in the prefrontal cortex. MS affected the microbiota at family level only while MFGM, the prebiotic blend and the combination influenced abundance at family and genus level as well as influencing beta diversity levels. In conclusion, intervention with MFGM and prebiotic blend significantly impacted the composition of the microbiota as well as ameliorating some of the long-term effects of early-life stress.

## Introduction

The occurrence of stressful episodes during critical windows of development can have long-lasting effects on the host which are seen in both clinical and preclinical studies (Heim et al., 2002). The early postnatal period is normally a stress hypo-responsive period when there is an intense phase of neuronal growth and myelination (Morgane et al., 2002). In humans, stress occurring during this critical time point has been linked to the development of both somatic and psychiatric phenotypes including depression, anxiety disorders and irritable bowel syndrome (Folks, 2004; Plotsky et al., 2005; Seckl, 2007). Maternal separation (MS) in rodents is a well-established animal model of early life stress and results in a variety of long-term behavioural and physiological effects in adult offspring (O'Mahony et al., 2011; Yi et al., 2017). Included in these alterations are stress hyper-responsivity, increased depressive-like behaviour, visceral hypersensitivity and reduced diversity of intestinal microbiota, thus demonstrating the face validity of this model (Bailey & Coe, 1999; O'Mahony *et al.*, 2009). While MS is just one model of early life adversity, other studies have shown that other stressors, including early life antibiotics (O'Mahony et al., 2014), neonatal colonic inflammation (Al-Chaer et al., 2000) and repeated neonatal colorectal distension (Lin & Al-Chaer, 2003) during this early stage of development also induce similar dysfunction as seen with MS (Pohl *et al.*, 2015).

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The milk fat globule membrane (MFGM) is the triple layer membrane that surrounds lipid droplets present in milk. MFGM is derived from the mammary epithelium and secreted in the alveolar lumen of the lactating mammary gland of both humans and bovine species (Cavaletto et al., 2008). MFGM consists of a complex system of proteins, enzymes, and lipids, with bioactive properties (Hernell et al., 2016). MFGM has been reported to play an important role in various cellular processes and defence mechanisms in the new-born infant (Cavaletto et al., 2008) and is thought to confer some of the health benefits associated with breast milk in humans (Bourlieu & Michalski, 2015). MFGM has been identified as potentially promoting beneficial health effects, particularly with respect to brain development and function (Spitsberg, 2005; Mudd *et al.*, 2016). Infants fed MFGM supplemented formula have demonstrated enhanced cognitive performance when compared to those fed standard formula (Timby et al., 2014). Furthermore, pre-school aged children given milk supplemented with MFGM concentrate showed lower behavioural problems as reported by parents (Veereman-Wauters et al., 2012). MFGM has also been shown to enhance sleep quality, which was related to changes in gut bacteria and it also modulated the impact of stress on sleep (Thompson et al., 2016). MFGM supplementation has also been associated with antipyretic effects, as it decreased the incidence of middle ear infections (Timby *et al.*, 2014) and is also associated with changes in the oral microbiota composition (Timby *et al.*, 2014) and gastrointestinal development (Bhinder et al., 2017; Demmelmair et al., 2017).

Prebiotics are non-digestible food ingredients, usually carbohydrates, which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host health (Gibson & Roberfroid 1995; Roberfroid 2007). More recently this definition has been updated to include non-carbohydrate substances, applications to body sites other than the gastrointestinal tract, and

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diverse categories other than food (Gibson et al., 2017). Recently we (Burokas *et al.*, 2017; McVey Neufeld *et al.*, 2017) and others (Tarr *et al.*, 2015; Thompson *et al.*, 2016; Williams *et al.*, 2016; Mika *et al.*, 2017) have shown that prebiotics can have a positive effect on physiology and behaviour and can reverse the effects of stress and immune activation in adult animals. Moreover, research now shows that a combination of prebiotics and MFGM improves sleep and attenuates the impact of stress in rats (Thompson *et al.*, 2016; Mika *et al.*, 2017), however, the ability of MFGM to counter the enduring effects of early life stress remains unexplored.

Given the promising results we have observed in attenuation of early-life stress effects through feeding of prebiotics, and the growing literature in support of MFGM as a potentially beneficial dietary supplement, the aim of this project was to examine the potential long-term benefits of feeding the prebiotic blend polydextrose/galactooligosaccharide (PDX-GOS) both with and without the addition of MFGM enriched whey protein concentrate to male rat offspring previously subjected to early-life MS. In addition, we sought to examine the possible mechanisms of action underlying any behavioural and physiological effects.

## Methods

### *Animals and housing*

Male and female Sprague-Dawley rats (approximately 8 weeks of age) were purchased from Harlan UK. These rats were mated in the Preclinical Research Facility, Biosciences Institute, University College Cork and the subsequent offspring were used in the following experiments. The resulting dams and litters were housed in large breeding cages in a temperature- and humidity- controlled room on a 12hr light, 12hr dark cycle (lights on from 0700-1900hr). All experiments were conducted in accordance with the European Directive 2010/63/EEC, the requirements of the S.I No 543 of 2012 and approved by the Animal Experimentation Ethics Committee of University College Cork.

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***Maternal Separation Model and Experimental Design***

Both MS and non-separated (NS) male rats were used for each intervention (n=12/group). MS protocol was carried out as described previously (O'Mahony *et al.,* 2009). Briefly, at birth [postnatal day 0 (PND0)] litters were randomly assigned to undergo MS or to remain as NS controls. At PND2 the litters assigned to MS were removed from the main colony room to an adjacent room (See Figure 1 for experimental design). Mothers were first removed from the home cage and placed into a smaller holding cage and following this the pups (entire litter) were gently placed into a small cage (e.g. mouse cage). Mothers were returned to the home cage and the main colony room without their pups. The cage containing the pups remained in the adjacent room (so no sound could be passed from mother to pup and *vice versa*) that was maintained at the same temperature (21ºC ± 2 ºC) and lighting conditions as the main colony room and placed on a heating pad set at 30-33 ºC for 3 hours. After the 3-hour separation the mothers were again brought into the adjacent room and placed briefly in a holding cage while pups were returned to the original home cage. This was followed immediately by the return of the mother.

Control litters were left undisturbed in the mothers' home cage except for routine cage cleaning performed once a week. This procedure was repeated from PND2 to PND12 (inclusive). The period of separation was carried out at the same time daily (9:00am-12:00pm). At PND21 male and female offspring were sexed and only male rats were used for the remainder of the experiment. These were randomly group-housed (3 per cage) with no littermates housed together.

Behavioural testing began at 8 weeks of age and between each test the rats were given 1 week of a washout period so as to reduce the impact on subsequent behavioural tests as much as possible.

***Control and Test Diets***

All experimenters were blinded to type of diet administered. The diets were formulated by Mead Johnson Nutrition (MJN, Evansville IN, USA) based on AIN-93G specifications. The control diet

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consisted of: Casein 200.00 g/Kg; L-Cysine 3.0 g/Kg; Corn Starch 392.374 g/Kg; Maltodextrin 132.00 g/Kg; Sucrose 100.00 g/Kg; Lactose monohydrate 7.5 g/Kg; Soybean Oil 64.7 g/Kg; Cellulose 50.0 g/Kg; Mineral Mix (without Ca and P) 13.4 g/Kg; Calcium Carbonate 7.2 g/Kg; Calcium Phosphate dibasic 7.0 g/Kg; Vitamin Mix AIN-93-VX 15.0 g/Kg; Choline Bitartate 2.5 g/Kg; Vitamin K1 0.002 g/kg; TBHQ (antioxidant) 0.014 g/Kg; and DHA/ARA Oil 5.3 g/Kg. The test diets differed from control diet by the inclusion of 1. GOS 20.86 g/Kg; PDX 6.44 g/Kg (Prebiotic); 2. Whey protein concentrate MFGM-10 15.9 g/Kg (MFGM) or 3. A combination of GOS/PDX and MFGM-10 (Prebiotic + MFGM). All diets were isocaloric. Levels of carbohydrate, protein, fat, vitamin and mineral levels were similar by adjustment of corn starch, casein, soybean oil, and calcium concentrations (Supplemental Table 1). Upon completion of the experiments and subsequent data analysis, experimenters were informed of the contents of diets. Diets continued throughout all behavioural testing. Body weight was measured weekly, water intake daily, and feed intake 3 times/week.

