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Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice

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Short title: “Prebiotics for Stress-Related Disorders”

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Keywords: prebiotics, animal behavior, anxiety, microbiota, SCFAs.
Abstract

Background: The realization that the microbiota-gut-brain axis plays a critical role in health and disease, including neuropsychiatric disorders is rapidly advancing. Nurturing a beneficial gut microbiome with prebiotics, such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) is an appealing but under-investigated microbiota manipulation. Here we tested whether chronic prebiotic treatment modifies behavior across domains relevant to anxiety, depression, cognition, stress response and social behavior.

Methods: C57/Bl6/J male mice were administered FOS, GOS, or a combination of FOS/GOS for 3 weeks prior to testing. Plasma corticosterone, microbiota composition and cecal short chain fatty acids (SCFAs) were measured. In addition FOS/GOS, or water treated mice were also exposed to chronic psychosocial stress and behavior, immune and microbiota parameters were assessed.

Results: Chronic prebiotic FOS/GOS treatment exhibited both antidepressant and anxiolytic effects. Moreover, the administration of GOS and the FOS/GOS combination reduced stress-induced corticosterone release. Prebiotics modified specific gene expression in the hippocampus and hypothalamus. Regarding SCFA concentrations, prebiotic administration increased cecal acetate and propionate and reduced iso-butyrate concentrations, changes that correlated significantly with the positive effects seen on behavior. Moreover, FOS/GOS reduced chronic stress-induced elevations in corticosterone and pro-inflammatory cytokines levels, depressive-like and anxiety-like behavior in addition to normalizing the effects of stress on the microbiota.

Conclusions: Taken together, these data strongly suggest a beneficial role of prebiotic treatment for stress-related behaviors. These findings strengthen the evidence base supporting therapeutic targeting of the gut microbiota for brain-gut axis disorders, opening new avenues in the field of nutritional neuropsychopharmacology.

Keywords: prebiotics, animal behavior, stress, anxiety, microbiota-gut-brain axis, SCFAs.
INTRODUCTION

Increasing evidence suggests that the microbiota-gut-brain axis plays a key-role in regulating brain functions, particularly emotional processing and behavior (1, 2). Indeed the microbiota plays an important role in neurodevelopment, leading to alterations in gene expression in critical brain regions, and resulting in perturbation to the programming of normal social and cognitive behaviors in mice (3-6). The gut microbiota has principally been exploited to yield positive effects on brain health via probiotics with various bifidobacteria and lactobacilli strains shown to have anxiolytic and pro-cognitive effects in both rodents (7-10) and humans (11-14). Although single or multi-strain probiotics have shown potential to modify behavior they also are limited by their ability to have relatively narrow spectrum effects on the microbiome. Moreover, given that they are live biotherapeutics, there are formulation and storage issues to consider.

An alternative but under-investigated strategy to target the microbiome is via dietary prebiotics. These are defined as selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (15). Unabsorbed/undigested carbohydrates in the small intestine, are fermented by the gut microbiota in the large bowel producing their main end products, short-chain fatty acids (SCFAs) and lactic acid (16), which may have multiple effects including the modulation of enteroendocrine serotonin secretion (17).

Fructo- (FOS) and galacto-oligosaccharides (GOS) are soluble fibers extensively used as prebiotics, traditionally associated with the stimulation of beneficial bacteria such as
bifidobacteria and lactobacilli among other gut members (18). Many beneficial effects on the gut and immune system have been associated with prebiotic use (19, 20). It has previously been shown that the prebiotic, sialyllactose, is able to diminish stress-induced alterations in colonic mucosa-associated microbiota community structure, anxiety-like behavior, and immature neuron cell numbers irrespective of immune or endocrine functionality in mice (21). Furthermore, oligosaccharides increased brain-derived neurotrophic factor (BDNF) expression and NMDA receptor signaling in rats (22). In a clinical setting, human subjects supplemented with GOS presented suppression of the neuroendocrine stress response and an increase in the processing of positive versus negative attentional vigilance, showing an early anxiolytic-like profile (23). However, the CNS effects of prebiotic administration have not been extensively explored and the links to a behavioral repertoire require extensive elaboration.

In the present study, we investigated whether administration of prebiotics, FOS, GOS or combination of both, affects behavior, specifically anxiety, depression-like, cognition and social behavior, in parallel with associated changes in discrete brain regions, gut microbiota composition and SCFAs produced, and endocrinology. Moreover, we assessed the impact of the combination prebiotic treatment on chronic psychosocial stress-induced changes in behavior, HPA axis, immune system and microbiota.
METHODS AND MATERIALS

Animals
In this study male C57BL/6J mice (n=69; Harlan, UK; 7 weeks of age on arrival) were used. (More details in Supplemental Information). All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork.

Prebiotic administration
Mice were administered the prebiotics (Healy Group Ltd., Dublin, Ireland) FOS, GOS, a combination of FOS and GOS (dissolved in drinking water for 0.3-0.4 g / per mouse / per day), or water during all of the studies. Duration of treatment was chosen based on previous studies in rodents showing behavioral and neurochemical effects following two-three weeks treatment with prebiotics (21, 22, 24, 25).

Anxiety-like behavior
Anxiety behavior was assessed using the open field, defensive marble burying and elevated plus maze and stress-induced hyperthermia as previously described (7) and detailed in Supplemental Information. The experimental design is presented in Figure 1.

Depressive-related behavior
Anhedonia was assessed using the female urine sniffing test (26) and antidepressant sensitive behaviors assessed with the tail suspension and forced swim tests as detailed previously (7, 27) (Supplemental Information).
**Social behavior**

Sociability was assessed by the three-chambered social approach task (28, 29) and resident-intruder test (30) with minor modifications (Supplemental Information).

**Cognition**

Cognitive function was assessed using the novel object recognition test (27, 31) and fear conditioning paradigm which allows differentiating between context and context/cue related behavioral responses in the same setting (9) with nociception assessed by the hot plate test to ensure specificity (Supplemental Information).

**Corticosterone, Tryptophan and Neurotransmitter levels**

Plasma Corticosterone and tryptophan levels as well as brain neurotransmitter were measured as previously described (32) and detailed in Supplemental Information.

**Social defeat/overcrowding procedure followed by social interaction test**

Chronic unpredictable social stress was carried out as described previously (26) and deficits in social interaction have been one of the most robust manifestations of chronic social defeat-induced anxiety in rodents (Supplemental Information).

**Spleen cytokine assay**

Spleens were collected immediately following sacrifice and cultured as previously described (33) (Supplemental Information).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**
Total RNA was extracted using the mirVana™ miRNA Isolation kit (Ambion/Life technologies, Paisley, UK) and DNase treated (Supplemental Information).

**DNA extraction from cecum content and amplicon sequencing**

Total DNA was extracted from the cecum contents of all the samples using the QIAamp DNA Stool Mini Kit (Qiagen, Sussex, UK) (Supplemental Information).

**Quantitative PCR Analysis for bacteria**

Absolute quantification of *Lactobacillus* spp., *Bifidobacterium* spp. and total bacteria numbers in cecum was carried out by qPCR as previously described (34) (Supplemental Information).

**SCFA concentration analysis from cecal content**

The analysis of SCFAs was carried out as previously described (35) (Supplemental Information).

**Bioinformatic and Statistical analysis**

Bioinformatics sequence analysis is outlined in Supplemental Information. Statistical analyses were conducted using SPSS software, version 22 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Bacterial compositional and non-parametric data was analyzed using the non-parametric Kruskal-Wallis and Mann-Whitney or Dunn’s tests. Changes in body weight, corticosterone and fear conditioning data were analyzed using a two-way repeated measures ANOVA. For all other data, a one-way ANOVA was conducted, followed by Fisher’s LSD post hoc test. Correlation analyses were
performed using a Pearson correlation co-efficient. Statistical significance was set at $p<0.05$. 
RESULTS

For space reasons detailed results and statistical analysis can be found in Figure Legends as detailed in Supplemental Information.

Study 1

General Effects of Prebiotic Administration

The prebiotic administration did not have any effect on body weight gain (Figure S1A, B) or on non-fasted glucose levels in plasma (Figure S2) and defecation patterns during behavioral tests (data not shown), but there were a significant effect on cecum weight that increased after 10 weeks of all prebiotic administrations (Figure S3).

16S Compositional Analysis of Cecal Microbiota

MiSeq sequencing generated a total of 6,874,289 reads; after quality control, denoising, and chimera removal, samples were rarefied to an even sampling depth of 63,000 reads. The analysis of beta-diversity showed a clear separation of the microbiota population of control mice group from that of groups fed with prebiotics (Figure 2A), suggesting that the cecal microbiota composition was altered following dietary supplementation with prebiotics. No statistical differences were shown in alpha diversity (Figure S4) (Supplemental Information).

