TREATING MATERNALLY-SEPARATED RATS WITH SHORT-CHAIN FATTY ACIDS: THE EFFECT OF SUPPLEMENTATION ON BEHAVIOR AND THE GUT MICROBIOTA

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Abstract

The human gut microbiota consists of thousands of species of microorganisms that communicate with the brain via the microbiota-gut-brain axis (MGBA) to influence host physical and mental health. Early life adversity (EA) disrupts the gut microbiota, possibly contributing to the development of depressive and anxiety-like behavior. Some of the microbiota that are disrupted by EA normally produce short-chain fatty acids (SCFAs) as metabolic products; without these SCFAs, healthy communication between the gut and the brain is altered. Here, we utilized maternal separation as a model of EA to investigate the capacity of an oral SCFA supplement to mitigate the behavioral and microbial effects of EA. Maternally separated (MS) rats were removed from their home cage and isolated for three hours daily from postnatal day (PND) 1-14. Non-separated (NS) control rats remained in their cage during this period. Beginning on PND21 and continuing throughout behavioral testing, rats were administered an SCFA solution or equimolar sodium chloride in their drinking water. Two groups of rats were selected for cecal sample collection on PND21 and 28, while a third group of rats underwent open field, forced swim, and sucrose preference tests to assess depressive and anxiety-like behavior. Results indicated that MS altered gut microbial composition but did not consistently induce depressive or anxiety-like behavior. SCFA supplementation failed to attenuate these microbial alterations and maladaptive behavior when it was observed. Future studies should employ larger sample sizes, control for handling of rat pups, utilize a lower concentration sucrose solution and administer the SCFA solution for longer than one week prior to initiation of behavioral testing.

Treating Maternally-Separated Rats with Short-Chain Fatty Acids: The Effect of Supplementation on Behavior and the Gut Microbiota

The human gut contains thousands of species of microorganisms that have colonized and coevolved with the human species (for review, see Sekirov et al., 2010). These bacteria, archaea, viruses, and unicellular eukaryotes, collectively known as the gut microbiota, perform physiological functions necessary for the host that the host themselves cannot perform(for review, see Xu et al., 2007). The cells of the gut microbiota outnumber the cells of all other human microbiota combined, with Bacteroidetes and Firmicutes as the dominant bacterial phyla (Xu et al., 2007). The gut microbiota influence the development of the immune system and gastrointestinal tract, protect the host from pathogens, and play a role in host metabolism and nutrition (Sekirov et al., 2010). More recently, researchers have begun to investigate how the gut microbiota communicate with the brain via neural, endocrine, and immune pathways to influence neurological outcomes and behavior. This bidirectional communication pathway, known as the microbiota-gut-brain-axis (MGBA), influences central nervous system (CNS) development and is thought to contribute to various psychiatric conditions (Cryan et al., 2019).

To study the impact of the gut microbiota on CNS development and psychopathological behavior, the microbiome of animal models can be manipulated in a controlled environment, allowing causal conclusions to be made. Studies employing animals raised without any microflora (germ-free, GF) or with select pathogenic bacteria eliminated (specific-pathogen free, SPF) (Neumann et al., 2019), offer proof-of-principle findings for a gut microbial influence on behaviors related to mood disorders. GF animals consistently display reduced anxiety in a variety of behavioral tests (Foster & McVey Neufeld, 2013). As compared to SPF rats, GF rats exhibited decreased anxiety-like behavior and altered signaling molecules previously associated with

anxiety, including brain-derived neurotrophic factor (BDNF) mRNA and monoamines (Bahi, 2017; Heijtz et al., 2011; Neufeld et al., 2011). GF mice similarly exhibited reduced anhedonia and despair behavior compared to SPF mice (Lukić et al., 2019). Microbial recolonization of the GF animals restored these behaviors to levels observed in SPF mice (Heijtz et al., 2011). In a separate study, rats whose microbiota had been depleted by antibiotics developed anhedonia and anxiety-like behavior after receiving a fecal microbial transplant (FMT) derived from a human participant with major depressive disorder (Kelly et al., 2016). The behavioral changes that resulted from specific manipulations of the microbiota via FMT provide further causal evidence of gut microbial involvement in psychopathological behaviors.

The gut microbiota is essential for proper physiological brain function and structural development. For instance, GF animals exhibited an exaggerated hypothalamic-pituitary-adrenal (HPA) axis response to stress, which was alleviated following colonization with *Bifidobacterium infantis* (Sudo et al., 2004). Indeed, proper modulation of the HPA axis by the gut microbiota may play an essential role in mental health throughout the lifespan (for review, see de Weerth, 2017). The gut microbiota is also essential for proper development of microglia, which are crucial for immune regulation and other functions in the CNS (Erny et al., 2015). The expression of genes related to synaptic plasticity differs between GF and SPF animals, but colonization of the GF animals attenuated these differences, suggesting that the gut microbiota is also involved in the development of neuronal circuits possibly related to anxiety-like behavior (Heijtz et al., 2011).

The early postnatal period is a vulnerable developmental period for both the microbiota and the brain; consequently, stressors during this period can disturb the developing gut or brain, altering MGBA signaling and potentially impacting CNS development and later life mental

health (Borre et al., 2014). Early life adversity (EA), such as maltreatment or experience of family conflict in childhood, is one such stressor that can have a profound, enduring effect on one's development and wellbeing. EA is correlated with adverse later-life outcomes such as lower educational attainment (Houtepen et al., 2020; Porche et al., 2011), economic hardship (Kim et al., 2020), and physical and mental health challenges (Benjet et al., 2010; Merrick et al., 2019). Indeed, EA increases one's risk of developing psychopathology such as major depressive disorder (MDD) and anxiety disorders (Benjet et al., 2010; Spatz Widom et al., 2007). One meta-analysis revealed that children who have experienced any type of childhood maltreatment are 2.03 times more likely to have depression and 2.70 times more likely to have anxiety than those who have not experienced childhood maltreatment (Li et al., 2016). In addition, childhood maltreatment (e.g., neglect), is associated with reduced response to treatment for depression, as well as an increased risk for enduring depressive episodes (Nanni et al., 2012). The strong evidence linking EA with the development of depression and anxiety and a subsequent diminished response to conventional treatment makes investigations into novel EA interventions of the utmost importance.

Investigating the biomarkers of EA, in both the gut and the brain, can allow for the development of targeted, effective EA interventions. Preliminary findings utilizing participants from different age groups indicated that EA impacts the gut microbiota in humans. One study found that adolescents who had experienced EA, operationalized as experiencing institutional or foster care, had altered gut microbial composition compared to those who had not (Callaghan et al., 2020). Furthermore, EA was associated with gastrointestinal problems, which in turn mediated the relationship between EA and anxiety. In a sample of children, parent-child dysfunction and socioeconomic risk were associated with an altered gut microbiota, which itself

was correlated with depressive behavior (Flannery et al., 2020). Lastly, in a psychiatrically healthy sample of women, experiencing EA was associated with an altered gut microbiota, implicating EA in gut disturbances independent of psychiatric conditions (Hantsoo et al., 2019). These studies provide preliminary evidence that the gut microbiota is disrupted in humans who have experienced EA, and thus presents a potential target for a novel EA intervention.

Potential EA biomarkers in the brain include alterations in physiological processes and neural structures which are also influenced by the gut microbiota (Luczynski et al., 2016; Sudo et al., 2004) and may underlie predisposition for various psychiatric conditions when disrupted (for review, see Tyrka et al., 2013). For example, EA has been found to alter HPA axis function in humans, resulting in altered levels of the resulting hormone cortisol (e.g., Koss et al., 2016), although the effects are inconsistent (Strüber et al., 2014). Specifically, the direction of the change in basal (resting) cortisol levels associated with EA varies between studies (Strüber et al., 2014), and childhood maltreatment, a form of EA, is associated with increased HPA activity in response to a stressor in some studies (Heim et al., 2000, as cited in O'Mahony et al., 2017) but not others (Carpenter et al., 2007).

People who have experienced EA are at an increased risk for generalized anxiety disorder (GAD) and major depressive disorder (MDD); hence, investigating the link between gut dysbiosis and these disorders may provide further evidence that the pathophysiological effects of EA are due, in part, to the observed gut microbiota disturbances. Correlational studies of gut dysbiosis in humans have revealed gut microbial alterations in individuals diagnosed with MDD and GAD that implicate the gut microbiota in the pathophysiology of these disorders. For example, the microbes altered in MDD could confer a proinflammatory dysbiotic state (Jiang et al., 2015). Inflammatory biomarkers such as cytokines are elevated in those with depression

(Simon et al., 2008; Sluzewska et al., 1996), and inflammation has been implicated in the pathogenesis of the disorder (for review, see Maes et al., 2009); thus, proinflammatory gut dysbiosis is one mechanism by which the gut microbiota may contribute to the development of depression, referred to as the microbiome-inflammasome hypothesis of major depression (Inserra et al., 2018).

Both species diversity and species abundance can be disrupted in individuals with MDD. Recently, the abundances of several genera were found to correlate with MDD symptom severity (Liu et al., 2020). Meta-analytic results confirmed a pattern of microbiota alterations in individuals with MDD at several taxonomic levels (Barandouzi et al., 2020). Differences were also observed when examining microbiota of people with GAD compared to healthy controls (Jiang et al., 2018). In individuals with GAD, the abundance of several genera were negatively correlated with symptom severity and positively correlated with anxiety reduction one month after anti-anxiety drug treatment (Chen et al., 2019).