**Recognition Memory**

***Novel object recognition*:** The protocol used was adapted from (Bevins & Besheer, 2006) and used to test recognition memory. It was conducted over a 3-day test period. On Day 1, the animals were allowed to acclimate to the testing environment for 10 minutes, which was a large container equipped with an overhead camera. No bedding was used, and the container was wiped with 70% ethanol between each animal. On Day 2, the animals were allowed to acclimate to the test apparatus for 10 minutes. Following this period, the animal was removed from the container and two identical objects were introduced to the environment. The animal was returned to the container and allowed to explore for a further 10 minutes. The objects were cleansed before each trial with a 70% ethanol solution. Following the training period, the rodent was removed from the environment for a delay period of 24 hours. On Day 3, the rodent was returned to the container, which this time contained only 1 "familiar" object from the day previous and 1 "novel" object. Activity of the animal with the 2 objects was

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recorded for 5 minutes. The amount of time that the rodent spent exploring each object was recorded by manual observation and a discrimination index (DI) value corresponding to time spent interacting with the novel object over total interaction time was generated. A decrease in DI compared to control rats indicates a deficit in this type of memory.

**Spatial and Reference Memory**

***Morris water maze:*** The Morris water maze (MWM) is a test of spatial learning and reference memory for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform (Vorhees & Williams, 2014). Spatial learning is assessed across repeated trials and spatial/reference memory is determined by preference for the platform area when the platform is absent. It was carried out as previously described (O'Mahony *et al*., 2014). The apparatus consisted of a circular tank of 180 cm diameter filled with water to a depth of 31 cm. A transparent platform with a diameter of 10 cm was placed in the middle of one of the quadrants so that it was slightly submerged below the water level and not visible from the surface. Distal cues were arranged around the maze to provide landmarks that the animals could use to navigate to the platform. Animals received 4 days of training that consisted of 4 trials/day (Acquisition Training). At the beginning of each trial the animal was placed in one of 4 start positions facing the wall of the tank and allowed to explore the maze for 120 seconds. A different starting position was used for each of the 4 trials on a given day arranged in a semi-random pattern. If the platform was not located within this time the animal was gently assisted to the platform by the experimenter and detained there for 30 seconds. On the fifth day (Probe Trial) of the procedure the platform was removed and the animals were placed in a novel starting position and allowed to freely explore the pool for 60 seconds. The amount of time spent in the quadrant originally hosting the platform was recorded via EthoVision (Noldus, Wageningen, The Netherlands) and scored by an observer. An increase in latency to locate the platform and a decrease in time spent in the platform quadrant when compared to control rats indicates a deficit in spatial memory.

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**Visceral Sensitivity Assessment**

***Colorectal distension:*** This was conducted as previously described (O'Mahony *et al*., 2010). Animals were fasted for 24 hours and then lightly anaesthetised using isofluorane. A 6cm polyethylene balloon with a connecting catheter was inserted in the distal colon 1cm proximal to the anus. The catheter was fixed to the tail with tape to avoid displacement. Animals were allowed to recover from anaesthesia for at least 15 minutes before commencing colorectal distension. A customized barostat was used to control air inflation and pressure during colorectal distension. The distension paradigm used was an ascending phasic distension from 0 to 80 mmHg. A trained observer scored each rat for the threshold, or pressure when the first pain behaviour was noted as well as the number of pain behaviours exhibited by each animal.

## Determination of Plasma Corticosterone

During a 30 min restraint stress, blood samples *via* tail incision were collected at five different time points; immediately before and after the restraint, as well as 1 h, 1.30 h and 2 h later. To this end, rats were restrained in Plexiglas restrainers and using a single edge razor blade a diagonal incision of 2 mm long was made at 15 mm from the end of the tail. Approximately 200µl 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 4 degrees for 15min. Plasma was carefully aspirated and stored at -80 °C. Corticosterone levels were assayed using a commercially-available ELISA kit (Corticosterone EIA Kit, ADI-900-097, Enzo Life Sciences) according to manufacturer instructions. Light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm.

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**RNA Isolation and Quantitative Real-Time PCR**

Total RNA was isolated from both the hippocampus and the prefrontal cortex of adult rats using the RNeasy Mini Kit as per the manufacturer's instructions (Qiagen). Briefly, both the hippocampus and the prefrontal cortex were dissected out from the rat brain and stored at 4 degrees celcius in RNAlater RNA Stabilization Reagent (Qiagen) for 24 hours, followed by storage at -80°C until further processing. Samples were then denaturized using the Qiazol lysis solution (Qiagen). The lysate was then transferred to a microcentrifuge tube and centrifuged. The filter was then washed and finally the RNA was eluted using Nuclease-Free Water. Isolated RNA was stored at -80°C until further processing. RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop), and RNA quality was assessed using the Agilent 2100 Bioanalyzer.

Analysis of RNA expression levels was carried out as previously described (Pusceddu et al., 2015b). Briefly, equal amounts of RNA were first reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). Real-time PCR was performed using TaqMan Universal Master Mix II, no UNG, and TaqMan Gene Expression Assays designed by Applied Biosystems for rat genes on the ABI7300 Real Time PCR machine (Applied Biosystems, Warrington, UK). Each sample was analyzed in triplicate for both target gene and endogenous control using the 7300 System SDS Software (Applied Biosystems, Life Technologies). All assays were controlled for the absence of genomic DNA amplification. β-actin (VIC/MGB Probe, Primer Limited, Applied Biosystems) was used as an endogenous control. Cycle threshold (Ct) values were recorded. The Ct value for the target gene in each sample was normalized to its endogenous control transformed to relative gene expression value using the ΔΔCt method (Simen et al., 2006). The genes analysed in the hippocampus included gamma amino butyric acid (GABA)α1, GABAβ1, GABAβ2, glucocorticoid receptor (GR), mineralocorticoid receptor (MR) (Mohler, 2012). While the target genes in the prefrontal cortex included myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), phosphoinositide 1 (PiP1), myelin associated oligodendrocyte basic protein (MOBP) and myelin associated glycoprotein (MAG).

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**Microbiota analysis**

Fresh caecal samples were collected from individual rats at the end of the study. At least 20mg of fresh caecal material were placed in a microcentrifuge tube and kept on ice until storage at -80$^{o}$C. DNA was extracted using the DNA Fast Stool DNA extraction kit (Qiagen) using the protocol for Gram positive bacteria and including an additional bead beating step at the beginning of the procedure. The microbiota composition of the samples was established by amplicon sequencing of a ~380bp fragment of the V3-V4 hypervariable region of the bacterial 16S rRNA gene as outlined in the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina). Amplicons were quantified, normalised and pooled using the Qubit® dsDNA HS Assay Kit (Life Technologies). Samples were sequenced on the MiSeq sequencing platform at Clinical Microbiomics, using a 2 x 300 cycle kit, following standard Illumina guidelines.

**Bioinformatics**

Forward and reverse reads were merged using USEARCH requiring a merged minimum length of 300 bp, maximum 600 bp and minimum 100 bp overlap. A maximum of one expected error was allowed per read. Primers were trimmed, discarding any sequences that do not have a perfect match to both forward and reverse primers. Sequences were dereplicated, discarding clusters smaller than 5 and clustered at 97 % sequence similarity using USEARCH (Edgar, 2010). Suspected chimeras were removed using UCHIME (Edgar et al., 2011). Taxonomy was assigned to each OTU using the RDP Classifier (Wang et al., 2007). To estimate alpha diversity, the data set was rarefied to the same number of sequences and the Shannon diversity index was calculated using the diversity function from the vegan package in R (Oksanen, 2011). Beta diversity was also calculated in R using Bray-Curtis distances and Principal Coordinate Analysis (PCoA) was performed using the vegan package.

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**Statistical Analysis**

Data were analysed using a repeated measures two-way ANOVA, a two-way ANOVA and LSD's *post-hoc* test where appropriate using the statistical software package SPSS 22.0 (IBM). A p-value of 0.05 was selected as the threshold of statistical significance. The rank Kruskal-Wallis test was used for comparison of taxa followed by Dunn's test with False Discovery Rate p-value adjustment for multiple comparisons.

The Mann U Whitney test was used to assess for differences between the various intervention groups. The Benjamini-Hochberg for False Discovery Rate was used to calculate q (Q was set at 10%). A two-way ANOVA was carried out on alpha diversity indices for diet and separation and the interaction between diet and separation. Beta diversity was compared between groups using the adonis function in the R vegan package at the genus level, using Bray-Curtis as distance measurement.