Taxonomic shifts were also investigated and at phylum level, the murine cecal microbiota was dominated by Firmicutes and Bacteroidetes, showing slight changes among the mice groups (Figure 2B). At family level the murine cecal microbiota were dominated by Lachnospiraceae and the group S24-7_Unclassified, both of these were higher in prebiotics groups than in control group (Figure 2C).
In accordance with these results, at genus level *Lachnospiraceae* _Unclassified and S24-7_Unclassified, were the dominant microbial groups (Table S1). The significant increase in *Verrucomicrobiaceae* family was attributed to a significant increase in relative abundance of *Akkermansia* in the FOS+GOS group compared with the control group ($p<0.01$) and the other two prebiotic groups ($p<0.05$) (Figure 3A). Significantly higher proportions of the strict anaerobes *Bacteroides* and *Parabacteroides* were found in the prebiotic groups compared with the control group, with slight differences among those three groups fed with prebiotics (Figure 3C, D). In addition, prebiotic administration resulted in a significant increase in the abundance of uncultured *Oscillibacter*, being higher in the FOS group (Figure 3B). Low abundances of *Desulfovibrio*, *Ruminococcus*, *Allobaculum*, *Turicibacter*, *Lactobacillus* and *Bifidobacterium* were detected in the prebiotic-fed mice, reaching significance in some cases compared with the control group (Figure 3). The qPCR results showed that prebiotic administration produced a significant increase in total bacteria numbers (Figure S5), while no significant differences in *Lactobacillus* and *Bifidobacterium* levels were found among the four groups in the study. This suggests that decrease in the relative abundance of *Lactobacillus* and *Bifidobacterium* observed in 16S compositional analysis is likely due to an increase in the relative abundance of other genera.

**Short Chain Fatty Acids (SCFAs)**

Prebiotic administration had a significant effect on cecum SCFAs production as shown in (Figure 4 as detailed in Supplemental Information).
Anxiety-like behavior

FOS+GOS administration significantly increased time in the center of the open field test and a tendency to make more entries into the center of the open field test, but there was no effect of prebiotic administration on latency to the center zone (Figure 5A, B, C).

There was no effect of prebiotic administration on percentage time spent in open arms in elevate plus maze test (Figure 5D) but a significant effect of prebiotic administration on percentage entries into open arms in the elevated plus maze was observed (Figure 5E).

There was a tendency of prebiotic administration to reduce the number of buried marbles in defensive marble burying test (Figure 5F).

Depressive-related behaviors

FOS+GOS administration significantly decreased immobility time in the tail suspension test (Figure 6C). All prebiotic administrations significantly decreased immobility time in the forced swim test (Figure 6D). However, there was no significant effect of prebiotic administration on anhedonia in the female urine-sniffing test. ANOVA did not reveal significant differences between water sniffing time and female urine sniffing time (Figure 6A, B).

Social behavior

Prebiotic administration had no effect on interaction between mouse and object in the three-chamber test and on interaction between mouse and novel mouse (Figure 7A, B). Animals did not present aggressive behavior in resident-intruder test. However,
prebiotic administration significantly increased bouts of prosocial behavior in resident-intruder test (Figure 7C).

**Cognition**

Prebiotic administration had no effect on discrimination index for memory in novel object recognition test (Figure 7D). There was no effect of prebiotic administration on acquisition, recall and extinction in fear conditioning test (Supplement Information) (Figure S6).

**Nociception**

The pain response was not modified by prebiotics (Figure 7E) in the hot plate test.

**Locomotor Activity**

Locomotor activity measured during 10 min of habituation phase for novel object recognition test was not affected by prebiotic administration (Figure 7F).

**Endocrine response**

Repeated measures two-way ANOVA revealed that prebiotic administration significantly decreased corticosterone levels (Figure 8A). Area under the curve for corticosterone levels was reduced in prebiotic administration groups (Figure 8B). Moreover, stress-induced corticosterone levels after 45 min were also reduced in prebiotic treated groups (Figure 8C). Stress-induced hyperthermia was reduced by FOS+GOS administration (Figure 8D) and stress-induced defecation was reduced by GOS and FOS+GOS administrations (Figure 8E).
Hippocampal & hypothalamic gene expression

Prebiotic administration had a significant effect on expression of several genes in the hippocampus. FOS+GOS administration significantly increased BDNF gene expression in hippocampus (Figure 9A), GABA B1 receptor gene (Figure 9C) and GABA B2 receptor gene (Figure 9D). GOS and FOS+GOS administrations reduced mRNA levels of corticotropin releasing factor receptor 1 (CRF1R) (Figure 9B). FOS administration increased and FOS+GOS administration decreased NMDA receptor 2A subunit (Figure 9E) but no effect on 2B subunit (Figure 9F). No changes were observed on NMDA subunit 1, cannabinoid type 1, GABA Aα2, metabotropic glutamate receptor 4, glucocorticoid, and mineralocorticoid receptors mRNA levels after prebiotic administration (Supplement information) (Figure S7). FOS+GOS administration significantly reduced mRNA levels of glucocorticoid receptor in hypothalamus, but not CRF1R or mineralocorticoid receptor (Figure 10).

Tryptophan and tryptophan metabolites

GOS and FOS+GOS administration reduced L-tryptophan levels in the plasma (Table 1).

Brain monoamines

FOS and FOS+GOS administration increased serotonin levels in the prefrontal cortex. FOS+GOS administration decreased dohydroxyphenylacetic acid (DOPAC) levels in brainstem. Conversely, GOS and FOS+GOS administration increased DOPAC levels in frontal cortex. (Table 2).
SCFAs levels correlate with behavior and gene expression

The altered concentrations of SCFAs in cecum correlates with observed behaviors and gene expression data (Figure 11).

Study 2

The impact of FOS/GOS on psychosocial stress-induced changes

Behavior

Three weeks of chronic social stress significantly reduced social interaction (Figure 12B) while FOS+GOS administration preserved from this effect. Stress significantly impaired long-term memory by decreasing the DI in the novel object recognition test (Figure 12C) whereas prebiotics had a tendency prevent from this impairment. Stress also had an effect on anhedonia-like behavior where the time for sniffing female urine was reduced but an effect was attenuated in mice treded with the prebiotics (Figure 12D). The number of buried marbles was increased by stress but not in those treated with prebiotics(Figure 12E). There was a significant effect of treatment on anxiety-like behavior in the elevated plus maze test as characterized by reduced number of entries in open arms (Figure 12F) and time spent there (Figure 12G). However, following post-hoc analysis revealed that animals with prebiotics spent more time in open arm than stressed ones (Figure 12G). Number of entries to the center of open field was also reduced by stress but was not reversed by prebiotic co-treatment (Figure 12H).

Stress significantly increased immobility time in the tail suspension test where FOS+GOS administration attenuated the effects of stress (Figure 13A). Similarly, stress significantly increased immobility time in the forced swim test, but animals with FOS+GOS had an attenuated response (Figure 13B). Stress also increased defecation in the forced swim test but not in the group with prebiotics (Figure 13C).
**Acute stress & endocrine response**

Animals administrated with FOS+GOS had lower stress-induced hyperthermia than control or only stressed animals (Figure 13D). Only stressed animals significantly increased basal corticosterone levels (Figure 13E). Similarly, stress also lead to higher levels of corticosterone 45min after beginning of forced swim test, this was attenuated by prebiotic treatment had lower levels than only stressed animals (Figure 13F).

**Spleen cytokine production after stimulation with ConA and LPS**

Only stress group presented a higher concentration of Interleukin 6 (IL-6) after stimulation with Concanavalin A (ConA) and animals with prebiotics had similar levels like controls (Figure 13G). Similarly, stress induced an increased concentration in TNF-α after ConA stimulation and in animals with prebiotics this had normalized to control levels (Figure 13H). No effects on IL-1β and IL-10 (See Supplemental Information).

**16S Compositional Analysis of Cecal Microbiota**

MiSeq sequencing generated a total of 1,961,122 reads. After quality control, denoising, and chimera removal, samples were rarefied to an even sampling depth of 20,000 reads. Principal Coordinates using weighted UniFrac analysis showed slight clustering of samples related to control and stress/FOS+GOS group, separated from stress group (Figure 14A).

The different caecal microbiota composition was reflected in significant differences at multiple taxonomical levels (Figure 14 & 15 as detailed in Supplemental Information). At genus level, the most interesting result is a decrease in relative abundance of
Bifidobacterium (p<0.01) and this effect was abolished by treatment with prebiotics (p<0.001) (Figure 15A). In addition, q-PCR results corroborate the higher concentration (cfu/g of cecum) of not only Bifidobacterium but also Lactobacillus in control and prebiotic administration group than in stressed animals (Figure S10).
DISCUSSION

Prebiotics are widely used as modulators of the intestinal and immune systems, and are an important component of infant milk formulas (36). However, limited studies have focused on the effects of prebiotics on the central nervous system (22, 24, 25) and behavior (21). In this study we report that prebiotics (i.e., FOS, GOS and combination of both) were able to markedly modify behavior and brain chemistry relevant to anxiety and depression in mice. Additionally, we report that microbial community structure in mice fed the FOS, GOS and FOS+GOS were altered in a parallel manner. Changes in microbial community, coupled with increased cecal weight and total bacterial numbers led to higher levels of SCFAs in the cecum. Moreover, FOS+GOS prevented the deleterious effects on behavior, cytokine release and microbiota induced by chronic psychosocial stress.