Unfortunately, because studies of the effects of EA in humans are necessarily correlational for ethical reasons, conclusions that can be drawn about causality are limited, thus necessitating the use of animal models. The maternal separation (MS) paradigm is often used as a rodent model of EA (O'Mahony et al., 2011). Typically, MS involves separating pups from their dam for three hours daily during the early neonatal period (O'Sullivan et al., 2011), beginning around postnatal day 2 (PND2) and continuing through PND12 or 14. MS produces a myriad of behavioral and physiological effects in rodents, collectively termed the "MS Phenotype" by O'Mahony et al. (2011), which includes increased anxiety-like behavior (Daniels et al., 2004; Donoso et al., 2020; Gracia-Rubio et al., 2016), despair behavior, and anhedonia (Gracia-Rubio et al., 2016). In adulthood, MS rats were found to have higher levels of

corticotropin releasing factor (CRF) mRNA, increased CRF release, and, consequently, increased corticosterone release in response to restraint stress compared to controls (Plotsky & Meaney, 1993). These findings are especially significant given that altered HPA axis activity is proposed to mediate the relationship between EA and depressive and anxiety-like behavior in animal models (Amini-Khoei et al., 2019; Marais et al., 2008).

Findings from studies utilizing MS as a model of EA demonstrate that EA can disturb the gut microbiota, contributing to the development of psychopathology. Gut microbiota alterations are observed in response to MS (Donoso et al., 2020; Moya-Pérez et al., 2017; O'Mahony et al., 2009), although the effects with regards to specific taxonomic classifications are inconsistent (Rincel & Darnaudéry, 2020). MGBA-targeted interventions, such as probiotics or prebiotics, in the MS model provide further evidence of a gut microbial component to maladaptive behavior. Probiotics are live microorganisms that, once administered, are beneficial for the host (The Food and Agriculture Association & The World Health Organization, 2001), and prebiotics are molecules used as a nutritional source by probiotic microorganisms in the gut (Ríos-Covián et al., 2016). Prebiotic and probiotic administration can mitigate anxious and depressive behaviors in animal models of EA, providing further evidence that gut microbiota alterations can induce these behaviors. For example, Desbonnet et al. (2010) found that supplementing adult MS rats with the probiotic *Bifidobacterium infantis* reduced the amount of floating time during the forced swim test (FST), a measure of depressive behavior, to levels seen in NS rats. *Lactobacillus* probiotics similarly reduced immobility time during the FST across a variety of studies utilizing various models of stress in rodents, lending further support to gut microbial involvement in depressive behavior (Mindus et al., 2021). Anxiety-like behavior in adult MS rats was attenuated by supplementation with probiotic *Bifidobacterium pseudocatenulatum* (Moya-Pérez et al.,

2017). These findings provide evidence for EA-induced gut dysbiosis, and the subsequent attenuation of the behavioral phenotype by probiotic supplementation further indicates that the psychological effects of EA may result from gut microbiota disruption.

The findings from both human and non-human animal studies of EA illustrate that gut dysbiosis is implicated in psychopathology, but understanding the mechanism by which the microbiota exerts its influence on behavior is essential to develop successful EA interventions. A major consequence of gut dysbiosis is disruption of the production of microbial metabolites such as short-chain fatty acids (SCFAs), which are themselves vital for MGBA communication (Dalile et al., 2019). While there is a paucity of studies exploring how SCFA content is altered in humans who have experienced EA, SCFA content is reduced in animal models of EA who also exhibit gut dysbiosis (Donoso et al., 2020; Qian et al., 2018). Gut-derived SCFAs, consisting predominantly of acetate, butyrate, and propionate, are produced primarily from the fermentation of dietary prebiotic fiber by the microbiota (Cummings et al., 1987). SCFAs can act locally to influence intestinal mucin production (Burger-van Paassen et al., 2009) and gut permeability (Mariadason et al., 1997), but they also impact CNS function via several pathways, including vagal, immune, endocrine, and humoral (Dalile et al., 2019).

In mice, depressive and anxiety-like behavior can be attenuated by orally administering SCFAs (van de Wouw, 2018). Prebiotic supplementation, which stimulates production of SCFAs by the gut microbiota, produced anxiolytic and antidepressant effects, which were proposed to be mediated by changes in cecal SCFA content (Burokas et al., 2017). Probiotic treatment is also accompanied by increased SCFAs measured in the cecum (Hao et al., 2019). However, the extent to which SCFA supplementation can mitigate the negative effects of gut dysbiosis in MS animals early in development is unknown.

SCFAs may mediate MGBA communication by neural or epigenetic mechanisms (for review, see Dalile et al., 2019). For instance, the mitigating effect of the SCFA butyrate on depressive behavior may be achieved via upregulation of brain-derived neurotrophic factor (BDNF) (Valvassori et al., 2014; Wei et al., 2015), which is significant given that BDNF is implicated in the pathophysiology of depression (for review, see Jin et al., 2019). SCFAs may also mediate gut-brain signaling by stimulating the vagus nerve, which has been shown to mediate communication between the gut and the brain (Bravo et al., 2011). SCFAs such as butyrate can directly activate vagal afferents (Lal et al., 2001), but they can also enhance synthesis of serotonin which can then bind to chemoreceptors on afferent terminals of the vagus nerve (Reigstad et al., 2015). Hence, the capacity of SCFAs to mediate stimulation of vagal afferents could impact depressive and anxiety-like behavior.

Supplementation with SCFAs may also indirectly affect mood by influencing gut microbial composition and gut barrier integrity. In studies of obesity, butyrate supplementation induced beneficial gut microbiota changes (Zhou et al., 2017) and attenuated differences between the gut microbiota of high-fat diet and control mice (Lu et al., 2016). If SCFAs are similarly able to remediate gut microbial perturbations in models of EA, they may in turn be effective in treating psychiatric effects of EA derived, in part, from abnormal gut-brain communication. SCFAs may also be able to heal hyperpermeable gut membranes (van de Wouw et al., 2018), which are linked to inflammation and the pathophysiology of depression (Maes et al., 2008). Supplementation with SCFAs could therefore restore healthy levels of permeability directly or indirectly by first altering the gut microbiota, which in turn influences permeability and supports gut barrier integrity (Guo et al., 2021). Such a modification in the intestinal wall could alleviate depressive symptoms. Positive correlations between gut permeability and depressive symptom

severity (albeit in a select sample), as well as associations between gut integrity and response to treatment, support this conclusion (Calarge et al., 2019; Liśkiewicz et al., 2021).

Novel MGBA-derived treatments for depression and anxiety, particularly in those who have experienced EA and are less responsive to conventional treatment, are beginning to show promise. Probiotic supplementation has been efficacious in treating symptoms of mood disorders (Liu et al., 2019; Pirbaglou et al., 2016). However, the precise mechanism of action remains unknown, and many studies do not evaluate the gut microbiota despite postulating that the observed effects following supplementation are driven by changes in the microbiota (Hooks et al., 2019). The evidence of SCFAs as mediators of gut-brain communication, as well as their diverse biochemical functions, make them an appealing target for investigation of therapeutic potential. Current literature focuses on injection of sodium butyrate, but oral supplementation of an SCFA cocktail (Smith et al., 2013) presents a non-invasive treatment option that has rarely been examined. Here, we investigate whether oral administration of an SCFA cocktail can attenuate the behavioral and microbial effects of MS. Successful attenuation of the MS phenotype by SCFA supplementation would provide a novel, MGBA-derived intervention for those who have experienced EA and are at increased risk for developing psychopathology.

Method

Animals

Twelve female and twelve male Long-Evans rats (Envigo, Inc.) were bred at Colorado College, and their offspring (n = 70 males and n = 69 females) were utilized for this study. Three male rats (two NS-NaCl and one MS-SCFA) were later excluded from the study for health concerns, their data is not reported here. Pups were cross-fostered no later than postnatal day 1 (PND1) to ensure that there were no litters that exceeded 10 pups. Rats were housed in a

humidity and temperature-controlled room on a 12-hour light/dark cycle, with lights on at 0800. Cages were lined with pine bedding, and food (Teklad Global 16% Rodent Diet; Envigo, Inc.) and water were provided *ad libitum*. Pups were weaned on PND21 and reassigned to cages with two or three same-sex rats. Animals were weighed on PND21, PND28, and one day prior to euthanasia. All procedures were performed in accordance with the Colorado College Institutional Animal Care and Use Committee-approved protocol (2017-001-LLD).

Maternal Separation

Pups in the separated group (n = 62) were subjected to three hours of maternal separation (MS animals) daily beginning on PND1 and continuing through PND14, from either 11:00-14:00 or 14:00-17:00. Control pups (n = 64) remained in their cages during this period (non-separated animals, NS). MS pups were removed from their cage and placed in individual coffee mugs filled with a layer of shavings. The mugs containing the pups were kept in a separate, brightly lit, temperature-controlled room (73 - 77 °F) with humidity ranging from 30 - 40%. At PND8 or 10, the pups were placed in a larger glass beaker or jar lined with shavings for the duration of the 3-hour period to allow the pups more space. A grid of cardboard walls was constructed between the clear vessels to prevent the pups from seeing each other. Following the 3-hour separation period, the pups were returned to their cage with the dam.

SCFA Supplementation

From weaning on PND21 and continuing throughout behavioral testing, rats were administered via their drinking water an SCFA cocktail solution (SCFA rats; Smith et al., 2013) or a sodium-matched solution (control rats). The SCFA cocktail consisted of 67.5 mM sodium acetate, 40 mM sodium butyrate, and 25.9 mM sodium propionate in water. The sodium concentration and pH of the drinking water for the control rats was identical to that of the SCFA-

supplemented rats and was prepared by combining 150mM sodium chloride (NaCl) in water.

Open Field Test

A total of 104 rats underwent the Open Field Test (OFT) for anxiety on PND28, one week after oral SCFA or sodium chloride supplementation began. The OFT is a commonly used measure of anxiety-like behavior in rodents (Seibenhenner & Wooten, 2015). This behavioral paradigm relies on the principle that rodents have evolved to have an aversion to open, brightly-lit spaces, and as such, they display a tendency to remain near the walls of the arena, a behavior known as thigmotaxis (Seibenhenner & Wooten, 2015). Thigmotaxis is used as an indicator of anxiety. In contrast, increased time spent in the center of the arena is indicative of reduced anxiety (Prut & Belzung, 2003). These behaviors are sensitive to administration of various drugs known to be anxiolytic in humans (for review, see Prut & Belzung, 2003). The open field arena was a black 122 cm x 183 cm tabletop surrounded by black foam board walls and enclosed by cloth bedsheets suspended from the ceiling. A white tape grid of 24 squares was constructed on the tabletop and a camera was mounted on the ceiling to record the animals' movements. Rats were released in the middle of the arena and left undisturbed for 20 min. The arena was wiped down with 70% isopropyl alcohol and cleared of fecal matter between trials. The video footage was scored using an automated application developed in Python for time spent in the center region of interest (TC for time center), which was defined as the inner eight squares. The 20 min test was segmented into four 5 min intervals (labeled as the within-subjects variable "bin") for analysis.