**Results**

**Dietary interventions altered stress-induced spatial learning and memory and showed no effect on recognition memory**

*Effects of administering prebiotic blend and/or MFGM on spatial learning and reference memory in the MWM*

Animals successfully learned the location of the platform over the 4 training days as shown by a 2-way repeated measures ANOVA-a main effect for time ($F_{(3,252)}$=215.589, p<0.0001) was noted. There was also a main effect for dietary intervention ($F_{(9,252)}$=2.649, p=0.006), but no effect for early life stress ($F_{(3,252)}$=1.181, p=0.317), for the training days (see Figure 2A). On day 2 of training there were differences between NS and MS control diet fed rats and the rats that received the dietary interventions. For example, NS prebiotic blend + MFGM fed rats showed increased spatial learning

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compared to NS controls (p=0.0229) and both MS MFGM and MS prebiotic blend + MFGM fed rats showed increased spatial learning compared to MS controls (p=0.0025 and p=0.0313 respectively) (see Figure 2A).

When assessing reference memory during the probe trail a significant main effect for dietary interventions ($F_{(3,93)}$=3.504, p=0.019) with regard to the percentage of time spent in the platform quadrant on the probe test day (see Figure 2B) was found. The dietary interventions prebiotic blend, MFGM alone and prebiotic blend + MFGM enhanced reference memory in the MWM as compared to MS controls (p=0.007, p<0.0001 and p=0.019 respectively). Moreover, there was a trend towards decreased performance and reference memory in the probe trial for MS controls compared to NS controls (p=0.1).

*Effects of administering prebiotic blend and/or MFGM on novel object recognition*

A significant main effect for early life stress ($F_{(1,94)}$=20.843, p<0.0001) on recognition memory was found, in the novel object recognition test, with MS rats showing significantly less ability to discriminate a novel object from a familiar one. There was no main effect for dietary intervention. Post hoc analysis showed a significant difference between NS and MS control rats (p=0.028) (data not shown).

**Effects of administering prebiotic blend and/or MFGM on visceral sensitivity**

During colorectal distension, which is used to determine visceral sensitivity with MS rats fed with the control diet showed lower pain thresholds than NS rats fed the control diet (p=0.005), which were ameliorated with both prebiotic blend + MFGM and MFGM dietary interventions (p=0.0001 and p=0.01 respectively). In addition, there was a significant increase in threshold in NS rats fed the

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MFGM only diet compared to the NS control diet fed rats, p=0.007. There was a main effect for dietary intervention ($F_{(3,90)}$=8.902, p<0.0001) (see Figure 3A).

Analysis of the number of pain behaviours exhibited by rats undergoing colorectal distension revealed a main effect for dietary intervention ($F_{(3,63)}$=3.834, p=0.014), and a trend for an early life effect ($F_{(1,63)}$=3.647, p=0.061) (see Figure 3B). More specifically, a difference was observed between NS rats fed the MFGM only diet and the NS rats fed the control diet, p=0.01. While the combination of prebiotic blend and MFGM reduced the number of pain behaviours seen in the MS rats, p=0.027.

**Temporal dynamics of stress-induced corticosterone levels**

Analysis of the effect of restraint stress on plasma cortisol levels indicated main effect for time only ($F_{(4,352)}$=575.961, p<0.0001) (Fig. 4A). At the 30 minute time point, which was immediately following restraint stress, a 2-way ANOVA revealed a significant main effect for early-life stress*diet ($F_{(3,96)}$=2.836, p=0.043) (Fig. 4B). Early-life stress induced a significant increase in the plasma corticosterone levels (p=0.031) when compared to the NS control group at time point 30 minutes (Fig. 4B). Both MFGM alone (p=0.032) and the combination of prebiotic + MFGM (p=0.027) appeared to facilitate the HPA axis feedback so the corticosterone levels retuned to baseline faster. Moreover, Fisher's *post hoc* test highlighted a significant difference between NS and the prebiotic fed NS rats (p=0.042) (Fig. 4B).

**Effects on administering prebiotic blend and/ or MFGM on hippocampal gene expression levels.**

In an effort to understand the mechanism of action of early life stress and of the dietary interventions on behavioural outcomes particularly related to spatial learning and memory we measured the expression of genes related to these in the hippocampus of the rats. We did not note any significant change induced by early life stress nor did we note a difference induced by the dietary interventions in the MS rats. MFGM did lead to a decrease in the glucocorticoid receptor in the hippocampus of the

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NS rats only ($F_{(3, 88)}$=4.08, p=0.035). There were no significant differences in any of the other genes analysed in the hippocampus which included GABAα1, GABAβ1, GABAβ2, and MR. (See Table 1).

**Effects on administering prebiotic and/ or MFGM on MR and on myelin-related gene expression levels in the prefrontal cortex.**

Other studies have indicated that an altered gut microbiota may be related to changes in the expression genes associated with stress as well as myelin-related genes, so here we investigated several genes known to be associated with these processes.

The most interesting finding with regard to the myelin gene expression analysis was seen on MAG gene expression, showing the interaction for early-life stress*dietary intervention ($F_{(3,96)}$=4.895, p=0.003). Fisher's *post hoc* showed statistical difference between NS Control *vs* MS Control (P<0.05) with the prebiotic blend (P < 0.01) and the combination of MFGM and prebiotic blend (P<0.001) ameliorating the impact of early life stress. While no significant differences were seen with regard to the other genes analysed in the prefrontal cortex which included MBP, MOG, PiP1, and MOBP. (See Table 2).

**Effects of MFGM with and without a prebiotic blend on relative abundance and diversity of gut microbiota of MS and NS rats.**

All significant differences between groups are noted in Table 3 below while Figure 6 gives an overall representation of relative abundance of bacteria in all groups. Analysis of the gut microbiota revealed that at family level, early life stress induced only one change as seen as a higher relative abundance of the family Paenibacillaceae_2.

Among NS rats, prebiotic blend supplementation led a decrease in the relative abundance of Marinilabiliaceae at family level while at the genus level the greatest effect was seen on *Barnesiella*. The greatest effect induced by MFGM at family level was an increase in Erysipelotrichaceae and at

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genus level an increase in *Ruminococcus*. The combination of MFGM and prebiotic blend had a significant effect at family level with a decrease in Marinilabiliaceae and at genus level with an increase in *Parabacteroides*.

In MS rats, supplementation of prebiotic blend, MFGM, or the combinations differently affected relative abundance of bacteria. Prebiotic blend supplementation effects were at family level, with the greatest effect seen in Bacteroidaceae decrease, and at the genus level, with the greatest impact of an increase in *Syntrophococcu*s. The dietary supplementation of MFGM had an impact on the family Gracilibacteraceae and an increase in *Barnesiella* at genus level.

And finally, the supplementation of the combination of MFGM and prebiotic blend influenced the relative abundance of bacteria at family level with the greatest difference being an increase in Clostridiaceae_1 and the greatest difference at genus level was an increase in *Clostridium* family IV.

With regard to alpha diversity, which is a measure of species richness and how evenly microbes are distributed, we found no effect of diet or early life stress on diversity estimates.

With regard to beta diversity, which is how different the microbial composition in one group environment compared to another, differences were observed between the NS groups (Figure 5A). Significant differences were seen between the control diet fed rats and the rats fed prebiotic blend alone ($p=0.002$, $R2=0.125$), also between the MFGM fed rats and the rats fed the prebiotic blend alone ($p=0.001$, $R2=0.156$) and also between the group fed the combination of prebiotic+MFGM and prebiotic blend alone ($p=0.001$, $R2=0.127$).

Among MS rats, a greater number of significant differences between groups were observed (Figure 5B). Significant differences were between the control diet fed rats and prebiotic blend fed group ($p=0.002$, $R2=0.114$), between control diet fed rats and the rats fed the diet of the combination of prebiotic+MFGM ($p=0.011$, $R2=0.102$). There was also differences between the rats fed the MFGM alone and the rats fed the prebiotic+MFGM combination ($p=0.001$, $R2=0.203$), between the rats fed diet containing the MFGM alone and the rats fed diet containing prebiotic blend alone ($p=0.001$, $R2=0.179$) and finally between the rats fed the combination of prebiotic+MFGM diet and the rats fed

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the diet containing the prebiotic alone (p=0.001, R2=0.116). When all groups were grouped into MS or NS there was a trend towards a significant difference induced by MS on beta diversity (p=0.07).