Prebiotic administration had a marked effect on reducing stress-induced plasma corticosterone levels with the combination of FOS+GOS administration being most potent. Alterations in the hypothalamic–pituitary–adrenal (HPA) axis have been linked to the development of mood disorders and have been shown to affect the composition of the microbiota in rodents (37). Our data are in line with previous studies showing that chronic treatment with probiotics can prevent forced swim stress-induced increases in plasma corticosterone in mice (9). Similar effects were seen in humans where the salivary cortisol awakening response was significantly lower after Bimuno®- GOS intake compared with placebo (23).

Moreover, L-tryptophan levels in plasma also were reduced by prebiotic administration and the strongest effect was by FOS+GOS combination although this alteration in the
supply of tryptophan to the CNS was not manifested as reductions in serotonin concentrations. Interestingly, multiple different alternative approaches to microbiota manipulation also demonstrate an impact on tryptophan availability including germ-free animals (32) antibiotic-mediated depletion of the gut microbiota (6) as well as probiotic administration (38). It is unclear whether the current alteration in tryptophan availability reflects increased bacterial utilization of this important precursor or arises as a consequence of bacterial metabolite mediated impact on local host tryptophan metabolism into serotonin (39, 40).

In line with our biochemical evidence suggesting prebiotics have beneficial effects on stress responses, we assessed whether these changes were associated with behavioral alterations. Prebiotic administration reduced anxiety levels measured in the open field and elevated plus maze tests. Interestingly, the strongest effect was observed in animals administered the combination of FOS+GOS. In line with this evidence, another prebiotic, sialyllactose, was also able to reduce anxiety-like behavior in mice after chronic stress (21). Moreover, Bimuno®- GOS normalized anxiety after injection of lipopolysaccharide in mice (41). Taken together, these data suggest an anxiolytic-like effect of prebiotics.

Animals administered prebiotics showed reduced depressive-like behavior measured in tail suspension and forced swim tests; these tests are widely used assays of antidepressant efficacy (42). Again, the strongest effect was observed in animals administered FOS+GOS, indicating an antidepressant-like response after chronic prebiotic exposure. The modulation of the intestinal microbiota composition by prebiotic administration may be an additional way to reduce the effects of stress, as
microbiota and its specific profiles of biodiversity in the gut, significantly influence behavioral, neurochemical and immunological measures that are relevant to stress-related psychiatric disorders (43). Taking these behavioral and neuroendocrine findings together, it is intriguing that administration of the combination of FOS+GOS had a different impact on animals than each prebiotic alone, with the combination treatment group achieving overall more positive results, indicating an additive response of prebiotic administration. This could be due to the fact that giving a mixture of two different prebiotics leads to a broader range of bacterial stimulation.

We also observed novel changes in microbiota composition, especially the increase of *Akkermansia* relative abundance. Recently, *Akkermansia* spp. has received a lot of attention for its beneficial role for the host like protection from diet-induced obesity, insulin resistance, intestinal inflammation (44-46), gut barrier impairment (47) and was also found to thicken the mucin layer (48). Abundance of *Bacteroides* was also increased with all prebiotic administrations, and this was related to an increase of propionate levels. *Bacteroides* are strict anaerobes with high importance from the beginning of life (34) and some strains have been used as probiotics. Previous studies have shown that *Bacteroides fragilis* could reverse autism-like behaviors in mice (49).

No major effects were observed on cognition, pain perception and sociability with the exception of blunted aggressive behavior and more prosocial approaches. It must be taken into consideration that the animals in the study 1 were healthy adults and it will be of interest to assess the ability of these prebiotics to modify behavior across these domains in a disease model.
The changes in behavior in mice administered with prebiotics coincided with gene expression and monoamines level alterations. Mice administered with FOS+GOS combination presented high levels of BDNF expression in the hippocampus. Previously, we showed that mice consistently exhibited heightened anxiety behavior and depression-like behavior which were associated with decreased hippocampal bdnf (50). Hippocampal mRNA levels for a subunit of the GABA$_B$ receptor were also increased in animals administered with FOS+GOS combination. Interestingly, probiotic lactic acid bacteria Lactobacillus rhamnosus (JB-1) administration could also alter GABA$_A$ and GABA$_B$ receptor subunit mRNA levels in different mouse brain areas (9). Another important observation to explain behavioral improvement by prebiotic administration could be elevation of serotonin in the prefrontal cortex and a tendency of elevated levels in the frontal cortex. Pharmacological and microdialysis studies on forced swim test have already demonstrated that higher levels of serotonin are associated with a reduction in immobility and an increase in the time spent on swimming (51) indicative of antidepressant-like activity.

Interestingly, the observed behavioral, neurochemical, genetic and neuroendocrine changes after prebiotic administration could be mediated partially by SCFAs. The correlation data (Figure 11) strongly supports this idea. Indeed, recently it has been demonstrated that SCFAs are key molecules that modulate microglia maturation, morphology and function (52). In fact, stress has been linked to the development of both depression and anxiety, with a key contribution of microglia activation, as well as of recruitment of peripheral macrophages into the brain to such events (53). In humans colonic propionate production may play an important role in attenuating reward-based
eating behavior via striatal pathways, independent of changes in plasma PYY and GLP-1 (54).

Being able to modify stress-related behaviors in normal animals is of interest but for further translational value it is important to test whether interventions can reverse the effects of chronic stress. As FOS+GOS combination revealed strongest effect we also tested these prebiotics in animals subjected chronic stress. Interestingly, animals receiving FOS+GOS had reduced anhedonia, anxiety- and depressive-like behavior, comparing with stressed animals. Moreover, FOS+GOS administration attenuated acute stress-induced corticosterone levels and hyperthermia in chronically stressed animals. These results support the anxiolytic and antidepressant-like potential of these prebiotics. Chronic social stress increased pro-inflammatory response that was normalized by FOS+GOS administration. Previous study showed that a specific bacterial strain \textit{Bifidobacteria infantis} attenuated the exaggerated IL-6 response to ConA stimulation in rats after early-life stress (55).

Intriguingly, FOS+GOS administration also protected from the impact of chronic stress on the microbiota. The ratio \textit{Actinobacteria}:\textit{Proteobacteria} was decreased after stress an effect that was normalized by prebiotic treatment. Moreover, the decreased \textit{Actinobacteria}:\textit{Proteobacteria} ratio was also observed in patients with major depressive disorder (56). Similarly to our results, previous studies showed an increase in \textit{Anaerotruncus} and \textit{Peptococcus} genera after prenatal stress in rats (57). The microbiota of mice after chronic social stress was similar to observed in previous study in rats that received fecal microbiota transplantation from patients with depression (58): the relative abundances of \textit{Actinobacteria} was decreased at phylum level, \textit{Bifidobacteriaceae},
Coriobacteriaceae were decreased and Propionibacteriaceae was increased at the family level, Bifidobacterium, Allobaculum were decreased and Peptococcus were increased at genus level. In addition, FOS+GOS administration prevented the reduction of Bifidobacterium and Lactobacillus concentration caused by chronic stress. In agreement, individuals with lower Bifidobacterium and/or Lactobacillus counts are more common in patients with major depressive disorder compared to controls (59). Indeed, Bifidobacterium longum 1714 reduced stress and improved memory in healthy volunteers (14).

Although the mechanisms by which FOS and GOS support behavior are not yet fully known, it is clear that prebiotics strongly modulates the ecology of the microbiota. There is still a lot to determine the role of the microbial composition and the vast quantity, diversity and the functional capabilities of all these gut microorganisms on brain and behavior (43). This complex network of communication between the gut microbiota and the brain comprises the CNS, and both the sympathetic and parasym pathetic branches of the autonomic nervous system and the enteric nervous system in addition to the neuroendocrine and neuroimmune systems, bacterial metabolites, such as SCFAs and serotonin metabolism (1).