Forced Swim Test (FST)

The FST, also known as the behavioral despair test, involves placing rats in a tank of water from which they cannot escape. Typically, rats are active upon first being placed in the

water, and their time spent immobile gradually increases. This phenomenon has been anthropomorphized as behavioral despair; the rats have given up hope of escaping their situation (Castagné et al., 2011). The FST has good predictive validity for evaluating the efficacy of antidepressants (Cryan et al. 2005): 87% of antidepressants reviewed by Borsini and Meli (1988) reduced immobility time in the FST. Furthermore, antidepressants that target different neurotransmitter systems differentially affect rats' active behaviors, known as climbing and swimming, in the FST (Cryan et al., 2005).

The FST was conducted for 104 rats in a clear cylindrical container with a diameter of 30.48 cm and a height of 45.72 cm, filled with 23 - 25 ℃ water and placed in an isolated testing room with a video camera. Each rat was placed in the cylinder for one 15 min habituation period and one 5 min testing period on PNDs 47 and 48, respectively. At the end of each session, the rat was toweled off and returned to its home cage. Any fecal matter was removed from the tank between trials. At a minimum, the water was replaced between litters, and additionally on an as-needed basis when the water became cloudy. The 5-min testing period was manually coded for time spent swimming, struggling, and floating. A tag number was used to conceal the rats' identity and ensure that the raters were blind to each rats' treatment group. Each behavior was coded by a separate rater who had achieved intra-rater reliability in scoring (Cronbach's alpha > .85). All three behaviors were defined by Cryan et al. (2005). Swimming was defined as horizontal movement in the tank. Struggling, also known as climbing, was defined as frantic vertical movement that involved thrashing of the front paws and was frequently directed at the sides of the tank. Behavior in which the rat was immobile or using the minimal amount of motion required to stay afloat was defined as floating.

Sucrose Preference

The sucrose preference task to assess anhedonia was conducted for 104 rats. According to the DSM-5, experiencing anhedonia, described as a "loss of interest or pleasure in activities," and/or depressed mood, is required for MDD diagnosis (American Psychiatric Association, 2013). When mice are presented with a bottle of sucrose solution and a bottle of water, reduced consumption of the sucrose solution is used as an indicator of anhedonia (Liu et al., 2018). A 24-hour period of water deprivation on PND 50 preceded the 48-hour sucrose preference task. Rats were housed individually. Food was provided *ad libitum*. Matched bottles of water and 5% (wt/vol) sucrose solution were placed in cages for 48 hrs. and the bottle weights were recorded at 24 and 48 hrs. Bottle placement with respect to food was counterbalanced between cages and switched at the 24-hour mark to avoid effects of a side preference. Some bottles leaked or spilled, and those data were eliminated from analysis. The dependent variable "Sucrose preference total" (SPT) was calculated for 91 rats as a ratio of sucrose solution consumed to total fluid consumption over the entire 48-hour period. Two other variables, "sucrose preference 24" (SP24) and "sucrose preference 48" (SP48), were calculated for 95 rats in the same manner for the 1st 24-hour period and the 2nd 24-hour period, respectively.

Gut Microbiome Analysis

On PND21, immediately following the separation or control protocol, 8 MS rats and 8 NS rats (littermates of the animals used for behavioral testing) were euthanized using CO_2 to extract cecal samples. An additional 16 littermates were treated with the SCFA cocktail (n = 4 MS-SCFA, n = 4 NS-SCFA) or NaCl solution (n = 3 MS-NaCl, n = 5 NS-NaCl) for one week, beginning on PND21. These animals were similarly euthanized on PND 29 or 30, after undergoing the OFT on PND 28. Cecal samples were collected immediately following euthanasia and were stored at -80 ºC until analysis.

Analysis of the gut microbiome using 16S rRNA gene sequencing was conducted by Microbiome Insights (Vancouver, BC, Canada). The V4 region of the bacterial 16S rRNA gene was amplified using polymerase chain reaction (PCR). Amplicons were then sequenced using Illumina MiSeq and organized into operational taxonomic units (OTUs) based on sequence similarity ≥ 97%. Taxonomic classifications were assigned by comparing sequences to the Silva (v.138) database. Alpha diversity was estimated using the Shannon Diversity Index, a measure of the richness and evenness of OTUs. Beta diversity, which assesses similarity in microbiome composition between samples, was measured by the Bray-Curtis dissimilarity index, which,, un turn, was calculated using OTUs; OTUs with less than three counts in 10% of the samples were excluded. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the Bray-Curtis dissimilarity matrix to assess differences in the whole microbiome between groups. A Principal Coordinate Analysis (PCoA) ordination was performed to visualize between-group differences in a two-dimensional plot. Differentially abundant taxa were determined using a likelihood ratio test.

Statistical Analysis

Statistical analyses of behavioral data were conducted using SPSS version 25 (IBM, Inc). All dependent variables were analyzed using sex, supplementation, and separation as the between-subjects factors. Targeted *t*-tests were conducted following significant interactions. If the assumption of equality of variances could not be met (Levene's $p < .05$), the Greenhouse-Geisser correction model was used. All means reported are raw means. For the OFT, a mixed-model ANOVA was conducted with bin (the variable representing each of the four 5 min time segments) as the within-subjects factor. The data presented here is from a larger laboratory study involving other student researchers and a faculty advisor.

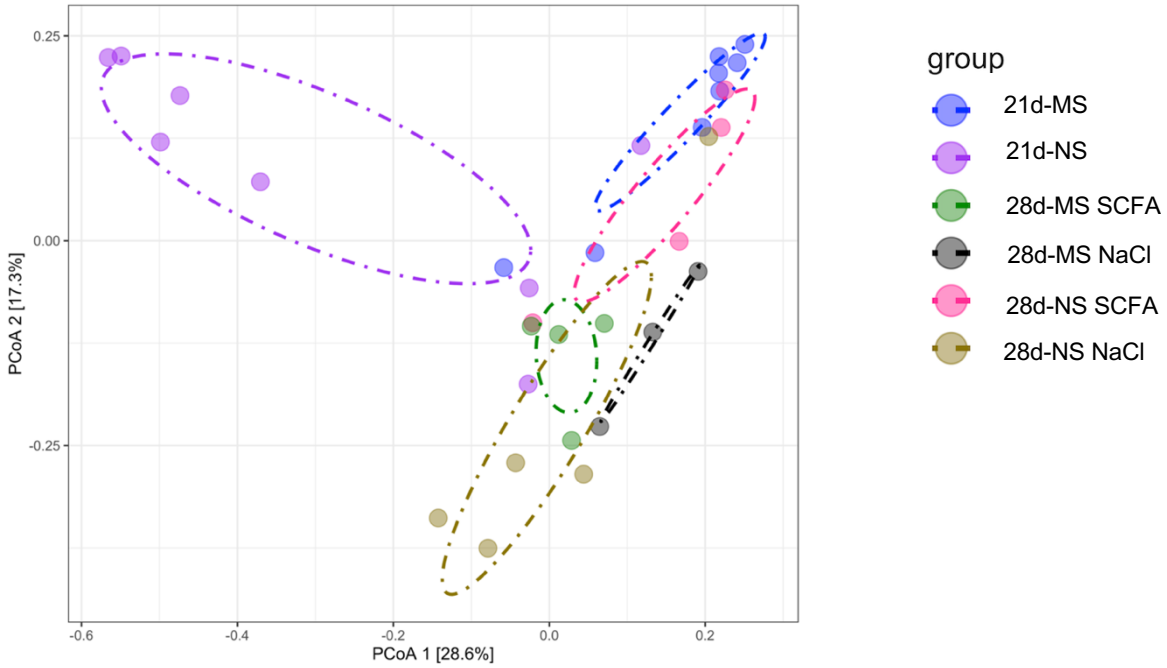
Results

Gut Microbiome

To analyze the effects of maternal separation alone on the gut microbiome, 21-day-MS and 21-day-NS rats were compared. A separate group of 28-day-MS-SCFA rats, 28-day-MS-control rats, 28-day-NS-SCFA rats, and 28-day-NS-control rats was also included to investigate whether any effects of MS on the gut microbiome could be normalized by one week of SCFA supplementation. Alpha diversity, as estimated by the Shannon Diversity Index, did not differ between groups. In contrast, analysis of beta-diversity (visualized in Figure 1) was significantly different between groups, $R^2 = .45$, $p < .001$. Post-hoc pairwise comparisons indicated that beta diversity was significantly different between all groups (adjusted $p < .05$) except for the 28-day-MS-control and 28-day-NS-SCFA rats, which were only marginally significantly different from each other, $R^2 = .34$, $p = .07$. Utilizing an alpha level of .05, 691 out of 1046 detected OTUs were found to be differentially abundant between groups.

Figure 1

Principle Coordinate Analysis Ordination of the Gut Microbiota of All Treatment Groups



Note. Ordination was performed using the Bray-Curtis dissimilarity matrix. Analysis was conducted and figure was compiled by Microbiome Insights (Vancouver, BC, Canada).

Post-hoc analysis of the 16 most abundant genera revealed significant differences in four genera between the 21-day MS and 21-day-NS rats, suggesting an effect of separation on the gut microbiome. Means and standard deviations are presented in Table 1. Compared to NS rats, MS rats had lower relative abundances of *Clostridiaceae_1_unclassified,* $t(7.011) = -2.62$, $p = .03$, *Clostridiales_unclassified,* $t(14) = -2.515$, $p = .03$), and *Alistipes*, $t(8.215) = -2.65$, $p = .03$. The relative abundance of *Bacteroides* was higher in MS rats than NS rats, $t(7.044) = 4.02$, $p = .005$.