## 4. Discussion

Targeting the microbiome to support brain health is an emerging concept in neuroscience. Here we show that early life stress-induced alterations in behaviour can be attenuated by supplementing the diet with either MFGM alone or in combination with a prebiotic blend. Notably, this is the first study to show that MFGM consumption results in a significant reduction of the early life stress-induced visceral hypersensitivity. While previous studies have shown that dietary supplementation with probiotics and prebiotics are effective in the reduction of visceral sensitivity (McKernan *et al.*, 2010; Kannampalli *et al.*, 2014; McVey Neufeld *et al.*, 2017; Wang *et al.*, 2017) the impact of MFGM has not been shown before.

Aspects of central nervous system development such as hippocampal learning are impacted upon by stressors in early life (McVey Neufeld et al., 2017). Here we noted that MFGM alone and combined with the prebiotic blend was sufficient to improve spatial learning in MS rats. In addition, animals in the NS group also benefitted from MFGM combined with the prebiotic blend as they too showed improvements in spatial learning during the acquisition period. There was also a trend towards impaired reference memory in MS rats, and our results indicate that dietary interventions at gut level can influence aspects of cognition and could have potential benefits supporting cognitive brain health as we noted an increase in reference memory also with the dietary interventions.

This beneficial effect of MFGM on aspects of cognition is confirmed in a different animal model, the rat model of restricted growth (Brink & Lonnerdal, 2018). Here cognitive function during a T maze was improved with the supplementation with MFGM. Our study is also in line with studies in human infants. Higher cognitive scores on the Bayley Scales and Toddler development were noted in infants receiving a low-energy, low-protein formula supplemented with MFGM compared with control-fed infants (Timby *et al.*, 2014; Mudd *et al.*, 2016).

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Furthermore, given that the dietary interventions in this study improved spatial memory in the Morris water Maze and not recognition memory in the novel object recognition test implies that they may have an impact on specific brain regions or neurotransmitter pathways associated with discrete aspects of cognition. This requires further investigation, and while there is considerable overlap with regard to brain areas and neurotransmitters systems for different types of memory, activation of the immediate early gene Arc in the prefrontal cortex and hippocampus has been specifically implicated in the spatial memory (Shanmugasundaram et al., 2015). Whilst hippocampal dopamine has been related to novel object recognition memory (Yang et al., 2017).

Enhanced or exaggerated sensitivity of the gastrointestinal tract can be very disabling and has been associated with an interruption of appropriate colonisation of the gut in early life (O'Mahony *et al.,* 2017). Colorectal distension is routinely used to assess visceral sensitivity both clinically and preclinically. In keeping with previously published work from our own laboratory, we saw an increased visceral sensitivity in MS rats (O'Mahony *et al.,* 2011; Felice *et al.,* 2014), which was improved with the consumption of MFGM. We and others have previously shown that dietary interventions targeting the gut microbiota reduce visceral sensitivity in several different animal models (Larauche *et al.,* 2012; Kannampalli *et al.,* 2014; McVey Neufeld *et al.,* 2017; Wang *et al.,* 2017). Yet here while we do not see striking differences in the microbiota profile of the control diet-fed MS and NS rats, the MS rats do display a significant increase in visceral sensitivity. The group with the greatest difference in behaviour from controls during colorectal distension was the MS group that received MFGM combined with the prebiotics which showed a significant decrease in sensitivity and pain behaviours. This is mirrored by the greatest number of changes in the gut microbiota compared to controls. Other factors such as gut barrier function and immune response which are both linked to changes in the gut microbiota are also likely to be involved in changes in gut sensitivity. Hence it is unlikely that changes to one system alone, as in the gut microbiota, are responsible for the difference in pain responses. Therefore, future studies investigating the exact mechanisms underpinning such effects should focus on illuminating the role of the gut barrier, local immune response as well as spinal and supra-spinal processes. These data support the proposition that such dietary interventions may be useful in disorders associated with abdominal discomfort or pain such as

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irritable bowel syndrome or infantile colic. Interestingly, MFGM diet reduced visceral sensitivity to colorectal distension in NS rats which indicates that supplementing with MFGM may also ease symptoms related to abdominal pain or discomfort that can occur from time to time in the healthy population.

It has been demonstrated previously that the hypothalamic–pituitary–adrenal (HPA) axis has been shown to be regulated by the microbiome (Sudo *et al.*, 2004; Foster *et al.*, 2017; Wiley *et al.*, 2017). Here we show that MFGM alone and in combination with the prebiotic blend modulates corticosterone release both in normal animals and in those exposed to early life stress. It is interesting that prebiotic blend alone or in combination with MFGM reduced corticosterone in the MS rats but not MFGM alone hence indicated that the prebiotic blend drives the response to stress. This effect can be viewed as beneficial as assisting with returning the HPA axis back to baseline after a stressful event which is a necessary component of coping with stress and adverse events. Conversely, we noted that the prebiotic intervention lead to an increase in cortisol at 30 minutes in the control rats. Further investigation is required in order to determine the true meaning of this effect.

Given our findings of altered stress reactivity, and in order to establish whether the glucocorticoid (GR) and/ or the mineralocorticoid receptors (MR) are involved in the prebiotic and prebiotic +MFGM effects on the HPA axis, we further investigated GR and MR gene expression levels in the rat hippocampus. However, their expression did not directionally parallel the changes in behavior suggesting that alternative mechanisms are underlying both the MS and dietary effects. We did note that MFGM alone led to a decrease in GR which may indicate a stress-modulatory effect which is line with the behavioral observations for MFGM alone in the visceral hypersensitivity test. In line with the cognitive improvement in the MWM, we investigated the effect of both early-life stress and dietary interventions on gene expression associated with learning and memory in the hippocampus and prefrontal cortex. No obvious trends in gene expression could be garnered to explain the behavioral effects observed with either stress or its counteraction by dietary manipulations. Reasons behind this could be a compensatory effect overtime or a functional readout or protein levels. These could be topics of further investigation. Intriguingly, MS rats showed an increase in MAG gene expression in

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the prefrontal cortex, which is associated with myelination. This was reduced by the prebiotic blend and the combination of prebiotic+MFGM. Myelination in the prefrontal cortex undergoes plastic changes and is influenced by environmental stimuli mediated by increased electrical activity in axons (Lee & Fields, 2009). Furthermore, we have shown that germ free mice have increased myelination related in genes in the prefrontal cortex also (Hoban et al., 2016) indicating that changes in the gut microbiota may play a role in myelination. Alterations in myelination during a specific time window of life can contribute to impairment of development of the central nervous system throughout the lifespan (Kikusui *et al.*, 2007; Hoban *et al.*, 2016). What is interesting here is that the change in MAG is related to changes in behavior in our MS model, specifically during the probe trial of the Morris water maze, but not to microbiota hence indicating that some of the impact on spatial memory of the stress and dietary interventions may be due to changes in myelination patterns. This requires further investigation in order to clarify the impact of MFGM and the prebiotic blend on myelination.

A growing body of evidence supports a role for the gut microbiota in modulating the effects of stress (Foster et al., 2017; O'Mahony et al., 2017; Rea et al., 2017) and here we show that the addition of MFGM to the diet was sufficient to influence the gut microbiota of both NS and to a greater extent, MS rats. We have previously shown that early life stress in rats reduced microbial diversity alongside behavioral changes (O'Mahony *et al.*, 2009; Pusceddu *et al.*, 2015a). While conversely we have also shown that disruption of the microbiota in early life using antibiotics leads to altered behavior with an apparently unaltered microbiota in adulthood (O'Mahony *et al.*, 2014). In another study, whilst germ free animals exposed to MS do not present behavioral impairment but do develop the behavioral deficits after colonization in adulthood (De Palma *et al.*, 2015). These studies and the differences between them highlight the importance of timing with regard to the interruption of the microbiota and the enduring effects of this disruption on the microbiota-gut-brain axis.