Taken together, these data provide further evidence for a beneficial role of prebiotics, and their effects on the microbiota-brain-gut axis in health and under stressful conditions and support the recent broadening of the definition of psychobiotic to include prebiotic-based strategy (60). Finally, this study supports the importance of new possible therapeutic targets in the field of nutritional neuropsychopharmacology.
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**Figure legends**

**Figure 1. Experimental schedule of study 1 during the 10 weeks.** Behavioral testing was conducted starting with the least to the most stressful test. Except for stress-induced hyperthermia, animals were brought to the experimental room 30 min prior testing, which occurred between 8.00 a.m. and 4.00 p.m. (8.00 a.m−12.00 p.m. for the forced swim test). Briefly, 40 adult (n=10) male mice had a battery of different behavioral tests during five weeks. Week 4: 3-CT; 3 chamber test, FUST; Female urine sniffing test, OF; Open field, NOR; Novel object recognition test. Week 5: MBT; Marble burying test; EPM; Elevated plus maze; SIH; Stress induced hyperthermia. Week 6: TST; Tail suspension test, RIT; Resident -intruder test. Week 7: FC; Fear conditioning. Week 8: HP; Hot plate, FST; Forced swim test and blood collection. Week 10: animals are culled and tissue is collected.

**Figure 2. Principal co-ordinate analysis (PCoA) (A).** PCoA based on unweighted UniFrac distances of cecum microbiota from the four mice groups of study. Mice groups colour coding: red, control group; blue, mice with FOS administration; orange, mice with GOS administration; green, mice with FOS+GOS administration. **Microbial distribution at phylum level (B).** Relative abundances of phylum level distributions of cecum microbiota. *Proteobacteria* and *Actinobacteria* were significantly decreased in the prebiotic groups compared with the control group ($p<0.5$) and FOS+GOS supplementation was associated with significantly increased *Verrumicrobia* levels compared with the other prebiotics and control groups ($p<0.05$, $p<0.01$ respectively). **Microbial distribution at family level (C).** The proportion of *Bifidobacteriaceae*, *Coriobacteriaceae*, *Clostridiaceae*, *Desulfovibrionaceae*, *Erysipelotrichaceae*, *Lactobacillaceae* and Family XIII were significantly decreased in the prebiotics groups compared with the control group. However, *Bacteriodaceae* and *Peptococcaceae* were increased significantly compared with the control group. GOS supplementation augmented *Ruminococcaceae* and FOS+GOS administration was associated with a significant increase in *Verrucomicrobiaceae*, compared with the other groups. Relative abundances of family level distributions of cecum microbiota in the four mice groups of study. All families comprising less than 1% of the total abundance were combined into the “Other” category.
Figure 3. Relative abundance of selected genera with significant differences among the four mice groups of study. Relative abundance of Akkermansia (A), Oscillibacter (B), Bacteroides (C), Parabacteroides (D), Lactobacillus (E), Bifidobacterium (F), Desulfovibrio (G), Ruminococcus (H), Allobaculum (I), and Turicibacter (J). The non-parametric Kruskal-Wallis test was used to analyze the differences among the mice groups and Mann–Whitney test was used in case of pairwise comparison. Statistical significance was accepted at $p<0.05$. Superscript symbols indicate statistically significant differences between: *, each group respect to control group; $, FOS+GOS vs GOS mice groups; #, FOS+GOS vs FOS mice groups; n=10; data represent mean ± SEM.

Figure 4. SCFAs concentrations in cecum. FOS and FOS+GOS administrations increased acetate levels in cecum (A) ($p<0.05$). All administrations increased propionate levels (B), but decreased iso-butyrate levels (C) while n-butyrate was not affected by any of the administrations (D). *$p<0.05$; **$p<0.01$; ***$p<0.001$; one-way ANOVA analysis followed by LSD post hoc test; n=8-10; data represent mean ± SEM.

Figure 5. Anxiety-like behavior. FOS+GOS administration increased time spent in the center of open field (A) and had a tendency to increase the number of entries into the center (B). The latency to enter into the center was not affected by any of the administrations (C). Percentage of time spent in the open arms was not affected by any of the administrations in elevated plus maze test (D), but increased the percentages of the entries into the open arms (E). The numbers of buried marbles in defensive marble burying test (F). *$p<0.05$; **$p<0.01$; one-way ANOVA analysis followed by LSD post hoc test (Mann–Whitney test in F); n=10; data represent mean ± SEM (median in F).

Figure 6. Depressive-like behavior. There was no any effect of prebiotic administration on anhedonia in female urine sniffing test: no effect on water sniffing time (A) or on female urine sniffing time (B). FOS+GOS administration decreased immobility time in the tail suspension test (C). All prebiotic administrations decreased immobility time in the forced swim test (D). *$p<0.05$; **$p<0.01$; one-way ANOVA analysis followed by LSD post hoc test; n=10; data represent mean ± SEM.
Figure 7. Social behavior and cognition. Prebiotic administration had no effect on interaction between mouse and object in the three-chamber test (A) and on interaction between mouse and novel mouse (B). Prebiotic administrations increased number of prosocial behavior events in resident-intruder test (C). Prebiotic administration had no effect on discrimination index for memory in novel object recognition test (D). The pain response was not modified by prebiotics in hot plate test (E) or total animal activity measured for 10 min (F). *p<0.05; one-way ANOVA analysis followed by LSD post hoc test; n=10; data represent mean ± SEM.

Figure 8. Endocrine response. Prebiotic administration decreased corticosterone levels after stressful event (forced swim test) (A). Area under the curve for corticosterone levels was reduced in prebiotic administration groups (B). Stress-induced corticosterone levels after 45 min were reduced in prebiotic treated groups (C). Stress-induced hyperthermia was reduced by FOS+GOS administration (D) and stress-induced defecation was reduced by GOS and FOS+GOS administrations (E). *p<0.05; **p<0.01; & p<0.05 comparing control to GOS and FOS+GOS groups; Repeated measures or one-way ANOVA analysis followed by LSD post hoc test; n=10; data represent mean ± SEM.

Figure 9. Hippocampal gene expression. FOS+GOS administration increased mRNA levels of BDNF not only compared with control group but also with other administrations as well (A). GOS and FOS+GOS administrations reduced mRNA of CRHR1 (B). FOS+GOS administration increased mRNA levels of GABA B1 receptor (C) and mRNA of GABA B2 receptor (D) compared with all the groups. FOS administration increased while FOS+GOS administration decreased mRNA levels of NMDA receptor 2A subunit (E) but no changes of mRNA for 2B subunits (F). *p<0.05; **p<0.01; ***p<0.001; one-way ANOVA analysis followed by LSD post hoc test; n=8-10; data represent mean ± SEM.

Figure 10. Hypothalamic gene expression. FOS+GOS administration decreased mRNA levels of glucocorticoid receptor (NR3C1) compared with control group (B). Prebiotics had no effects on mRNA levels of corticotrophin-releasing hormone receptor 1 (CRHR1) (A) or mineralocorticoid receptor (NR3C2) (C) in hypothalamus. **p<0.01;
one-way ANOVA analysis followed by LSD post hoc test; n=8-10; data represent mean ± SEM.

**Figure 11. SCFAs levels correlate with behavior and gene expression.** The color and size of the circles in the matrix code for level of correlation; red represents negative correlation and blue represents positive correlation. A correlation analysis revealed a significantly positive association of acetate concentration and sniffing time in female urine test to measure anhedonic behavior. For propionate, a negative association was revealed with immobility time in forced swim test and tail suspension test, buried marbles, rectal temperature increase in stress-induced hyperthermia, corticosterone elevation 45 min after stress or overall corticosterone response (AUC). The same effect was also revealed for mRNA levels of mineralocorticoid receptor, NMDA receptor 2A subunit, GABA receptor Aα2 subunit and a tendency on corticotropin releasing factor receptor 1 in hippocampus. A significantly positive association of propionate concentration was revealed with social behavior in resident-intruder test and sniffing time in female urine test. Reduced concentrations of iso-butyrate after prebiotic administration had significantly positive association with reduced immobility time in forced swim test, latency to enter into the center of open field test, corticosterone levels 45 min after stress and mRNA levels of mineralocorticoid receptor in the hypothalamus. In contrast, significantly negative association of iso-butyrate was revealed with sociability (preference for mouse vs object in three-chamber test), sniffing time in female urine test, percentage of entrance into open arms, number of enters into the center, time in the center in open field test and mRNA levels of NMDA receptor 2B subunit in hippocampus. n-Butyrate levels had a significantly positive association with anhedonic behavior in female sniffing urine test, corticosterone levels 90 min after stress and a negative association with the latency to enter into the center of open field test.