Table 1

Relative Abundance (proportions) of 16 Most Abundant Genera in 21-day rats

Genus	NS		MS	
	M	*SD*	*M*	*SD*
Lactobacillus	.50	.15	.51	.18
Lachnospiraceae_unclassified	.09	.07	.04	.03
Bacteroidales_unclassified	.09	.06	.14	.05
Muribaculaceae_ge	.04	.04	.08	.04
Bifidobacterium	.02	.02	.01	.02
Clostridiaceae_1_unclassified	**.01**	**.01**	**.001***	**.0004**
Ruminococcaceae_unclassified	.05	.05	.02	.01
Bacteroides	.0035	.003	.08	.06
Turicibacter	.005	.01	.0002	.0001
Peptostreptococcaceae_unclassified	.004	.004	.004	.003
Clostridiales_unclassified	**.01**	**.01**	**.005***	**.004**
Bacteria_unclassified	.01	.01	.01	.003
Ruminococcaceae_UCG-014	.01	.01	.005	.01
Verrucomicrobiae_unclassified	.003	.01	.0002	.0004
Lachnospiraceae_UCG-006	.03	.05	.001	.001
Alistipes	**0.02**	**0.01**	**0.003***	**0.004**

*$p < .05$

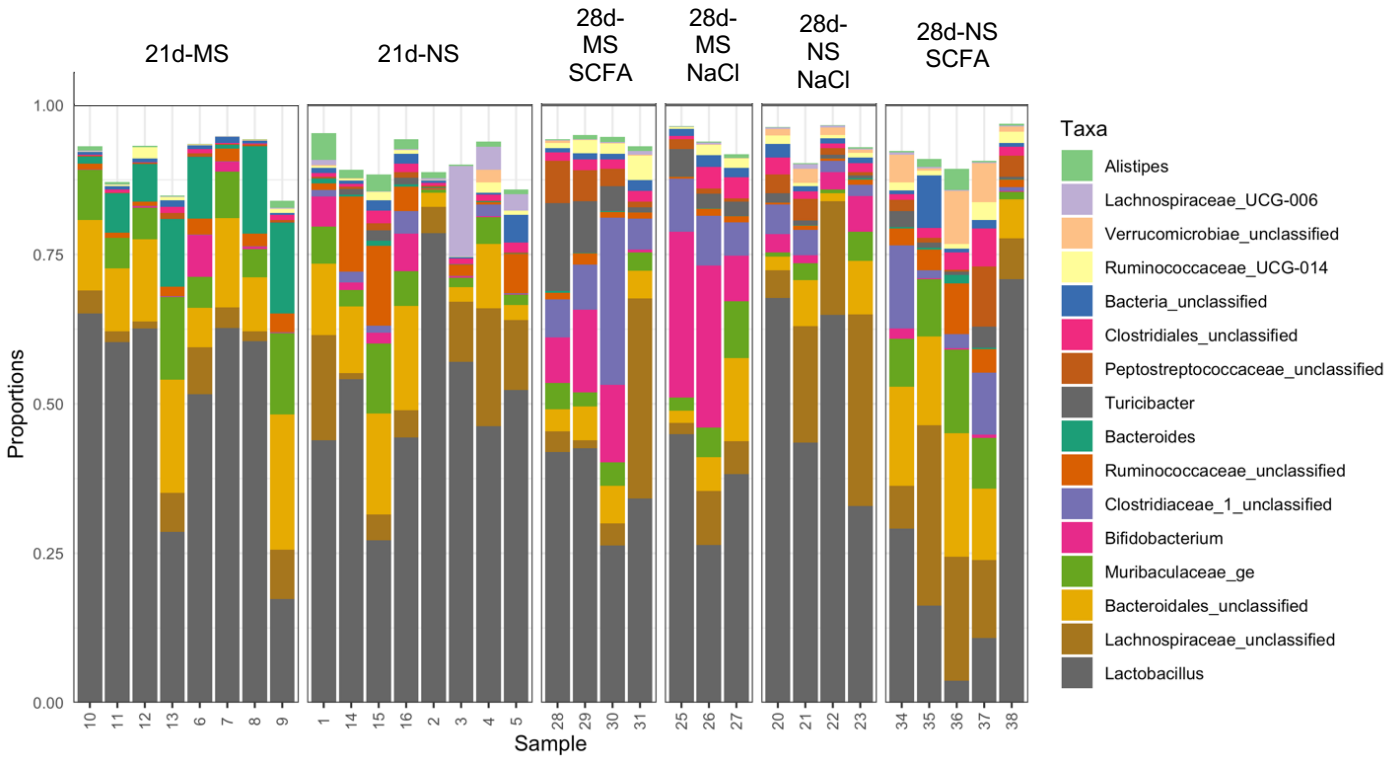
To examine the lasting effects of MS and the effect of SCFA supplementation on the microbiome, the 16 most abundant genera were examined in the older, 28-day rats using two-

way analysis of variance (ANOVA) and assessing for main effects of separation and supplementation. Means and standard deviations of the four treatment groups are presented in Table 2 and taxonomic composition at the genus level is visualized in Figure 2. Results indicated significant effects of separation on three genera, collapsed across supplementation status. *Bifidobacteria,* $F(1,12) = 19.86$, $p = .001$, $\eta^2 = .62$, and *Turicibacter,* $F(1,12) = 5.87$, $p = .03$, $\eta^2 = .33$, were increased in MS rats relative to NS rats. The relative abundance of *Verrucomicrobiae_unclassified* was lower in MS rats than it was in NS rats, $F(1,12) = 6.11$, $p = .03$, $\eta^2 = .34$. *Bifidobacteria* and *Verrucomicrobiae_unclassified* had not been differentially abundant when examined in the 21-day rats. Although the difference in relative abundance of *Turicibacter* between the 21-day-MS and 21-day-NS rats trended towards significance, the pattern was different at 28 days, with the MS rats harboring increased *Turicibacter* compared to the NS rats. These data suggest that the effect of MS on the gut microbiome is qualitatively different at 28 days than 21 days.

There was a significant main effect of supplementation for *Bacteroidales_unclassified,* $F(1,120) = 6.26$, $p = .03$, $\eta^2 = .34$, and *Muribaculaceae_ge,* $F(1,12) = 6.15$, $p = .03$, $\eta^2 = .34$. For both genera, the relative abundance was lower in SCFA animals than controls. Additionally, there was a significant interaction effect for *Bifidobacteria,* $F(1,12) = 6.65$, $p = .02$, $\eta^2 = .36$. Specifically, among non-supplemented rats, the separated animals had a higher relative abundance of *Bifidobacteria* than the non-separated animals, $t(2.01) = -3.08$, $p = .09$, but this difference was attenuated by SCFA supplementation. There was also a significant interaction for *Ruminococcaceae_unclassified,* $F(1,12) = 5.50$, $p = .04$, $\eta^2 = .31$. Post-hoc *t*-tests revealed that this interaction was driven by a higher relative abundance in the NS-control animals ($M = .04$, $SD = .03$) compared to all other groups.

Figure 2

Genus Level Taxonomic Composition of the Gut Microbiota of All Treatment Groups



Note. Unfilled portions of the bars represent lower-abundance taxa. "Sample" refers to the number assigned to each cecal sample from one rat. Analysis was conducted and figure was compiled by Microbiome Insights (Vancouver, BC, Canada).

Table 2

Relative Abundance (proportions) of 16 Most Abundant Genera in 28-day rats

Genus	NS				MS			
	NaCl		SCFA		NaCl		SCFA	
	M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
Lactobacillus	.26	.26	.52	.17	.36	.09	.36	.08
Lachnospiraceae_un.	.16	.11	.19	.11	.05	.04	.11	.15

Bacteroidales_un.[b]	**.14**	**.05**	**.05**	**.04**	**.07**	**.06**	**.05**	**.01**
Muribaculaceae_ge[b]	**.08**	**.05**	**.02**	**.02**	**.06**	**.04**	**.03**	**.01**
Bifidobacterium[a,c]	**.01**	**.01**	**.03**	**.02**	**.21**	**.11**	**.09**	**.06**
Clostridiaceae_1_un.	.06	.06	.03	.02	.08	.02	.12	.11
Ruminococcaceae_un.[c]	**.04**	**.03**	**.01**	**.003**	**.01**	**.005**	**.01**	**.004**
Bacteroides	.005	.005	.0001	.0001	.001	.0004	.001	.001
Turicibacter[a]	.02	.01	.01	.005	.03	.01	.07	.06
Peptostreptococcaceae_un.	.03	.04	.02	.01	.01	.01	.04	.03
Clostridiales_un.	.03	.02	.02	.01	.03	.02	.02	.002
Bacteria_un.	.02	.04	.01	.001	.02	.004	.01	.004
Ruminococcaceae_UCG-014	.02	.01	.01	.004	.01	.01	.02	.01
Verrucomicrobiae_un.[a]	**.04**	**.04**	**.01**	**.01**	**.0004**	**.0003**	**.002**	**.002**
Lachnospiraceae_UCG-006	.002	.001	.004	.003	.002	0.001	.002	.003
Alistipes	.01	.01	.001	.001	.003	.003	.006	.003

[a] Main effect of separation [b] Main effect of supplementation [c] Interaction effect