In the current study we note a greater impact of all dietary interventions on the gut microbiota than early life stress. Others have shown differences in the composition of the microbiota on postnatal day 20 that were amenable to microbial manipulation but these were no longer evident in adulthood (Fukui *et al.*, 2018). Similar to our study, differences in behavior between MS and NS rats were noted

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in adulthood without linking to large differences in the microbiome. This indicates that it is possible for the stress-associated hypersensitivity of the brain-gut axis to remain, despite apparent recovery of the gut microbiota composition. We also noted this in an early life antibiotic study, where the gut microbiota was changed considerably at one week post antibiotic but then by the time we analysed behavior from 8 week of age the microbiota of control and early life antibiotic treated rats appeared very similar (O'Mahony et al., 2014). Furthermore, events in the postnatal period play a fundamental role in the initiation of exaggerated response to stress and differences in the microbiota during this stage of life impacts on development of the central nervous system that remains without any great persisting differences in the microbiota. Other studies have shown that adverse early life events including MS leads to changes in several species of bacteria in a sex-dependent manner (Rincel *et al.*, 2019). This model was far more strenuous than the one we use here as it included maternal immune activation maternal separation (3hr per day from postnatal day (PND)2 to PND14) and maternal unpredictable chronic mild stress and was carried out in mice and hence is difficult to compare to ours. Furthermore, studies on the impact of MS in germ free mice have also identified a unique subset of bacterial species that are altered due to the adverse event (De Palma *et al.*, 2015). Yet again this is not a comparable study to ours given it was carried out in specific pathogen free and germ free mice. Given the many factors that influence the gut microbiota, replication of studies or comparison to others should be done with care and understanding of these influences.

Another possible reason for the lack of differences between the MS and NS control rats was the base dietary components. All diets contained docosahexaenoic acid (DHA), an omega-3 fatty acid. DHA is known to impact positively on the gut microbiota and is associated with the enhanced production of short-chain fatty acids (Costantini *et al.*, 2017). Despite this DHA in the control diet, it was not sufficient to ameliorate the enduring effects of early life stress on behavior. In contrast, the addition of MFGM to the DHA based diet was successful in ameliorating the impact of MS on memory related-behaviour and pain behaivours as well as influencing the microbiota.

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Also, important to note was that dietary interventions were initiated after weaning hence the impact of MS and hypothetical changes to the microbiota during the stress hyporesponsive persisted into adulthood despite the almost full recovery of the microbiota.

Specifically, with regard to the gut microbiota in this study we observed an effect of MS at family level with an increase in Paenibacillaceae_2. This bacteria was noted to be increased in the appendix of children who suffered from appendicitis compared to those that did not (Jackson et al., 2014) indicating a role in immune regulation. While the role of this bacteria has not been described with regard to pain or stress-associated disorders, MS rats do display exaggerated immune response to ex vivo stimulus (O'Mahony *et al.*, 2009).

Previous studies have indicated that MFGM significantly impacts on the gut microbiota (Thompson *et al.*, 2016; Bhinder *et al.*, 2017; Le Huerou-Luron *et al.*, 2018). In this study MFGM significantly affected relative abundance levels of different bacteria, with the addition of the prebiotic blend further enhancing this impact. We did not note changes to the same bacterial species as others. This may be due to the fact that we have used a different animal model and that the base/control diet contained DHA. Nonetheless we did see substantial changes to the gut microbiota induced by MFGM as well as the prebiotic blend alone, as well as the combination of both. The greatest impact on the gut microbiota was noted in the MS rats that received the combination of MFGM and the prebiotic blend and as mentioned above this group also showed the largest difference from the control group, particularly during colorectal distension.

The composition of the gut bacteria was altered by MFGM and the other dietary interventions whilst producing differential effects in NS and MS. Albeit no causal relationship can be completely drawn from the current study, these observations offer some indication on correlations between modulated composition with certain physiological conditions. In our study, within the MS animals, we saw *Barnesiella* abundance increased by MFGM. This bacterium has been shown to have beneficial effects on the gut (Ubeda *et al.*, 2010; Ubeda *et al.*, 2013) as it confers resistance to pathogenic infection. *Flavonifractor* was increased by MFGM and the combination MFGM and prebiotic blend.

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This bacterium consumes GABA which may have relevance to brain function as well as local nociception. The relative abundance of *Lachnospiraceae* was significantly reduced in animals fed with MFGM compared to control diet. Increases in this bacteria have been previously noted in an animal model for chronic stress (Li et al., 2017). *Lachnospiraceae* has an impact on lifespan and the immune system (Maldonado-Contreras et al., 2011) and is associated with disease such as ulcerative colitis, Crohn's and celiac disease.

*Clostridium* cluster IV and *Sporobacter* was increased in animals fed with the combination of MFGM and prebiotic blend. Some types of clostridia are associated with key functions and homeostasis in the gut (Lopetuso et al., 2013) and hence an increase may confer health benefits. Increases in *Sporobacter* have also been reported in piglets fed a fibre-containing diet, the alfalfa diet (Zhang et al., 2016). The relative abundance of *Ruminococcaceae* was also noted to be increased in this group and a higher abundance of this bacteria was previously seen in controls in comparison to patients with major depression (Jiang et al., 2015).

The alpha and beta diversity of the bacterial populations present were also investigated. No significant impact was observed for alpha diversity. Yet, beta diversity, was influenced by MS as well as MFGM and prebiotic administration. MFGM alone and in combination with prebiotic impacted on the beta diversity of both MS and NS groups. There was a trend towards a significant difference between the control MS and NS groups.

In conclusion, supplementation of early life stressed rats with prebiotic and MFGM alone, or its combination (prebiotic + MFGM) resulted in improved hippocampal-dependent spatial learning when compared to early life stressed rats fed a control diet. In addition, providing early life stressed rats with the combination of prebiotic + MFGM, or with MFGM alone ameliorated stress-induced visceral hypersensitivity. MFGM also improved visceral pain scores in control rats which may have implications for pain associated with bloating in the healthy population. These results have translational connotations as clinical studies have shown that stress in early life in children is associated with altered stress signalling, increased pain responses and cognitive deficits (Clarke et al.,

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2014). Moreover, the impact of adverse events in early life on the gut microbiota may be involved in the manifestation of the above deficits (O' Mahony et al., 2015). Here, we have shown that the most significant effect of the dietary interventions in the mechanistic analysis was indeed changes in the gut microbiota composition and interestingly these changes were different albeit not directly correlated to exposure to early life stress. Hence, we speculate that the ingredients investigated can influence behaviour through changes in the gut microbiota and to a greater extent in the maternal separation, early life stress model. Observation of microbiota changes and immune response at the time closer to the hyporesponsive period would enhance the understanding/resolution of this speculation.

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**Conflict of Interest Statement**

There is no conflict of interest.

**Author Contributions**

Siobhain M. O'Mahony designed the study and wrote the manuscript; Karen-Anne McVey Neufeld, Matteo M. Pusceddu, Kiera Murphy, Conall Strain, Mamen C. Laguna, Veronica L. Peterson all assisted with the experimental analysis; Rosaline V. Waworuntu, Sarmauli Manurung, Catherine Stanton, Brian M. Berg, Timothy G. Dinan and John F. Cryan were involved in study design and edited the manuscript.

**Data Accessibility Statement**

We will make our data available should it be required.

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**Abbreviations**

DI, discrimination index; FOS, fructo-oligosaccharide; GABA, gamma amino butyric acid; GOS, galacto-oligosaccharide; GR, glucocorticoid receptor; HPA axis, hypothalamic-pituitary-adrenal axis; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MFGM, milk fat globule membrane; MOBP, myelin associated oligodendrocyte basic protein; MOG, myelin oligodendrocyte glycoprotein; MR, mineralocorticoid receptor; MS, maternal separation; MWM, Morris Water Maze; NS, non-separated; PDX, polydextrose; PND, postnatal day; Pip1-phosphoinositide 1.

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**Figure legends**

**Figure 1:** Experiment was conducted as outlined above, PND is postnatal day.

**Figure 2. Prebiotic blend and/or MFGM intervention improve spatial learning and reference memory** (A) In NS groups, prebiotic blend + MFGM improved spatial learning on Day 2 of the acquisition training compared to NS controls, while in MS groups both prebiotic + MFGM and MFGM alone improved spatial learning on Day 2 as compared to MS rats on the control diet. (B) In the Probe Trial, prebiotic blend, prebiotic + MFGM and MFGM dietary interventions all resulted in increased time spent in the "probe" quadrant compared to MS rats on the control diet. * denotes vs respective control; # denotes *vs* MS control, t denotes trend towards difference compared to NS control, p=0.1.

**Figure 3. Prebiotic blend and MFGM feeding reduce stress induced visceral pain hypersensitivity** (A) Overall, MS rats showed lower pain thresholds which were ameliorated with both the MFGM diet alone and the combination of prebiotic blend + MFGM. MFGM diet also reduced visceral pain sensitivity to colorectal distension under NS conditions (B) Feeding NS rats MFGM resulted in fewer pain behaviours exhibited as compared to NS controls (, while feeding MS rats prebiotic+MFGM also resulted in fewer pain behaviours during colorectal distension. * denotes *vs* NS Control, # *vs* MS Control.