**Figure 12.** Experimental schedule of study 2 (A). 29 adult mice were used (n=9-10). Behavioral testing was conducted in same way as in the first study only with fewer tests. Chronic social unpredictable stress was applied during all 6 weeks and the group with prebiotics received FOS+GOS throughout the experiment. Behavioral tests were conducted during last 3 weeks of the study. Stress group showed reduced interaction ratio in social interaction test but not stress/FOS+GOS group (B). Stress and
stress/FOS+GOS groups presented lower discrimination index for memory in novel object recognition test (C), but stress/FOS+GOS groups showed a tendency to increase the DI compared with only stress group. Also stress and stress/FOS+GOS groups reduced female urine sniffing time, though group with FOS+GOS showed higher time than only stress group (D). The numbers of buried marbles in defensive marble burying test were increased only in stress group (E). Animals from stress and stress/FOS+GOS groups reduced entries to the open arms (F) and time spend there (G), however, group administered with prebiotics spend more time in open arms compared with only stress group (G). The number of entries into the center (H) was reduced in both stress groups compared with control group. *p<0.05; **p<0.01; ***p<0.001; comparing to the control group. #p<0.05; comparing to the stress group. One-way ANOVA analysis followed by LSD post hoc test; n=9-10; data represent mean ± SEM.

**Figure 13.** Stress group presented increased immobility time in the tail suspension test (A) and in the forced swim test (B), whereas stress group with prebiotics presented lower increment in immobility time compared with only stress group. Stress-induced defecation in forced swim test was increased only in the stress group (C). Stress-induced hyperthermia was reduced only in stress/FOS+GOS group (D). Chronic stress increased basal corticosterone levels (E) and corticosterone levels 45 min after stressful event (forced swim test) (F). Stress group with prebiotics presented lower increment in corticosterone levels 45 min after stressful event (F). Spleen cytokine production without stimulation (vehicle) or following stimulation with lipopolysaccharide (LPS) and concanavalin A (ConA). Stress group presented increased levels of released IL-6 (G) and tumor necrosis factor (TNFa) (H) after ConA stimulation. *p<0.05; **p<0.01; ***p<0.001; comparing to the control group. #p<0.05; ##p<0.01; comparing to the stress group. One-way ANOVA analysis followed by LSD post hoc test; n=9-10; data represent mean ± SEM.

**Figure 14.** Principal co-ordinate analysis (PCoA) (A) in study 2. PCoA based on weighted UniFrac distances of cecum microbiota from the three mice groups of the study. Mice groups colour coding: red, control group; blue, mice from stress group; yellow, stress/FOS+GOS group. Actinobacteria:Proteobacteria ratio (B). Microbial distribution at phylum level (C). Relative abundances of phylum level distributions of cecum microbiota in the three mice groups of the study.
Figure 15. Relative abundance of selected genera in study 2. At genus level, relative abundance of *Bifidobacterium* is decreased in the stressed mice and the abolition of the effect by treatment with prebiotics (p<0.001) (A). A similar opposite effects were observed in relative abundance of *Alloprevotella, Peptococcus, Anaerotruncus, Blautia* where stress increased but stress/FOS+GOS group presented similar to the control or sometimes lower relative abundance (B, C, D, F). Only stress reduced relative abundance of *Allobaculum* (p<0.01) (E). Low abundances of *Prevotella* and *Enterorhabdus* were observed in both stress groups compared with control group (G, H). On the other hand, only stress/FOS+GOS group showed a decrease in *vadinBB60* uncultured bacterium, *Defluviitaleaceae_Incertae_Sedis* and *Ruminococcaceae_Incertae_Sedis* (I, J, M) and an increase in *Parabacteroides* (p<0.01) (L). S24-7_uncultured made up 46% of relative abundance in stress/FOS+GOS group, whereas only stressed animals displayed 34%, which was significantly lower (p<0.05) (K). Similarly to the results of the study 1, FOS+GOS administration even under the stress conditions had a tendency to increase relative abundance *Akkermansia* and decrease of *Desulfovibrio* (p<0.01) (N, O). The non-parametric Kruskal-Wallis test was used to analyze the differences among the mice groups and Dunn’s test was used in case of pairwise multiple comparison. *p*<0.05; **p**<0.01; ***p**<0.001; comparing to the control group. #p<0.05; comparing to the stress group. n=8-10; data represent mean ± SEM.
Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects in Mice

Supplemental Information

Supplemental Methods and Materials

Animals
In this study, male C57BL/6J mice (n=69; Harlan, UK; 7 weeks of age on arrival) were used. One week after arrival animals were singly housed. Water and food were available ad libitum to all mice throughout the whole study. The holding room was temperature (21±1 °C) and humidity (55±10%) controlled and under a 12-h light/dark cycle (lights on 7:00 am). All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork. All efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were weighed at the start and end of experiments and once every week during the experiment.

Open field
To assess the response to a novel stressful environment and locomotor activity, mice were placed into open arena (40 × 32 × 23 cm, L × w × h) with ~60 lux lighting and allowed to explore for 10-mins. Experiments were videotaped using a ceiling camera for further parameter analysis using Ethovision software (3.1 version, Noldus, TrackSys, Nottingham, UK). The distance travelled and the latency to enter a virtual central zone (defined at 50% away from the edges) was scored.

Defensive marble burying
A higher number of marbles buried represents higher levels of anxiety. Mice were individually placed in a novel plexiglas cage (35 × 28 × 18.5 cm, L × W × H), filled up with sawdust (5-10 cm) and 20 marbles on top of it (five rows or marbles regularly spaced 2 cm away from the walls and 2 cm apart). Thirty minutes later, the number of marbles buried for more than 2/3 of their surface was counted.
**Elevated plus maze**

The set up was made of a grey plastic cross-shaped maze 1 m elevated from the floor, comprising two open (fearful) and two closed (safe) arms (50 × 5 × 15 cm walls or 1 cm no wall). Experiments occurred under red light (~5 lux). Mice were individually placed into the center of the maze facing an open arm (to avoid direct entrance into a closed one) and were allowed 5-min free exploration. Experiments were videotaped using a ceiling camera for further parameters analysis using Ethovision software (3.1 version, Noldus, TrackSys, Nottingham, UK). The percentage of time spent, distance moved and the number of entries in each arm were measured, for anxiety behavior and locomotor activity, respectively (entrance in an arm was defined as all four paws inside the arm).

**Female urine sniffing test**

On the morning of the test, vaginal smears from 20 C57BL/6J female mice were taken and analyzed for the cycle stage of the animal. Only urine from mice in estrus was collected for the test. Male mice were transferred to a quiet, dimly lit room prior to the test, and habituated to an empty cotton tip applicator inserted into their homecage. One hour later a cotton tip dipped in sterile water was presented to the animal for three minutes and sniffing time was measured. After a 45-min intertrial interval, during which mice were left undisturbed, presentation of a cotton tip infused with 60 μl of fresh urine from a female mouse in estrus was carried out for three minutes and sniffing duration was timed.

**Tail suspension test**

Mice were individually hung by the tail with adhesive tape (2 cm from tail tip) to a grid bar 30-cm elevated from the floor and the test lasted 6 min. Experiments were videotaped using a numeric tripod-fixed camera and data were further scored twice using the videos (Video Media Player software) and averaged by an experimenter blind to conditions. The time spent immobile (s) was scored; lower percentage of immobility reflecting lower depression-like behavior; immobility is defined as the absence of voluntary or escape-orientated movement.

**Forced swim test**

Mice were individually placed in a clear glass cylinder (24 × 21 cm diameter), containing 15-cm-depth water (25 ± 0.5 °C). Water was changed between each animal
tested to remove odors. The test lasted 6 min and experiments were videotaped using a numeric tripod-fixed camera; data were further scored twice using the videos (Video Media Player software) and averaged by an experimenter blind to conditions. The latency to immobility was scored. The time of immobility (s) was measured for the last 4 min of the test, with immobility being defined as a total absence of movement except slight motions to maintain the head above the water.

**Three-chambered social approach task (three-chamber test)**

The social testing apparatus was a rectangle, three-chambered box. Each chamber was 20 cm L × 40 cm W × 22 cm H. Dividing walls were made with small circular openings (5 cm in diameter) allowing access into each chamber. Two identical wire cup-like cages, with a bottom diameter of 10 cm, 13 cm in height and bars spaced 1.2 cm, allowing nose contact between the bars, but prevented fighting, were placed inside each side chamber in bilaterally symmetric positions. The test has three phases of 10 min each: 1) habitation 2) mouse *versus* object 3) novel mouse *versus* familiar mouse. Experiments were videotaped using a ceiling camera for further parameters analysis using Ethovision software (3.1 version, Noldus, TrackSys, Nottingham, UK). For the first phase the test mouse was placed into the middle chamber and allowed to explore the entire box with empty small wire cages inside for a 10-min habituation session. After the habituation period, the test mouse is removed from the testing box for a short interval while an object is placed in one side chamber and an unfamiliar conspecific male mouse (no prior contact with the test subject) in the other side chamber, both enclosed in a wire cup-like cage. During phase two, the test mouse is placed in the middle chamber and allowed to explore the entire box for 10 min. The amount of time spent exploring the object or mouse in each chamber and the number of entries into each chamber were evaluated. The location of the unfamiliar mouse in the left vs right side chamber was systematically alternated between trials. An entry was defined as all four paws in one chamber. During the third phase an object was replaced with an unfamiliar mouse serving as a novel mouse and in the other chamber the mouse used in phase two was kept the same, now serving as familiar mouse. After every trial, all chambers and cup-like wire cages were cleaned with 10% ethanol, dried and ventilated for a few minutes to prevent olfactory cue bias and to ensure proper disinfection. Lack of innate side preference was confirmed during the initial 10 min of habituation to the entire arena.
**Resident-intruder test**
Intruder animals of a similar weight were housed four per cage. Each session consisted in placing an intruder mouse into the resident's home cage for a period of 4 minutes. The latency to first approach, aggressive (attack or menace like tail-beating) interactions, as well as non-aggressive (general sniffing, anogenital sniffing, grooming and rearing) interactions of the resident mouse with its counterpart were quantified.