Sucrose Preference

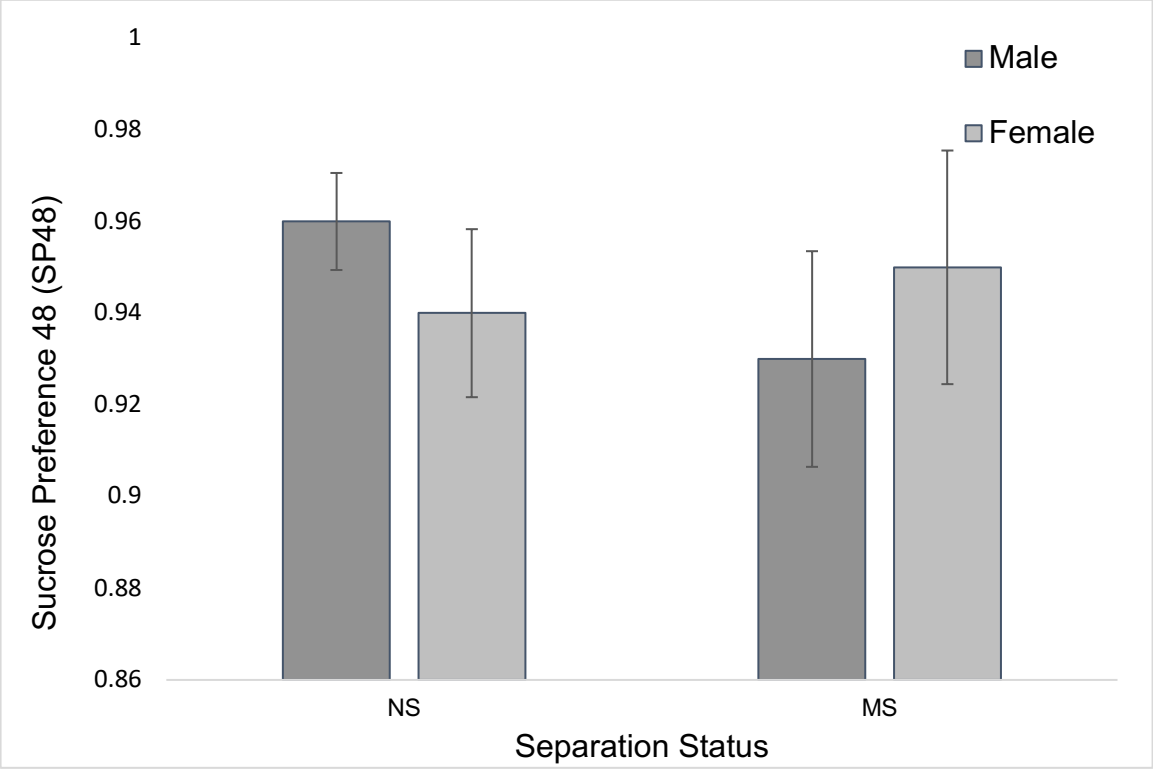
Two rats were excluded from sucrose preference analyses based on high *z*-scores for fluid consumption of 3.97 and 3.15, possibly indicating a leaky bottle that did not accurately reflect consumption. Factorial ANOVA results yielded a significant main effect of separation on total fluid consumption, $F(1,83) = 5.89$, $p = .02$, with the MS rats drinking less ($M = 102.00$ g, $SD = 23.80$) than the NS rats ($M = 116.38$ g, $SD = 27.61$). There were no significant effects of

supplementation or sex on total fluid consumption.

There were no significant effects of separation, supplementation, or sex on sucrose preference for the entire 48-hour period (SPT) or for the first 24 hours (SP24). However, there was a marginally significant sex x separation interaction for the second 24-hour period (SP48), $F(1,87) = 3.77$, $p = .06$. Further analysis of this interaction depicted in Figure 3 revealed that there was a significant effect of separation on SP48 for male rats, $t(33.018) = -2.19$, $p = .04$, but not female rats, such that MS males had a lower sucrose preference ratio ($M = .93$, $SD = .06$) than NS males ($M = .96$, $SD = .03$).

Figure 3

Differential Effect of Separation for Male and Female Rats on Sucrose Preference Ratio



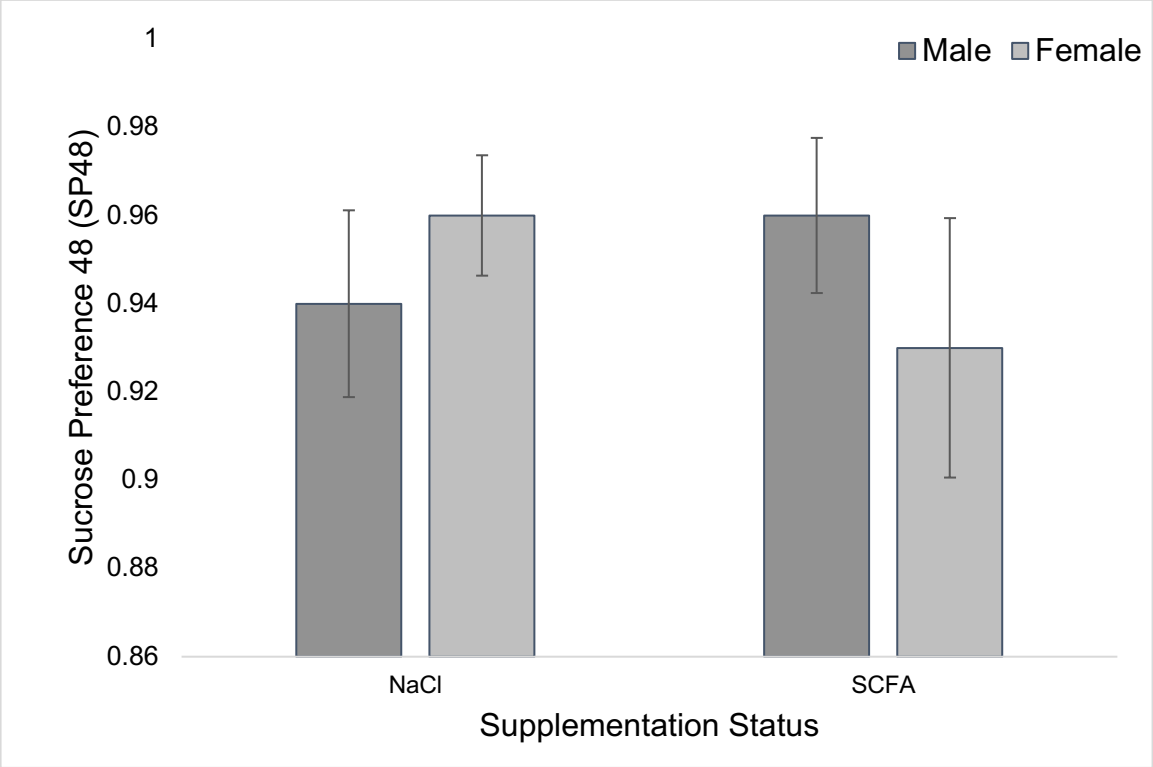
Note. Error bars represent a 95% confidence interval. Sucrose Preference 48 refers to the ratio of sucrose solution consumed to total fluid consumed during the 2nd 24-hour period.

There was also a marginally significant sex x supplementation interaction for SP48

depicted in Figure 4, $F(1,87) = 3.62$, $p = .06$. Control female rats had a marginally higher sucrose preference ratio ($M = .96$, $SD = .04$) than SCFA female rats ($M = .93$, $SD = .07$), $t(45) = -1.77$, $p = .08$. There was no effect of supplementation for male rats.

Figure 4

Differential Effect of Supplementation for Male and Female Rats on Sucrose Preference Ratio



Note. Error bars represent a 95% confidence interval. Sucrose Preference 48 refers to the ratio of sucrose solution consumed to total fluid consumed during the 2nd 24-hour period.

Open Field Test

A mixed factorial ANOVA was conducted with sex, separation, and supplementation as the between-subjects independent variables and bin as the within-subjects variable. There were rats who spent extensive time in the center region of interest (high TC scores) in Bin 2; to mitigate the influence of these extreme outliers, the data were transformed by reassigning TC scores with *z*-scores higher than 3.0 (4.36, 3.95, 3.25, and 3.14) for Bin 2 to the next highest

value (36.8 s). Results revealed a significant effect of bin on TC, $F(2.387, 233.964) = 20.64$, $p < .001$, $\eta^2 = .17$. Pairwise comparisons revealed that rats spent significantly more time in the central region of the arena in Bin 2 than in Bins 1 and 3, which, in turn, was significantly greater than TC in Bin 4. Means and standard deviations are presented in Table 3.

Table 3

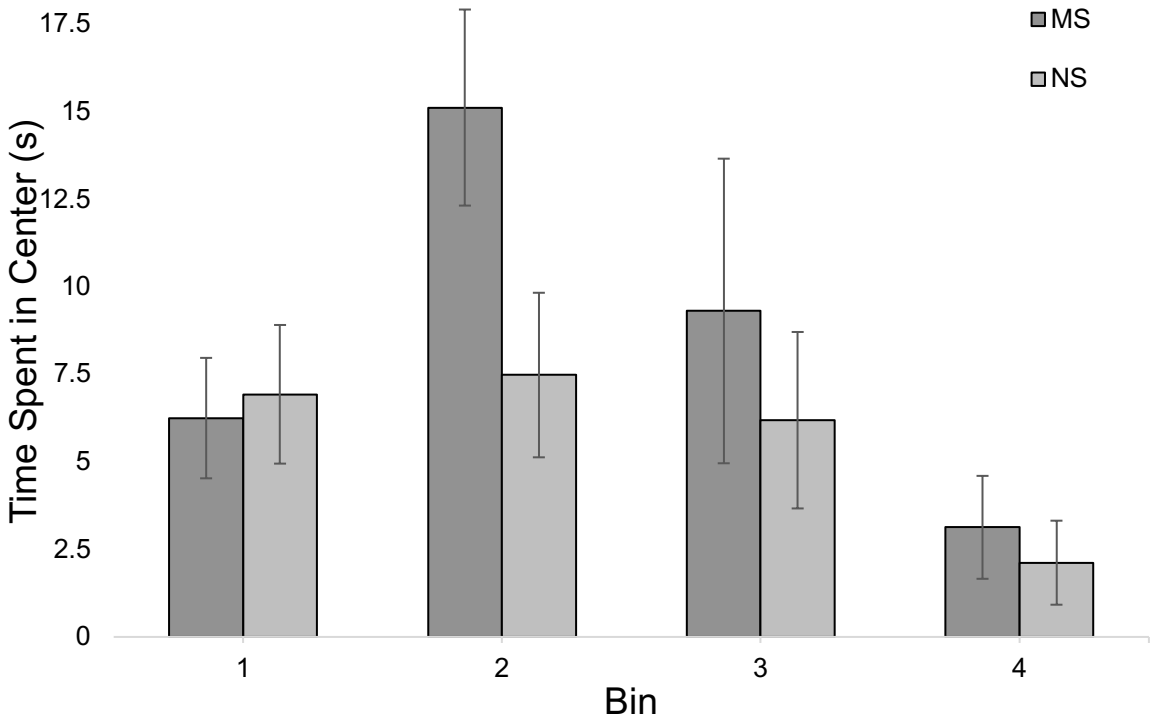
Means and Standard Deviations (in seconds) of Time Spent in the Center Region of Interest