**Figure 4. Prebiotic blend and the combination of prebiotic blend +MFGM facilitate HPA axis feedback on early-life stress-induced corticosterone levels.** (A) Temporal dynamic of stress-induced corticosterone levels. (B) At 30 minutes' time point both prebiotic alone and the combination of prebiotic+MFGM showed a quicker return to baseline of corticosterone levels induced by early-life stress. *$P$<0.05 denotes significant difference vs NS-Control; \$$P$<0.05 denotes significant difference vs MS-Control. Data are presented as mean ± SEM.

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**Figure 5. Impact of early-life stress, MFGM, and prebiotics on the beta-diversity of the caecal microbiome.** Principal coordinates analysis (PCoA) plots based on Bray-Curtis dissimilarity. PC1, PC2, PC3 represent the first, second and third principal

components, respectively. The distance between samples indicates the similarity of the distribution of functional classifications in the sample. The closer the distance, the higher the similarity.

a) Non-separated (NS) rats; Red = Control, Blue = Prebiotic+MFGM, Yellow = Prebiotic, Green = MFGM.

b) Maternally separated (MS) rats; Red = Control, Blue = Prebiotic+MFGM, Yellow = Prebiotic, Green = MFGM.

**Supplemental Figure 1: The relative abundance of caecal microbiota.** MS and the dietary interventions, MFGM and prebiotic and the combination, significantly yet differentially changed the relative abundance of the microbiota.

**Supplemental Table 1:** The breakdown of all components within each diet administered to the difference groups.

**Graphical abstract legend:** MS-induced changes in the brain-gut microbiota axis. Intervention with MFGM and prebiotic blend significantly impacted the composition of the microbiota as well as ameliorating some of the long-term effects of early-life stress.

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| Group | GABAα1 | GABAβ1 | GABAβ2 | GR | MR |
|---|---|---|---|---|---|
| NS-control | 0.893 ± 0.06 | 1.000 ± 0.08 | 1.000 ± 0.06 | 1.000 ± 0.08 | 0.090 ± 0.07 |
| NS-prebiotic | 0.852 ± 0.08 | 1.048 ± 0.10 | 1.109 ± 0.08 | 0.991 ± 0.06 | 0.789 ± 0.05 |
| NS-MFGM | 0.637 ± 0.03 | 1.148 ± 0.16 | 1.023 ± 0.12 | 0.799 ± 0.04 * | 1.006 ± 0.09 |
| NS-prebiotic+MFGM | 0.845 ± 0.09 | 1.048 ± 0.14 | 1.050 ± 0.11 | 0.848 ± 0.04 | 0.814 ± 0.10 |
| MS-control | 0.805 ± 0.09 | 1.003 ± 0.14 | 1.002 ± 0.10 | 0.848 ± 0.05 | 0.756 ± 0.05 |
| MS-prebiotic | 0.928 ± 0.10 | 0.993 ± 0.08 | 0.945 ± 0.05 | 0.892 ± 0.05 | 0.926 ± 0.08 |
| MS-MFGM | 0.979 ± 0.10 | 0.982 ± 0.11 | 1.060 ± 0.13 | 0.989 ± 0.06 | 0.902 ± 0.80 |
| MS-prebiotic+MFGM | 0.935 ± 0.13 | 1.154 ± 0.14 | 1.045 ± 0.12 | 0.936 ± 0.05 | 1.044 ± 0.10 |

**Table 1. Dietary interventions both with and without early life stress on hippocampal gene expression.** No differences were observed in $GABAA_{\alpha 1}$, $GABAA_{\beta 1}$ and $GABAA_{\beta 2}$ gene expression levels in any MS or diet groups. MFGM diet reduced GR gene expression levels as compared to NS control only *$P$<0.05 denotes significant difference vs NS-Control. Data are presented as mean ± SEM. GR-glucocorticoid receptor, MR-mineralocortocoid receptor, GABA-gamma amino butyric acid.

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| Group | MBP | MOG | PiP1 | MOBP | MAG |
|---|---|---|---|---|---|
| NS-control | 0.99±0.11 | 1.00±0.09 | 0.99±0.12 | 1.00±0.11 | 1.00±0.13 |
| NS-prebiotic | 0.44±0.09 | 1.0± 0.08 | 0.8± 0.11 | 0.93±0.07 | 0.91±0.07 |
| NS-MFGM | 0.87±0.14 | 0.89±.08 | 0.73±0.87 | 0.91±0.08 | 0.94±0.06 |
| NS-prebiotic+MFGM | 0.96±0.05 | 1.03±0.07 | 0.81±0.05 | 1.04±0.07 | 1.18±0.12 |
| MS-control | 0.78±0.11 | 0.93±0.09 | 0.82±0.07 | 1.01±0.06 | 1.27±0.12* |
| MS-prebiotic | 0.69±0.08 | 0.86±0.06 | 0.78±0.14 | 1.06±0.10 | 0.87±0.08# |
| MS-MFGM | 0.87±0.13 | 1.12±0.09 | 1.16±0.20 | 1.22±0.10 | 1.09±0.08 |
| MS-prebiotic+MFGM | 0.50±0.09 | 0.81±0.08 | 1.05±0.17 | 0.95±0.12 | 0.77±0.07# |

**Table 2. Expression levels of myelination-related genes in the prefrontal cortex**. Prebiotic diet decreased MBP gene expression levels compared to NS control and NS prebiotic+MFGM, respectively; MFGM diet increased MOG gene expression levels compared to prebiotic+MFGM in the MS group; No differences were observed in both MOBP and PiP1 gene expression levels in any diet groups. Intergroup comparisons: *$P<$ 0.05 denotes significant difference vs NS-prebiotic; *#P<0.05* denotes significant difference vs MS-control. Data are presented as mean ± SEM. MBP-myelin basic protein, MOG-myelin oligodendrocyte glycoprotein, Pip1-phosphoinositide 1, MOBP-myelin associated oligodendrocyte basic protein.

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**NS ctrl Vs MS ctrl**

| *Family* | P | NS Ctrl | MS Ctrl | q |
|---|---|---|---|---|
| **Paenibacillaceae_2** | **0.044** | 0.00% | 0.03% | q=0.005 |

**NS ctrl Vs NS+pre**

| *Family* | p | NS Ctrl | NS Pre | q |
|---|---|---|---|---|
| **Marinilabiliaceae** | **0.013** | 0.67% | 0.25% | 0.0053 |
| ***Genus*** | | | | |
| **Barnesiella** | **0.001** | 16.46% | 11.67% | 0.0021 |
| **Lachnoanaerobaculum** | **0.004** | 3.82% | 0.42% | 0.0042 |
| **Pseudoflavonifractor** | **0.009** | 5.55% | 3.67% | 0.0063 |
| **Parabacteroides** | **0.016** | 0.91% | 1.83% | 0.0083 |
| **Anaerostipes** | **0.044** | 0.46% | 3% | 0.0104 |
| **Syntrophococcus** | **0.044** | 1.91% | 4.75% | 0.0125 |

**NS ctrl Vs NS+MFGM**

| *Family* | p | NS Ctrl | NS MF | q |
|---|---|---|---|---|
| **Erysipelotrichaceae** | **0.019** | 30.97% | 35.40% | 0.0053 |
| **Peptostreptococcaceae** | **0.032** | 2.47% | 1.49% | 0.0106 |
| ***Genus*** | | | | |
| **Ruminococcus** | **0.023** | 3.27% | 6.83% | 0.0021 |
| **Barnesiella** | **0.027** | 16.46% | 13% | 0.0042 |

**NS ctrl Vs NS+Pre+MFGM**

| *Family* | p | NS Ctrl | NS MF + Pre | q |
|---|---|---|---|---|
| **Marinilabiliaceae** | **0.019** | 0.67% | 0.25% | 0.0053 |
| **Ruminococcaceae** | **0.047** | 30.97% | 36.40% | 0.0106 |
| ***Genus*** | | | | |
| **Parabacteroides** | **0.002** | 0.91% | 2.27% | 0.0021 |
| **Alistipes** | **0.028** | 0.27% | 0.81% | 0.0042 |
| **Flavonifractor** | **0.034** | 2.91% | 4.27% | 0.0063 |

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| | | | | |
|---|---|---|---|---|
| **Clostridium_IV** | **0.047** | 3.36% | 4.64% | 0.0083 |