**Novel object recognition test**
Mice were placed in the middle of a grey plastic rectangular box (40 × 32 × 23 cm, L × W × H) under a dimly light, 60 lux at the level of the arena, for 10 min. 24 h after mice were placed in the box with the two identical objects for a total time of 10 min (acquisition phase). After a 24 h, one of the two identical objects were substituted with a novel object and mice were placed in the middle of the box at the mid-point of the wall opposite the sample objects for a total time of 10 min (retention phase). Animals were acclimatized to the testing room for 30 min prior each experiment. Box and objects were cleaned with alcohol 10% to avoid any cue smell between each trial. Experiments were videotaped using a ceiling camera for further parameter analysis. Directed contacts with the objects, include any contact with mouth, nose or paw or minimal defined distance (<2 cm), were scored using a stop watch. Any contact in which the animal is standing or leaning on the object as a way of exploring other aspects of the chamber were not interpreted as directed contact with object. Discrimination index: (novel object exploration time – familiar object exploration time) / total exploration time.

**Fear conditioning**
Training (day 1) involved 3 min of baseline recording, followed by 6 light/tone conditioned stimulus (CS) and shock [unconditioned stimulus (US)] pairings with an interval of 1 min. Pairings consisted of the cue [e.g., a combined light (260 lx) and tone exposure (70 dB)] for 20 s and an electric foot shock (0.4 mA) during the last 2 s of the cue. Mice were returned to their home cage 2 min after the last pairing. At 24 and 48 h after conditioning (days 2 and 3, respectively), the same experimental procedure was repeated in absence of shocks to test for memory and extinction of the conditioned fear response. The procedure lasted 12 min per mouse per day and was performed between 8.00 and 15.00 h in an experimental room different to the housing room.
**Stress-induced hyperthermia**

Animals were acclimatized to the testing room for 30 min prior each experiment. The body temperature was measured at T1 (T = 0) and T2 (T = 15 min). A sterile mouse thermometer was gently inserted 20 mm in the rectum of mice hung by the tail until stable thermometer measurement was reached (~15 s). Body temperature was measured to the nearest 0.1 °C; difference between T1 and T2 (ΔT) reflected the stress-induced hyperthermia.

**Plasma collection**

Mice were not restrained and the end of the tail was held with two fingers. Using a single edge razor blade a diagonal incision of 2-5 mm long was made from the end of the tail. Approximately 100 μl 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 °C temperature for 15 min. Plasma was carefully aspirated and stored at −80 °C. The same animal was sampled at all time points.

**Corticosterone assay**

Samples were analyzed in duplicate in a single assay using 20 μL plasma per sample; the threshold detection was less than 32 pg/mL; coefficient of variation limit=20%; the concentrations are expressed in ng/mL. Light absorbance was read with a multi-mode plate reader (Synergy HT, BioTek Instruments, Inc.) at 405 nm.

**Tissue collection**

Animals were sacrificed in a random fashion regarding treatment and testing condition; sampling occurred between 9.00 a.m. and 1:00 p.m. Trunk blood was collected in potassium EDTA (Ethylene Diamine Tetra Acetic Acid) tubes and spun for 15 min at 4000 g. Plasma was isolated and stored at −80 °C for further tryptophan and kynurenine analysis. The cecum was removed, weighted and stored at −80 °C for further microbiota and SCFAs analysis. The brain was quickly excised, dissected and each brain region was snap-frozen on dry ice and stored at −80 °C for further analysis.
Neurotransmitter concentrations

HPLC analysis was conducted in 3 different brain areas: brain stem, frontal cortex and prefrontal cortex. Briefly, brain tissues were sonicated in 500 μl of chilled mobile phase spiked with 4 ng/40 μl of N-Methyl 5-HT (Sigma Chemical Co., UK) as internal standard. The mobile phase contained 0.1 M citric acid, 5.6 mM octane-1-sulphonic acid (Sigma), 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork) and 9% (v/v) methanol (Alkem/Reagecon), and was adjusted to pH 2.8 using 4 N sodium hydroxide (Alkem/Reagecon). Homogenates were then centrifuged for 15 min at 22,000 × g at 4 °C and 40 μl of the supernatant injected onto the HPLC system which consisted of a SCL 10-Avp system controller, LECD 6A electrochemical detector (Shimadzu), a LC-10AS pump, a CTO-10A oven, a SIL-10A autoinjector (with sample cooler maintained at 40 C) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Kinetex 2.6 u C18 100 × 4.6 mm, Phenomenex) maintained at 30 °C was employed in the separation (Flow rate 0.9 ml/min). The glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimadzu) operated a +0.8 V and the chromatograms generated were analyzed using Class-VP 5 software (Shimadzu). The neurotransmitters were identified by their characteristic retention times as determined by standard injections, which run at regular intervals during the sample analysis. The ratios of peak heights of analyte versus internal standard were measured and compared with standard injection. Results were expressed as ng of neurotransmitter per g fresh weight of tissue.

HPLC assay for tryptophan and kynurenine

HPLC analysis involved using a system comprising a Waters 510 pump (Waters Ireland, Dublin, Ireland), 717plus cooled autosampler, a 996 PDA detector, a Hewlett Packard 1046A Fluorescent Detector (Waters Ireland, Dublin, Ireland), a waters bus SAT/IN module and a croco-cil column oven. System components were used in conjunction with Waters Empower software (Waters Ireland, Dublin, Ireland). All samples were injected onto a reversed phase Luna 3μ C18(2) 150 × 2 mm column (Phomenex, Macclesfield, UK), which was protected by Krudkatcher disposable precolumn filters and security guard cartridges (Phomenex). HPLC grade acetonitrile, acetic acid, and perchloric acid were obtained from Fisher Scientific Ireland (Dublin, Ireland). The analysis method was based on that by Herve et al. (1996). The mobile phase consisted of 50 mmol L−1 acetic acid, 100 mmol L−1 Zinc Acetate with 3% (v/v)
acetonitrile and was filtered through a 0.45 μm Millipore filter (AGB, Dublin, Ireland) and vacuum degassed prior to use. Separations were achieved by isocratic elution at 0.3 mL min⁻¹. The fluorescent detector was set to an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The PDA detector start wavelength was 210 nm and the end wavelength was 400 nm with chromatogram extraction at 330 nm. Working standard dilutions were prepared from millimolar stock solutions of each standard and stored at −80°C until required for analysis. Samples were deproteinized by the addition of 20 μL of 4 mol L⁻¹ perchloric acid to 200 μL of plasma spiked with 3-nitro-L-tyrosine as internal standard. Twenty microliters of either sample or standard was injected onto the HPLC system and chromatograms generated were processed using Waters Empower software. Analytes were identified based on their characteristic retention time and their concentrations determined using Analyte:Internal standard peak height ratios; these were measured and compared with standard injections which were run at regular intervals during the sample analysis. Results were expressed at ng analyte per mL of supernatant/plasma.

Social defeat/overcrowding procedure
Prior to the first stress day, all CD1 mice received aggression tests on three individual days. Briefly, the CD1 mouse was exposed to other CD1 mouse until the first attack. The 40 CD1 mice with the shortest attack latencies were selected for the social defeat procedure. For each social defeat session, stress mice were pseudo-randomly assigned to a different aggressor-CD1 mouse counterbalanced by diet group. All C57BL/6J mice were stressed according to a temporally unpredictable mixed schedule of social defeat and overcrowding sessions over a 6 week period. In social defeat sessions mice were exposed to the aggressive male CD1 mice and interaction was permitted until the first attack by the CD1 mouse occurred followed by a defeat posture from the stress animal. Mice were then separated by a perforated plexiglass wall that allowed visual, auditory and olfactory but not physical contact for two hours. Subsequently, the separator was removed and, after another defeat, stress mice were transferred back to their home cage. For overcrowding sessions, stress mice of one group (n=9-10) were placed into a standard holding cage for 24 or 48 h.
**Social interaction test**

In the first 2.5-min trial (‘no target’), the mouse was placed into a plastic box (39 × 31 × 24 cm) containing an empty wire mesh cage (9.5 × 7.5 × 7.0 cm) opposed to one side and allowed to explore freely. The mouse was then removed and transferred back to its home cage for 1 min. For the second 2.5-min trial (‘target’), the mouse was placed back into the box now containing within the wire mesh cage an unfamiliar aggressor CD1 mouse that had been previously been used as resident aggressor over the stress procedure with another SD/OC mouse. The test mouse could freely explore for another 2.5 min. At the end of the test, both mice were returned to their home cages and the arena and mesh cage were cleaned with 70% ethanol. All testing was carried out between 10:00 and 12:00 h under red light to reduce interference of potential anxiogenic factors with social interaction behavior. Trials were recorded by a camera mounted on the ceiling above the box and were tracked and evaluated using Ethovision 3.1 (Noldus, Wageningen, Netherlands). The time spent in the ‘interaction zone’ around the wire mesh cage during the first and the second trial was scored and the interaction ratio was calculated as the ratio of the time in the interaction zone in the target condition over the no-target condition (expressed as a percentage).