Bin	*M*	*SD*
Bin 1	6.31	7.16
Bin 2	11.08	11.04
Bin 3	6.31	7.16
Bin 4	2.62	4.70

There was a significant main effect of separation on TC, $F(1,98) = 5.19$, $p = .03$, $\eta^2 = .05$. Contrary to the hypothesis, MS rats spent significantly more time in the center region of interest than the NS rats ($M = 8.75$ s, $SD = 6.59$ s and $M = 5.83$ s, $SD = 5.84$ s, respectively). Mixed factorial ANOVA yielded a significant bin x separation interaction, $F(2.387, 233.964) = 6.05$, $p = .001$, $\eta^2 = .06$. Further analysis revealed that the effect of separation on TC depended on bin such that MS rats spent more time in the center ($M = 15.12$ s, $SD = 10.83$ s) than NS rats ($M = 7.48$ s, $SD = 9.22$ s) in Bin 2 only, $t(114) = -4.10$, $p < .001$. The bin x separation interaction and main effect of separation are depicted in Figure 5.

Figure 5

Time Spent in the Center Region of Interest by Separation Status

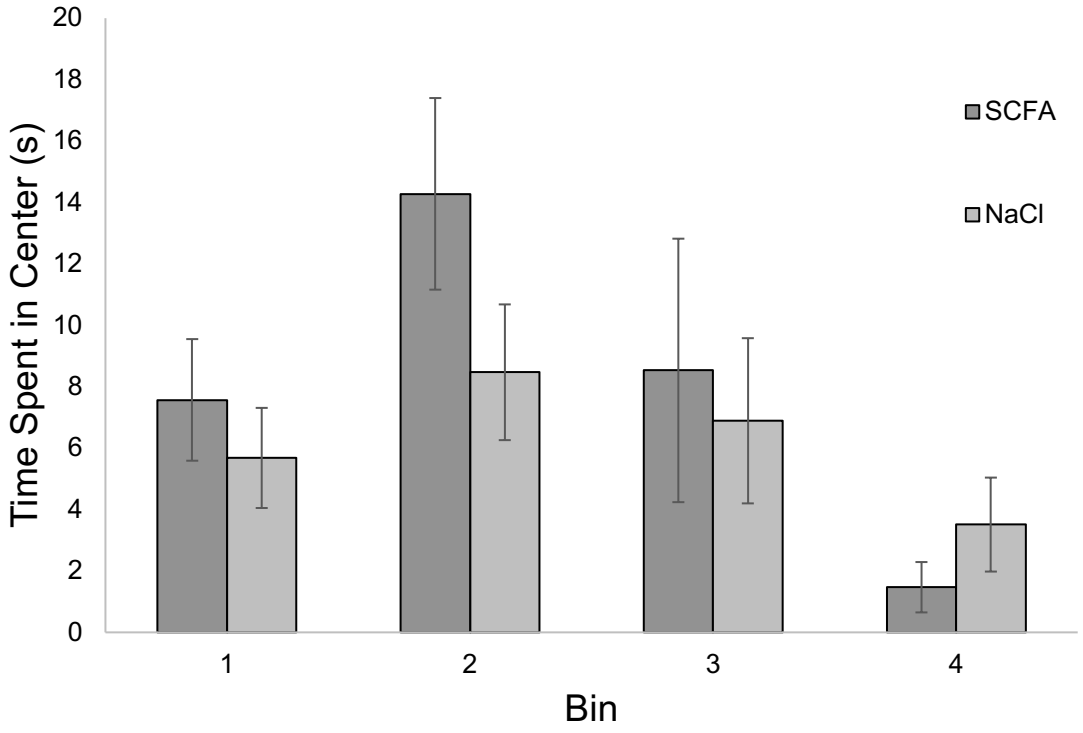


Note. Error bars represent a 95% confidence interval.

There was no main effect of supplementation, but there was a significant bin x supplementation interaction, $F(2.387, 233.964) = 3.60$, $p = .02$, $\eta^2 = .04$, that was due to differences in Bins 2 and 4, as shown in Figure 6. In Bin 2, SCFA rats spent significantly more time in the center ($M = 14.28$ s, $SD = 11.80$ s) than control rats ($M = 8.47$ s, $SD = 8.86$ s), $t(99.681) = -2.967$, $p = .004$. Conversely, in Bin 4, the SCFA rats spent less time in the center than the control rats: $M = 1.47$ s, $SD = 2.92$ s and $M = 3.51$ s, $SD = 5.99$ s, $t(87.640) = 2.23$, $p = .024$. There was no main effect of sex on TC, nor were there any other interactions.

Figure 6

Time Spent in the Center Region of Interest by Supplementation Status



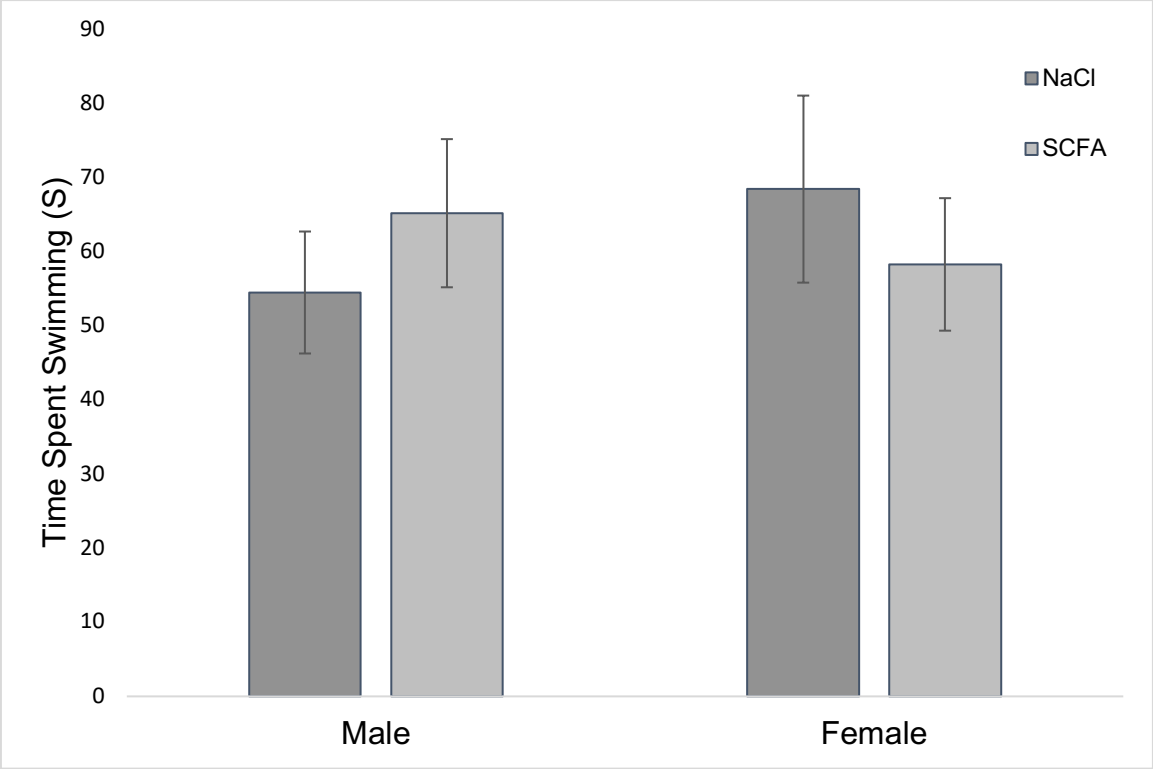
Note. Error bars represent a 95% confidence interval.

Forced Swim Test

Between-subjects factorial ANOVAs were conducted to analyze the effects of sex, separation, and supplementation on time spent swimming (SM), floating (FL) and struggling (SG) in the FST. The data for two litters was lost, so the data for 90 rats were analyzed. There were no main effects of sex, separation, or supplementation on SM. Figure 7 depicts the significant sex x supplementation interaction, $F(1, 82) = 3.97$, $p = .05$, $\eta^2 = .05$. Specifically, the effect of supplementation on SM depended on sex, such that supplementation increased SM for male rats but decreased SM for female rats. Despite a significant interaction, supplementation did not have a significant effect in males or females when analyzed separately.