**MS Ctrl Vs MS+Pre**

| *Family* | p | MS Ctrl | MS Pre | q |
|---|---|---|---|---|
| **Bacteroidaceae** | **0.005** | 7.33% | 4.77% | 0.0053 |
| **Rikenellaceae** | **0.012** | 0.38% | 0.64% | 0.0106 |
| **Deferribacteraceae** | **0.012** | 0.50% | 0.25% | 0.0160 |
| **Streptococcaceae** | **0.02** | 0.04% | 0.02% | 0.0213 |
| **Peptostreptococcaceae** | **0.039** | 1.88% | 2.93% | 0.0266 |
| ***Genus*** | | | | |
| **Syntrophococcus** | **0** | 1.50% | 6% | 0.0021 |
| **Parabacteroides** | **0.007** | 1% | 2.10% | 0.0042 |
| **Bacteroides** | **0.01** | 7.25% | 4.67% | 0.0063 |
| **Coprococcus** | **0.014** | 2.75% | ND | 0.0083 |
| **Clostridium_IV** | **0.017** | 2.42% | 4% | 0.0104 |
| **Alistipes** | **0.039** | 0.17% | 0.67% | 0.0125 |

**MS Ctrl Vs MS+MFGM**

| *Family* | p | MS Ctrl | MS MF | q |
|---|---|---|---|---|
| **Gracilibacteraceae** | **0.004** | 0.10% | 0.05% | 0.0053 |
| **Lachnospiraceae** | **0.044** | 23.75% | 18.62% | 0.0106 |
| ***Genus*** | | | | |
| **Barnesiella** | **0.004** | 13.17% | 17.82% | 0.0021 |
| **Flavonifractor** | **0.016** | 2.25% | 3.55% | 0.0042 |

**MS Crtl Vs MS+Pre+MFGM**

| *Family* | p | MS Ctrl | MS MF + Pre | q |
|---|---|---|---|---|
| **Clostridiaceae_1** | **0** | 0.38% | 0.54% | 0.0053 |
| **Eubacteriaceae** | **0.004** | 0.09% | 0.23% | 0.0106 |
| **Peptostreptococcaceae** | **0.013** | 1.88% | 4.25% | 0.0160 |
| **Ruminococcaceae** | **0.016** | 28.75% | 35.87% | 0.0213 |
| **Rikenellaceae** | **0.019** | 0.38% | 0.78% | 0.0266 |

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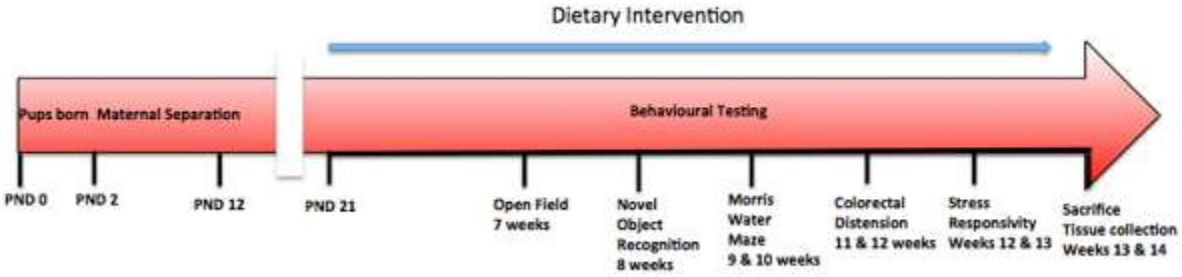
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|---|---|---|---|---|
| **Coriobacteriaceae** | **0.023** | 0.02% | 0.06% | 0.0319 |
| **Bacteroidaceae** | **0.032** | 7.33% | 5.14% | 0.0372 |
| ***Genus*** | | | | |
| **Clostridium_IV** | **0** | 2.42% | 6.50% | 0.0021 |
| **Flavonifractor** | **0.007** | 2.25% | 3.82% | 0.0042 |
| **Clostridium_XI** | **0.016** | 2% | 4.27% | 0.0063 |
| **Clostridium_sensu_stricto** | **0.023** | 0.17% | 0.73% | 0.0083 |
| **Cellulosibacter** | **0.023** | 0.17% | 0.72% | 0.0104 |
| **Sporobacter** | **0.032** | 0.83% | 1.73% | 0.0125 |
| **Parabacteroides** | **0.037** | 1% | 1.64% | 0.0146 |
| **Bacteroides** | **0.044** | 7.25% | 5.18% | 0.0167 |
| **Alistipes** | **0.044** | 0.17% | 0.82% | 0.0188 |

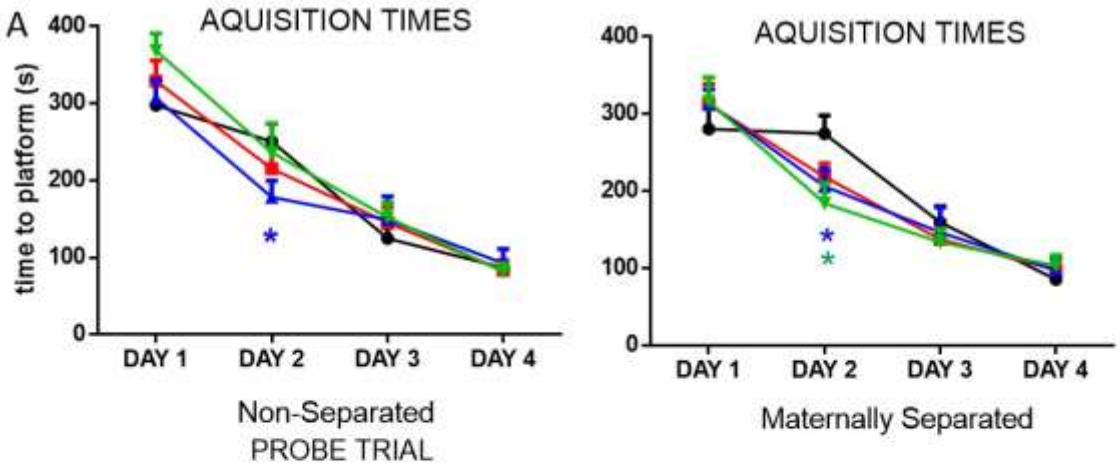
**Table 3:** The taxa that were significantly different between groups at phylum, family and genus level. % refers to percentage of relative abundance of bacteria. Q value is the adjusted p value for false discovery rates.

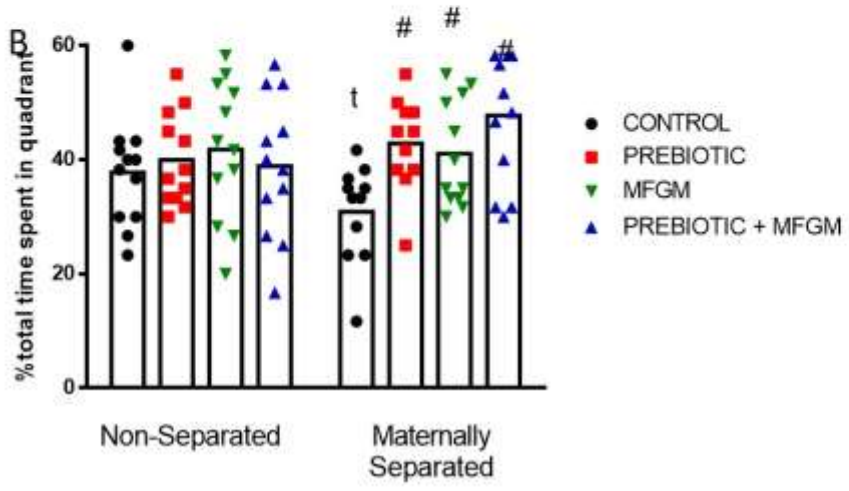
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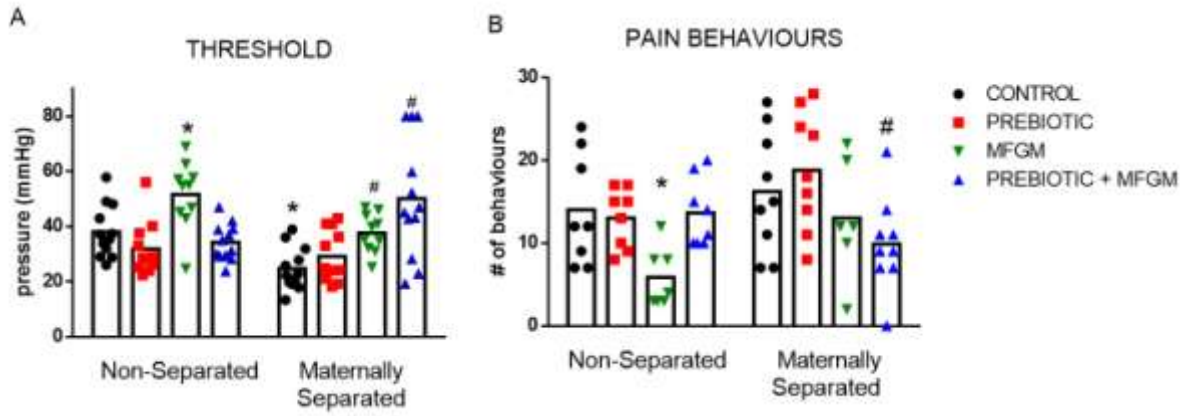