**Spleen cytokine assay**

Spleens were collected immediately following sacrifice and cultured. To culture spleen cells, first the spleens where homogenised in media [RPMI (with l-glutamine and sodium bicarbonate, R8758 Sigma) + FBS (F7524, Sigma) + Pen/Strep]. The homogenate was then filtered over a 70um strainer, centrifuged at 200 g for 5 min and resuspended in media. Cells were counted and seeded (4,000,000/mL media). After 2.5 h of adaptation, cells were stimulated with lipopolysaccharide (LPS-2 μg/ml) or concanavalin A (ConA-2.5 μg/ml) for 24 h. Following stimulation, the supernatants were harvested to assess the cytokine release using Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, Maryland, USA) for TNFα, IL-10, IL-1β and IL-6. The analyses were performed using MESO QuickPlex SQ 120, SECTOR Imager 2400, SECTOR Imager 6000, SECTOR S 600.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using the mirVana™ miRNA Isolation kit (Ambion/Llife technologies, Paisley, UK) and DNase treated (Turbo DNA-free, Ambion/life
technologies) according to the manufacturers recommendations. RNA was quantified using NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA) according to the manufacturer's instructions. RNA quality was assessed using the Agilent Bioanalyzer (Agilent, Stockport, UK) according to the manufacturer's procedure and an RNA integrity number (RIN) was calculated. RNA with RIN value >7 was used for subsequent experiments. RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Briefly, Multiscribe Reverse Transcriptase (50 U/µL) (1)(2)(1)(10) was added as part of RT master mix, incubated for 25°C for 10 min, 37°C for 2 h, 85°C for 5 min and stored at 4°C. Quantitative PCR was carried out using probes (6 carboxy fluorescein - FAM) designed by Applied Biosystems to mouse specific targeted genes, while using β-actin as an endogenous control. Amplification reactions contained 1 µl cDNA, 5 µl of the 2X PCR Master mix (Roche), 900 nM of each primer and were brought to a total of 10 µl by the addition of RNase-free water. All reactions were performed in triplicate using 96-well plates on the LightCycler®480 System. Thermal cycling conditions were as recommended by the manufacturer (Roche) for 55 cycles. To check for amplicon contamination, each run contained no template controls in triplicate for each probe used. Cycle threshold (Ct) values were recorded. Data was normalized using β-actin and transformed using the $2^{-\Delta\Delta Ct}$ method.

**DNA extraction from cecal contents and amplicon sequencing**

Total DNA was extracted from the cecal content of all samples using the QIAamp DNA Stool Mini Kit (Qiagen, Sussex, UK) according to manufacturer’s instructions, with the addition of an initial bead-beating step. Isolated DNA was frozen at -20°C until analysis. The V3-V4 hypervariable region of the 16S rRNA gene was amplified as outlined in the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina). Briefly, DNA was amplified using the primers F (5’-TCGTCGCGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3’) and R (5’-GTCTCGTGGGCTCGGAGATGTGTATAAGACAGCAG-3’) and products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). Dual indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA), according to manufacturer’s instructions and a second clean-up step using
the Agencourt AMPure XP system was performed. Library quantification, normalization, pooling and denaturation were performed as per manufacturer’s protocol (Illumina). Samples were sequenced at Clinical-Microbiomics, Denmark on the Illumina MiSeq platform using a 2 x 300 bp kit.

**Bioinformatics sequence analysis**

300 bp paired-end reads were assembled using FLASH with parameters of a minimum overlap of 20 bp and a maximum overlap of 120 bp (2). The QIIME suite of tools, v1.8.0, was used for further processing of paired-end reads, including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds (3). Denoising, chimera detection and operational taxonomic unit (OTU) grouping were performed in QIIME using USEARCH v7 (4). Taxonomic ranks were assigned by alignment of OTUs using PyNAST to the SILVA SSURef database release 111 (5, 6). Alpha and beta diversities were generated in QIIME and calculated based on unweighted UniFrac distance matrices (7). Principal coordinate analysis (PCoA) plots were visualized using EMPeror v0.9.3-dev (8).

**qRT-PCR analysis for bacteria**

Absolute quantification of *Lactobacillus* spp., *Bifidobacterium* spp. and total bacteria numbers in cecum was carried out by qPCR using the Roche LightCycler 480 platform (Roche Diagnostics, West Sussex, United Kingdom). The reactions were performed in a 10 µl volume using the KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPA Biosystems, Boston, Massachusetts, United States) and the manufacturers recommended protocol. The primers used for the quantification of *Lactobacillus* spp. were F (5’-GCAGCAGTAGGGAATCTTCCA-3’) and R (5’GCATYYCACCCTCTACATG-3’) (9); for *Bifidobacterium* spp. F (5’-CTCCTGGAAACGGGTGGT-3’) and R (5’-GCTGCCTCCCGTAGGAGT-3’) (10); and for total bacteria F (5’-ACTCCTACGGGAGGCAGCAG-3’) and R (5’-ATTACCGCGGCTGCTGG-3’) (11). Thermal cycling consisted of an initial cycle of 95 °C 5 min, followed by 40 cycles of 95°C 10s, 60°C for 20s and 72°C for 1s followed by melting curve analysis of 95°C for 5s, 65°C for 1 min and 97°C continuously and a final cooling at 40°C for 10min. Standard curves for each microbial group were established using 10^3 to 10^8 copies 16S rRNA/µl. Samples were analysed in duplicates in at least two independent PCR runs.
Short chain fatty acids concentration analysis from cecum content

Cecum content was mixed and vortex with MilliQ water and incubated at room temperature for 10 min. Supernatant were obtained by centrifugation (10000 g, 5 min, 4 °C) to pellet bacteria and other solids and filtration by 0.2µm. It was transferred to a clear GC vial and 2-Ethylbutyric acid (Sigma) was used as the internal standard. The concentration of SCFA was analyzed using a Varian 3500 GC flame-ionization system, fitted with a with a ZB-FFAP column (30 m x 0.32 mm x 0.25 mm; Phenomenex). A standard curve was built with different concentrations of a standard mix containing acetate, propionate, iso-butyrate and n-butyrate (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. All SCFA data are expressed as µmol/g.
Supplemental Results

Study 1
General Effects of Prebiotic Administration

The prebiotic administration did not have any effect on body weight gain. There was an overall effect of time within subjects (two-way ANOVA repeated measures; $F_{10,360}=148.041, p<0.001$), and an interaction effect of groups with time ($F_{30,360}=1.768, p<0.01$). However, post-hoc analysis revealed no significant differences between groups at any of the individual time points (Figure S1A). FOS and/or GOS administration did not affect increase in body weight over the 10 week period (one-way ANOVA; $F_{3,39}=1.543, p=n.s.$) (Figure S1B). After 10 weeks of prebiotic administration there was no effect on non-fasted glucose levels in plasma (one-way ANOVA $F_{3,39}=0.163, p=n.s.$) (Figure S2) and defecation patterns during behavioral tests (data not shown), but there were a significant effect on cecum weight after 10 weeks of all prebiotic administrations (one-way ANOVA; $F_{3,39}=8.814, p<0.001$) (Figure S3).

16S Compositional Analysis of Cecal Microbiota

MiSeq sequencing generated a total of 6,874,289 reads; after quality control, denoising, and chimera removal, samples were rarefied to an even sampling depth of 63,000 reads. Chao 1 and Observed Species, estimators of species richness, did not show significant differences between the four groups. This was also observed using Simpson and Shannon diversity estimators (Figure S4), suggesting that dietary supplementation with prebiotics did not influence alpha diversity of the cecal microbiota in mice. Beta-diversity was analysed using unweighted UniFrac distances and principal coordinate analysis showed a clear separation of the microbiota population of control mice group from that of groups fed with prebiotics (Figure 2A). This suggests that the cecal microbiota composition was altered following dietary supplementation with prebiotics.