Figure 7

Effect of Supplementation for Male and Female Rats on Time Spent Swimming



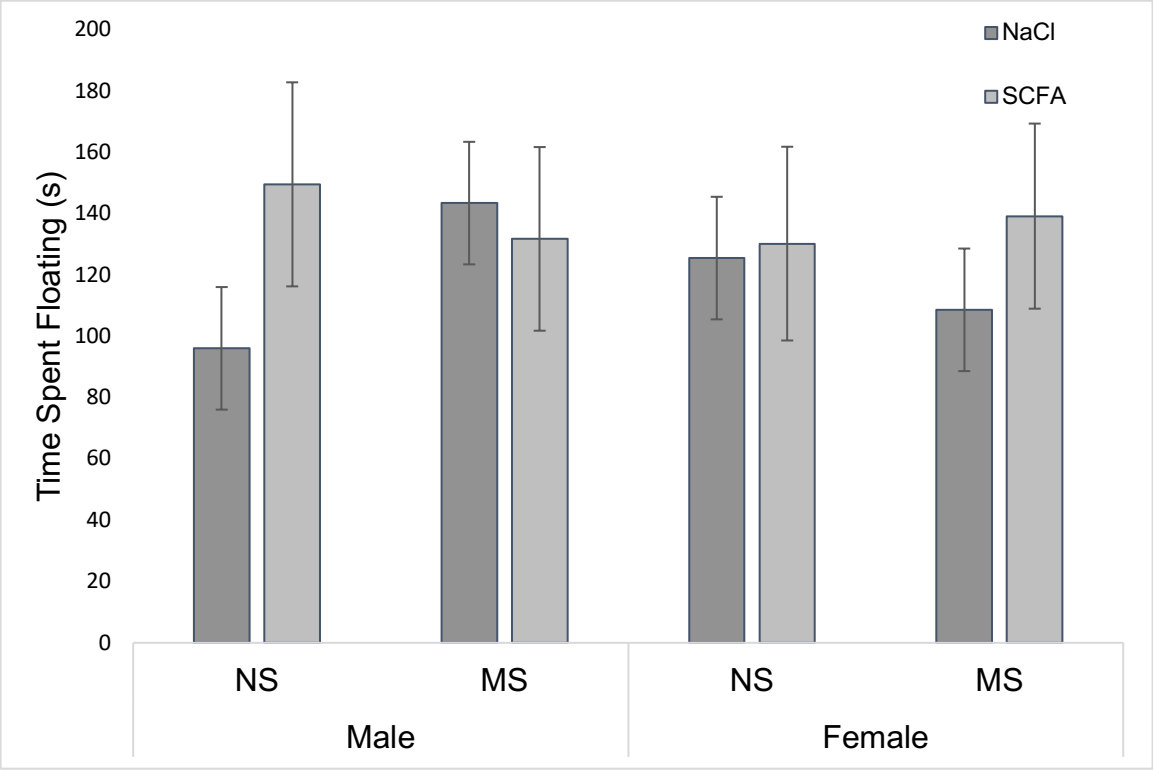
Note. Error bars represent a 95% confidence interval.

There were no main effects of sex, separation, or supplementation, or two-way interactions between any of these variables, on FL. However, there was a significant three-way interaction, $F(1, 82) = 4.82$, $p = .03$, $\eta^2 = .06$. When analyzing the effects of separation and supplementation for male rats, the NS-NaCl males floated less ($M = 96.11$ s, $SD = 49.75$ s) than the other treatment groups: they differed significantly from both the NS-SCFA and the MS-NaCl groups and were trending toward significantly different from the MS-SCFA rats. This three-way interaction is shown in Figure 8 and the means and standard deviations of the other three treatment groups are listed in Table 4. For females, the MS-SCFA rats floated more than the MS-NaCl rats, although these groups were not significantly different from each other when compared

using a *t*-test.

Figure 8

Three-Way Interaction Between Separation, Supplementation, and Sex for Time Spent Floating



Note. Error bars represent a 95% confidence interval.

Table 4

T-tests Comparing Time Spent Floating (in seconds) for NS-NaCl Male Rats to Other Males

Group	*M*	*SD*	*t*(df)	*p*
NS-SCFA	149.63*	56.35	*t*(20) = -2.36	.03
MS-NaCl	143.51*	45.49	*t*(24) = -2.52	.02
MS-SCFA	131.83	49.46	*t*(20) = -1.67	.11

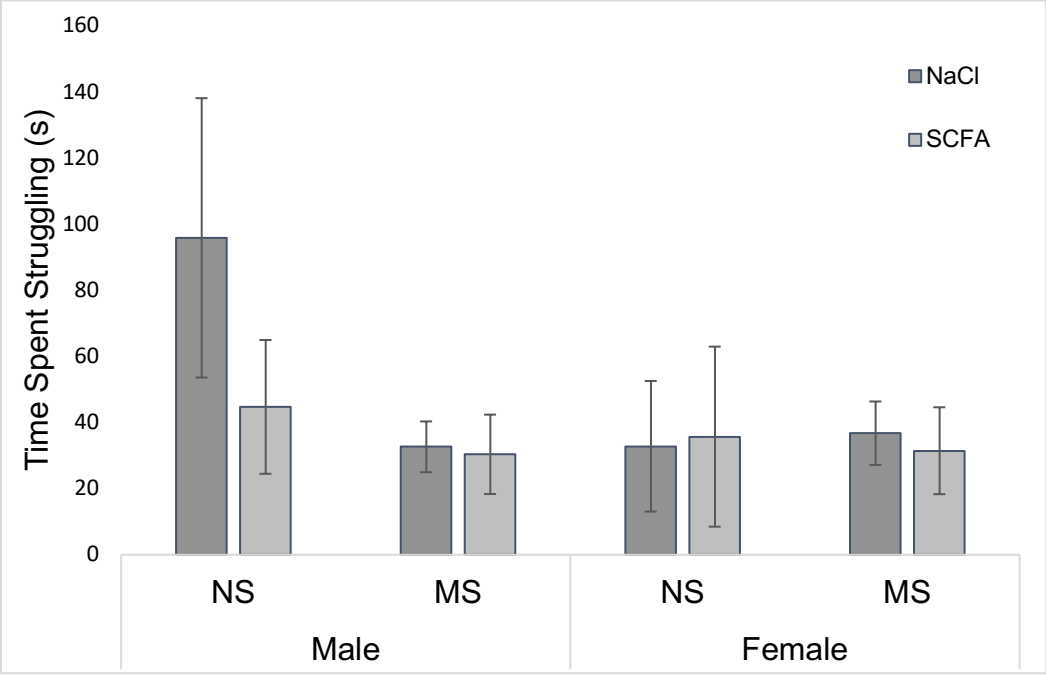
*$p < .05$

There were several significant findings for SG, which are depicted in Figure 9. There was

a significant main effect of sex on SG, $F(1, 82) = 4.75$, $p = .03$, $\eta^2 = .06$, such that SG was greater for male rats than female rats ($M = 49.40$ s, $SD = 46.70$ s and $M = 34.20$ s, $SD = 29.56$ s, respectively). There was a main effect of separation for SG, $F(182) = 6.44$, $p = .01$, $\eta^2 = .07$, such that the NS rats struggled more than the MS rats ($M = 52.17$ s, $SD = 53.19$ s and $M = 32.87$ s, $SD = 17.32$ s, respectively). Results indicated a significant sex x separation interaction, $F(1,82), = 6.34$, $p = .01$, $\eta^2 = .07$: the effect of separation on SG differed by sex such that SG was greater for NS males ($M = 70.32$ s, $SD = 60.69$ s) than MS males ($M = 31.7$ s, $SD = 17.20$ s), $t(23.86) = 2.89$, $p = .01$, whereas there was no significant difference for females. The sex x supplementation interaction neared significance, $F(1, 82) = 2.77$, $p = .10$, $\eta^2 = .03$. Supplementation decreased SG for male rats ($M = 37.56$ s, $SD = 28.45$ s and $M = 59.24$ s, $SD = 56.50$ s), $t(38.15) = 1.73$, $p = .09$, while no such effect was observed for female rats.

Figure 9

Effect of Separation and Supplementation on Time Spent Struggling for Male and Female Rats



Note. Error bars represent a 95% confidence interval.

There were three rats with high SG values resulting in z-scores greater than 3.0 (4.47, 3.52, and 3.36). All three rats were males in the same treatment group (NS-NaCl). To assess whether the previous findings persisted when mitigating the influence of these outliers, the data were transformed by reassigning the SG value for these three rats to the next highest value of 136.21 s. After transforming the data, there were still main effects of sex, $F(1,82) = 3.75$, $p = .06$, $\eta^2 = .04$, and separation, $F(1,82) = 5.55$, $p = .02$, $\eta^2 = .06$. The sex x separation interaction was also still significant, $F(1,82) = 5.44$, $p = .02$, $\eta^2 = .06$.

Discussion

The gut microbiota influence the brain through neural, endocrine, and immune mechanisms to ultimately impact a variety of behaviors, including social, anxiety-like, depressive, and mnemonic (for review, see Cryan et al., 2019). In turn, the gut microbiota is susceptible to environmental factors such as early life adversity (EA), producing a dysbiotic state that may contribute to psychopathology (Borre et al., 2014). Elucidating the relationship between the gut microbiota and psychopathology would allow for the development of novel, MGBA-derived interventions for people who have experienced EA and are at an increased risk of developing disorders such as MDD and GAD. The microbial metabolites, SCFAs, are emerging as mediators of gut-brain communication, and production of these metabolites is impacted by EA (Donoso et al., 2020; Qian et al., 2018); consequently, we investigated their potential to ameliorate behavioral deficits in a rodent model of EA.

In the current study, male and female rats underwent EA in the form of maternal separation (MS), and MS and NS rats were administered an oral SCFA cocktail or sodium-balanced control solution in the drinking water. The microbiota of all treatment groups were different from each other, indicating that both maternal separation and supplementation resulted

in significant shifts in the composition of the gut microbiota. 1046 OTUs were found to be differentially abundant between groups. This is in contrast with previous work that did not find strong microbial alterations in response to adult social stress or SCFA supplementation (van de Wouw et al., 2018). Hence, stress early in life may have a more significant impact on the gut microbiota than adult stress. When examining the effects of MS on the gut microbiota, the genera that were differentially abundant between the NS and MS animals differed depending on age group. Our finding that the microbiota alterations were qualitatively different in the 28-day rats than the 21-day rats is in agreement with previous research (Barouei et al., 2012; García-Ródenas et al., 2006; Zhou et al., 2016). Among the 21-day rats, the relative abundance of *Bacteroides* was significantly higher in the MS rats than the NS rats. Other studies have consistently found alterations in *Bacteroides* with MS*,* although the precise direction of the change differs between studies (Rincel & Darnaudéry, 2020). No clear trend emerged regarding the effect of MS on the relative abundance of SCFA-producing bacteria, as some genera that are regarded as SCFA producers were enriched in MS rats (Fernandez-Julia et al., 2021; Russell et al., 2011) while the opposite trend was observed for other SCFA-producing genera (Bultman & Jobin, 2014; Parker et al., 2020). However, classifying a genus as SCFA-producing becomes difficult when the microbial sequences are similar enough to be grouped as a genus within a certain family, but specific characteristics of that particular genus remain unexplored. Our data confirm that both MS and supplementation impact the gut microbiota, although the extent of the changes observed, as well as the nuanced role of different microbial species, prevent us from classifying the microbiota of one treatment group as “worse” than that of another.