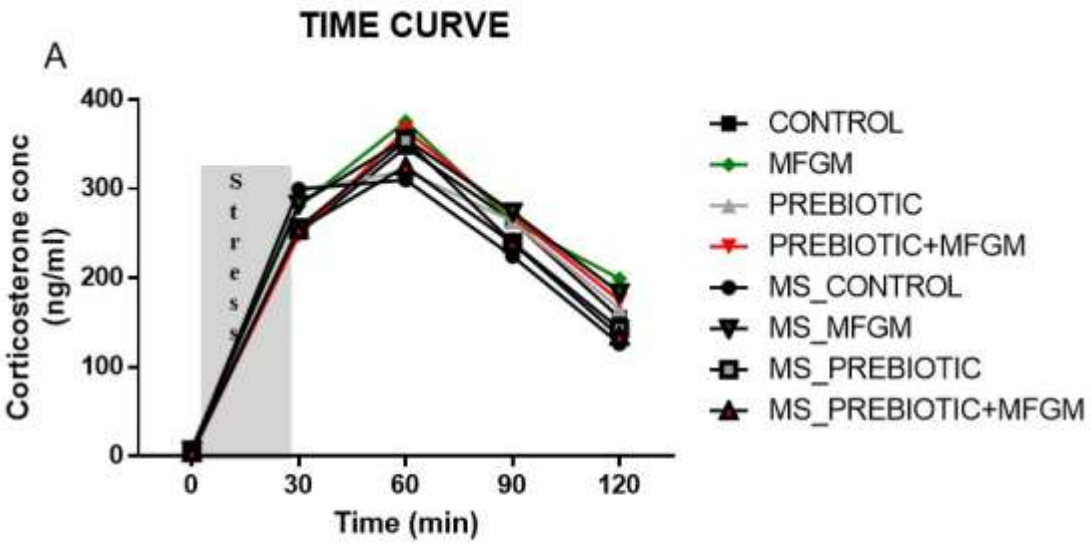

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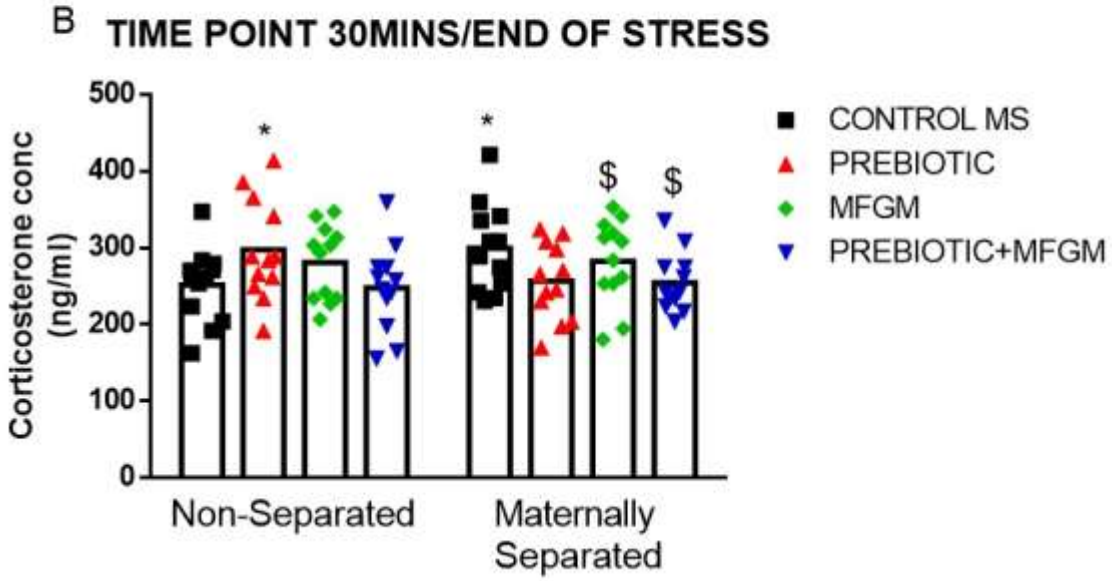
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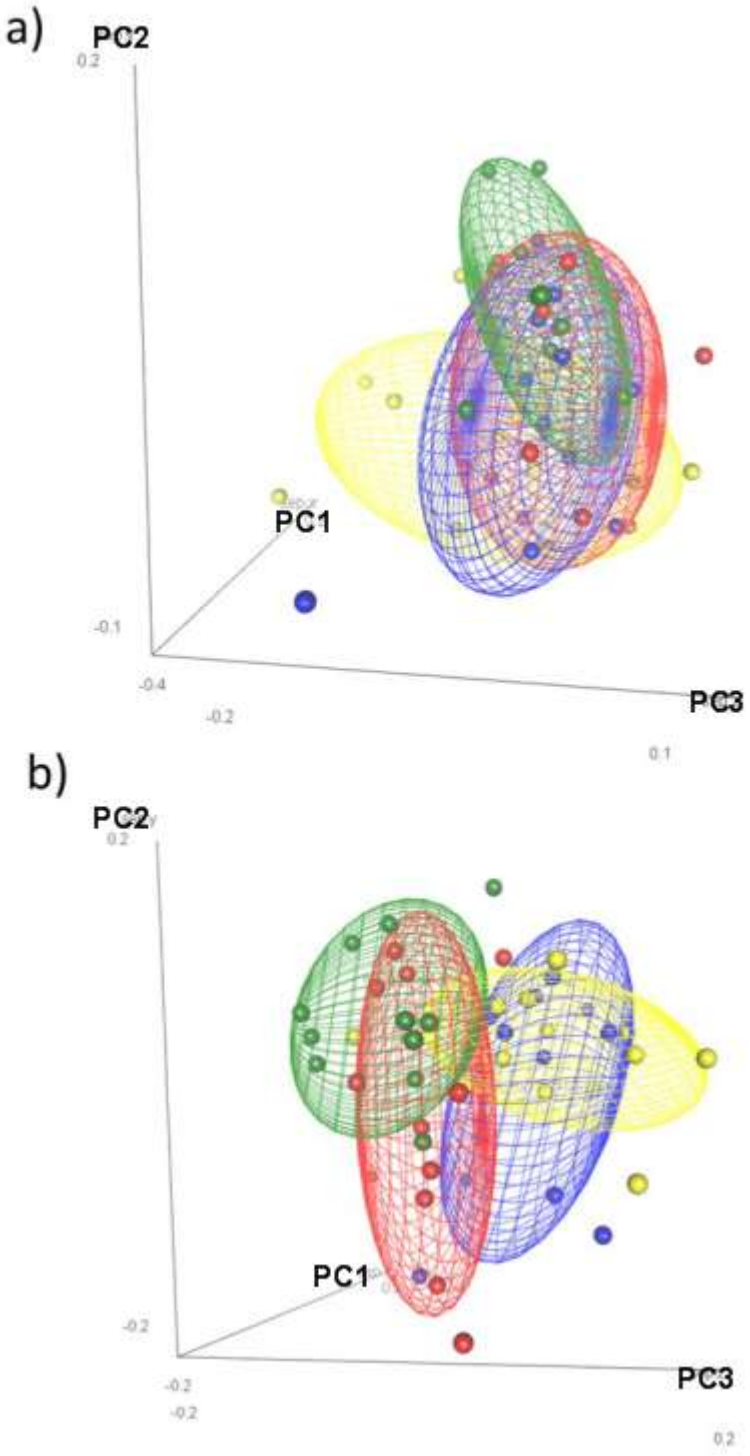
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