Our hypothesis that differences in the gut microbiota caused by MS would be attenuated by SCFA supplementation was not supported. The microbiota of the MS-SCFA rats was still

significantly different from that of the NS-NaCl rats. Although supplementation did attenuate the difference in abundance of *Bifidobacteria* between the MS and the NS animals, this change resulted not from increasing the abundance of *Bifidobacteria* in the NS animals but by reducing *Bifidobacteria* abundance in the MS animals. Given the putatively beneficial role of this SCFA-producing genus (for review, see Russell et al., 2011), as well as previous findings that supplementation with various *Bifidobacteria* species attenuates behavioral deficits (Desbonnet et al., 2010), the direction of the change observed in our study suggests that SCFA supplementation could be harmful. It is interesting to note that the MS animals, with increased abundance of *Bifidobacteria,* also exhibited reduced anxiety-like behavior in the OFT.

Our study was the first, to our knowledge, to investigate the effects of SCFA supplementation on microbial changes caused by MS. Previous studies investigating other MGBA-targeted interventions such as prebiotics and probiotics have found normalization of the gut microbiota following supplementation; however, the animals in these studies were supplemented for 3 weeks or 19 days, respectively (Burokas et al., 2017; Moya-Perez et al., 2017). Therefore, it is possible that the one week of SCFA supplementation in our study was insufficient to normalize the composition of the gut microbiota of the MS rats, although the SCFAs did significantly alter the gut microbiota compared to the control treatment during this time. To address this limitation, future studies should collect cecal samples before behavioral testing (one week after supplementation initiation) and after behavioral testing as well, to verify whether a longer duration of supplementation is more effective at attenuating behavioral deficits.

We hypothesized that MS rats would exhibit increased anxiety-like, depressive, and anhedonic behavior, and that such maladaptive behavior would be promoted by gut dysbiosis. Despite the MS rats at both 21 and 28 days harboring different gut microbiota than their NS

counterparts, the results of this study largely fail to support our hypothesis, as concurrent behavioral changes were not consistently observed. Contrary to our prediction and inconsistent with previous literature (e.g., Wang et al., 2020), the MS rats exhibited reduced anxiety-like behavior in the OFT compared to the NS rats. Metanalytic results indicate anxiogenic effects of MS in the OFT, although there is considerable variation between studies that likely arises from a lack of a standardized MS protocol and small sample sizes (Wang et al., 2020). In our study, 57 NS and 49 MS rats were utilized in our OFT analysis, while Wang et al. (2020) recommend 100 rats per group to have a sufficiently powered study. Nonetheless, our unexpected results could be interpreted as increased risk-taking behavior in the MS rats (Colorado, 2006). Maladaptive risk-taking behavior is correlated with EA in human studies (Birn et al., 2017), hence the increased exploratory behavior of the MS animals could reflect this correlation. The translational validity of the MS paradigm has also been contested as EA in humans is not uniform; the assumption that one behavioral paradigm mimics the effects of a heterogenous category of stressors that may differentially impact humans is dubious (for review, see Brenhouse & Bath, 2019 and Murthy & Gould, 2018). Lastly, it is also possible that our results are confounded by the increased handling that the MS rats experienced during the MS protocol: this group might have been more accustomed to strange situations and handling, resulting in less anxiety during the OFT.

Our hypothesis that SCFA supplementation would ameliorate behavioral deficits in the MS animals was contingent upon the MS rats exhibiting increased anxiety-like behavior, but our model of EA diverged from previous literature in this manner. Nonetheless, supplementation failed to increase exploratory behavior for the NS rats, who displayed more anxiety-like behavior than the MS rats. This is inconsistent with previous findings that SCFA supplementation

increases exploration in the OFT (van de Wouw et al., 2018). Our results indicate that SCFA supplementation is inadequate to mitigate the anxiety of the more-anxious rats.

While previous literature has found that MS rats exhibit increased anhedonia as measured by the SPT (e.g., Masrour et al., 2018), our study yielded mixed results as this finding was corroborated only when analyzing the male rats in the second 24-hour period. The SPT is more sensitive to differences in anhedonia if conducted over the course of four or six days instead of two (Tordoff & Bachmanov, 2002); future research adopting this protocol might reveal more significant changes in anhedonia. It is also interesting to note that the MS males, who displayed mild anhedonia, also struggled less and floated more in the FST compared to the NS males, possibly suggesting a depressive phenotype. However, there was no effect of supplementation for the male rats in the second 24-hour period, indicating that SCFA supplementation failed to mitigate the anhedonia when it was observed.

The SPT was conducted after a variety of stress-inducing tests such as the OFT, FST, and Morris Water Maze. The order of our behavioral testing battery could have induced stress in the rats of the NS group, preventing any differences in hedonic behavior from manifesting in the SPT. Additionally, it has been hypothesized that anhedonic behavior may be "overridden" by utilizing a too-high sucrose concentration (Schalla et al., 2020); it is possible that this mitigated anhedonia that otherwise would have occurred as we utilized a 5% (wt/vol) sucrose solution in our study, while many researchers define the optimal concentration as ranging from 1% - 2% (wt/vol) (Liu et al., 2018).

Instead of predisposing the MS rats for anxiety-like behavior and anhedonia indicative of depressive-like behavior, it is possible that the MS protocol induced an optimal level of stress that confers resilience to future stressors. The relationship between adversity and resilience may

be visualized as a U-shaped curve, in which experiencing moderate levels of adversity, as opposed to no adversity or extreme adversity, results in better mental health and well-being later in life (for review, see Seery, 2011). Hence, the mild anhedonia and reduced anxiety-like behavior observed in the MS rats, as opposed to the more significant maladaptive behavior predicted, could be indicative that the MS rats were better able to cope with the stress of the behavioral testing battery because they had experienced moderate levels of adversity previously. Indeed, unlike rats that experienced unpredictable MS, rats that were subjected to MS on a predictable schedule, such as the protocol employed here, failed to exhibit increased anxiety-like and depressive behavior and were more resilient to restraint stress as adults (Shi et al., 2021). Various factors contributing to the heterogeneity of MS protocols, such as species, strain, timing, and environment, can all moderate the effects of MS and contribute to resilience in certain experimental situations (Brenhouse and Bath, 2019). By disentangling the relationship between these variables and adaptive or maladaptive outcomes, the MS paradigm can be better employed to study EA in animal models (Brenhouse and Bath, 2019).

Consistent effects of MS and supplementation were not observed for the active or passive behaviors on the FST. Our data suggests that sex influences behavior in the FST, as sex interacted with other variables for all three behaviors. Male rats struggled more than female rats in general, a finding that is consistent with previous literature (Drossopoulou et al., 2004). The most intriguing results were observed for the male rats: Contrary to our hypothesis, the NS male rats struggled more during the FST than the MS male rats. This finding is inconsistent with previous literature (e.g., Amini-Khoei et al., 2019). Supplementation produced conflicting effects for male rats by increasing time spent swimming and decreasing time spent struggling, which are both indicative of an antidepressant effect, while simultaneously increasing time spent floating,

which can be interpreted as despair behavior.

Although the FST has been effective at predicting the efficacy of antidepressants, the validity of anthropomorphizing the behaviors displayed in the FST as indicative of depression has sparked debate (Cryan et al., 2005). Specifically, it has been proposed that interpreting the motivational state of the FST subjects should be avoided altogether, and instead the observed active behaviors such as climbing and swimming can be interpreted as active coping, while floating is a passive coping strategy that can also be advantageous in some situations (Cryan et al., 2005, Thierry et al., 1984). In this view, the NS rats exhibited increased active coping as indicated by greater SG than the MS rats.

In conclusion, maternal separation did not cause consistent behavioral changes in our study, and supplementation was not effective at normalizing the maladaptive behavior when it was observed (regardless of separation status). While NS rats exhibited increased anxiety-like behavior in the OFT, only male MS rats demonstrated anhedonic behavior, and neither behavior was mitigated by SCFA supplementation. Furthermore, although the male SCFA rats did struggle less in the FST than the NaCl males, supplementation increased time spent floating for both sexes, although the effect was not significant for female rats, suggesting a negative effect of SCFAs. Our study presented limited opportunities to assess the effectiveness of SCFA treatment on stress-induced behaviors because our maternal separation paradigm failed to produce the behavioral phenotype typically observed. Hence, future studies are needed to more accurately determine whether SCFAs are helpful in mitigating adverse behaviors.

There are several limitations that should be considered when interpreting the results of our study. Our sample sizes for the microbiome analysis were very small, ranging from three to eight per cell. Such a small sample size increases the influence of within-group variability and

impedes our ability to conclusively determine effects of separation and supplementation on the gut microbiome. The inconsistent effects of MS in our study conflicts with extensive previous work and calls attention to the need to standardize the MS protocol. This can be achieved by setting a specific duration of separation (both daily and long-term), controlling for potential confounding factors such as handling, initiating the protocol on a specific postnatal day, and increasing sample size to avoid under-powered studies (Wang et al., 2020).

While the results presented here do not offer strong support for SCFAs as potential therapeutic agents to mitigate negative behavioral effects, the MGBA is still a promising avenue for future EA intervention research. Preliminary metanalytic results of controlled, clinical trials suggest anxiolytic and antidepressant effects of probiotics (Liu et al., 2019; Pirbaglou et al., 2016), which is significant given data indicating that a history of EA is associated with reduced response to both pharmacological and psychological treatment (Nanni et al., 2012). Furthermore, given the effects of MS on SCFA production (Donoso et al., 2020; Qian et al., 2018), and the potential mediating effect of SCFAs in pre and probiotic supplementation (Burokas et al., 2017; Hao et al., 2019), the efficacy of MGBA-derived interventions, specifically the role of SCFAs in this bidirectional pathway, warrants further investigation. Future research delving into the mechanisms of MGBA communication is essential for deriving new treatments that can ameliorate the enduring effects of EA.

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