Concomitant with the observations at phylum level, at family level the murine cecal microbiota were dominated by *Lachnospiraceae* and the group S24-7_Unclassifified, both of these were higher in prebiotics groups than in control group (Figure 2C). The proportion of *Bifidobacteriaceae*, *Coriobacteriaceae*, *Clostridiaceae*, 


*Desulfovibrionaceae*, *Erysipelotrichaceae*, *Lactobacillaceae* and Family XIII were significantly decreased in the prebiotics groups compared with the control group. However, *Bacteriodaceae* and *Peptococcaceae* were increased significantly compared with the control group. GOS supplementation augmented *Ruminococcaceae* and FOS+GOS administration was associated with a significant increase in *Verrucomicrobiaceae*, compared with the other groups (Figure 2C).

**Short Chain Fatty Acids (SCFAs)**

Prebiotic administration had a significant effect on cecum SCFAs production, which suggests that microbial fermentation in the cecum was enhanced by dietary supplementation with prebiotics. FOS and FOS+GOS significantly increased acetate levels in the cecum (*F*$_{3,39}$=3.007, *p*<0.05) (Figure 4A). Propionate levels were also increased by prebiotic administrations in cecum (*F*$_{3,39}$=6.001, *p*<0.01) (Figure 4B), but iso-butyrate levels were decreased by all prebiotic administrations (*F*$_{3,39}$=4.801, *p*<0.05) (Figure 4C). Prebiotics did not alter n-butyrate levels in the cecum (*F*$_{3,39}$=0.176, *p*=n.s.) (Figure 4D).

**Anxiety-like Behavior**

FOS+GOS administration significantly increased time in the center of the open field test (*F*$_{3,39}$=3.320, *p*<0.05) (Figure 5A) and a tendency to make more entries into the center of the open field test (*F*$_{3,39}$=2.702, *p*=0.060) (Figure 5B), but there was no effect of prebiotic administration on latency to the center zone (*F*$_{3,39}$=2.020, *p*=n.s.) (Figure 5C).

There was no effect of prebiotic administration on percentage time spent in open arms in elevate plus maze test (*F*$_{3,38}$=1.636, *p*=n.s.) (Figure 5D) but a significant effect of prebiotic administration on percentage entries into open arms was observed in the elevated plus maze (*F*$_{3,38}$=3.395, *p*=0.05) (Figure 5E).

Only data from Marble burying test (no other behavioral tests) failed normality testing. There was a tendency of prebiotic administration to reduce the number of buried marbles in defensive marble burying test (*χ*$_{3}^{2}$=6.951, *p*=0.073) (Figure 5F).
Depressive-related Behaviors
FOS+GOS administration significantly decreased immobility time in the tail suspension test ($F_{3,39} = 5.249, \ p < 0.01$) (Figure 6C). All prebiotic administrations significantly decreased immobility time in the forced swim test ($F_{3,39} = 5.403, \ p < 0.01$) (Figure 6D). However, there was no significant effect of prebiotic administration on anhedonia in the female urine-sniffing test. ANOVA did not reveal significant differences between water sniffing time ($F_{3,39} = 0.588, \ p = \text{n.s.}$) (Figure 6A) and female urine sniffing time ($F_{3,39} = 2.651, \ p = \text{n.s.}$) (Figure 6B).

Social Behavior
Prebiotic administration had no effect on interaction between mouse and object in the three-chamber test ($F_{3,39} = 2.421, \ p = \text{n.s.}$) (Figure 7A) and on interaction between mouse and novel mouse ($F_{3,38} = 1.565, \ p = \text{n.s.}$) (Figure 7B). Animals did not present aggressive behavior in resident-intruder test. However, prebiotic administration significantly increased bouts of prosocial behavior in resident-intruder test ($F_{3,38} = 4.123, \ p < 0.05$) (Figure 7C).

Cognition
**Novel object recognition test**
Prebiotic administration had no effect on discrimination index for memory in novel object recognition test ($F_{3,39} = 2.357, \ p = 0.088$) (Figure 7D).

**Fear conditioning**
Prebiotic administration had no effect on acquisition, recall and extinction in fear conditioning test (Figure S6). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252} = 126.877, \ p < 0.001$), but no effect of prebiotic administration ($F_{3,36} = 1.906, \ p = \text{n.s.}$), or interaction between these two factors ($F_{21,252} = 0.736, \ p = \text{n.s.}$) during acquisition depending on context (Figure S6A). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252} = 7.163, \ p < 0.001$), but no effect of prebiotic administration ($F_{3,36} = 2.787, \ p = \text{n.s.}$), or interaction between these two factors ($F_{21,252} = 0.986, \ p = \text{n.s.}$) during memory recall (day 2) depending on context (Figure S6B). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252} = 7.738, \ p < 0.001$), but no effect
of prebiotic administration \((F_{3,36}=1.358, \ p=n.s.)\), or interaction between these two factors \((F_{21,252}=1.162, \ p=n.s.)\) during extinction (day 3) depending on context (Figure S6C). During acquisition, depending on cue, repeated measures two-way ANOVA revealed that there was a significant effect of time \((F_{5,175}=69.390, \ p<0.001)\), but no effect of prebiotic administration \((F_{3,35}=0.922, \ p=n.s.)\), or interaction between these two factors \((F_{15,180}=0.573, \ p=n.s.)\) (Figure S6D). Repeated measures two-way ANOVA revealed that there was a significant effect of time \((F_{5,180}=2.294, \ p<0.05)\), but no effect of prebiotic administration \((F_{3,36}=1.490, \ p=n.s.)\), or interaction between these two factors \((F_{15,180}=0.556, \ p=n.s.)\) during memory recall (day 2) depending on cue (Figure S6E). Repeated measures two-way ANOVA revealed that there was a significant effect of time \((F_{5,180}=2.922, \ p<0.05)\), but no effect of prebiotic administration \((F_{3,36}=2.314, \ p=n.s.)\), or interaction between these two factors \((F_{15,180}=0.713, \ p=n.s.)\) during extinction (day 3) depending on cue (Figure S6F).

**Nociception**
The pain response was not modified by prebiotics \((F_{3,31}=0.870, \ p=n.s.)\) (Figure 7E) in the hot plate test.

**Locomotor Activity**
Locomotor activity measured during 10 min of habituation phase for novel object recognition test was not affected by prebiotic administration \((F_{3,39}=0.252, \ p=n.s.)\) (Figure 7F).

**Endocrine Response**
Repeated measures two-way ANOVA revealed that prebiotic administration significantly decreased corticosterone levels. There were significant effects of time \((F_{3,108}=146.535, \ p<0.001)\) and prebiotic administration \((F_{3,36}=3.906, \ p<0.05)\), but no interaction between these two factors \((F_{9,108}=0.944, \ p=n.s.)\) (Figure 8A). Area under the curve for corticosterone levels was reduced in prebiotic administration groups \((F_{3,39}=4.974, \ p<0.05)\) (Figure 8B). Moreover, stress-induced corticosterone levels after 45 min were also reduced in prebiotic treated groups \((F_{3,39}=3.148, \ p<0.05)\) (Figure 8C). Stress-induced hyperthermia was reduced by FOS+GOS administration \((F_{3,39}=4.666, \ p<0.01)\) (Figure 8D) and stress-induced defecation was reduced by GOS and FOS+GOS administrations \((F_{3,39}=3.729, \ p<0.05)\) (Figure 8E).
Hippocampal Gene Expression
Prebiotic administration had a significant effect on expression of several genes in the hippocampus. FOS+GOS administration significantly increased Bdnf gene expression in hippocampus ($F_{3,31}=4.665$, $p<0.01$) (Figure 9A), GABA B1 receptor gene (Gabbr1) ($F_{3,31}=16.887$, $p<0.001$) (Figure 9C) and GABA B2 receptor gene (Gabbr2) ($F_{3,31}=3.606$, $p<0.05$) (Figure 9D). GOS and FOS+GOS administrations reduced mRNA levels of corticotropin releasing factor receptor 1 (Ctfr1) ($F_{3,31}=6.917$, $p<0.01$) (Figure 9B). FOS administration increased and FOS+GOS administration decreased NMDA receptor 2A subunit (Grin2a) ($F_{3,31}=8.439$, $p<0.001$) (Figure 9E) but no effect on 2B subunit (Grin2b) ($F_{3,31}=1.743$, $p=n.s.$) (Figure 9F). No changes were observed on several other receptors mRNA levels after prebiotic administration: glucocorticoid receptor (Nr3c1) (one-way ANOVA $F_{3,31}=1.296$, $p=n.s.$) (Figure S7A), mineralocorticoid receptor (Nr3c2) (one-way ANOVA $F_{3,31}=2.004$, $p=n.s.$) (Figure S7B), NMDA receptor subunit 1 (Grin1) (one-way ANOVA $F_{3,31} = 1.868$, $p = n.s.$) (Figure S7C), cannabinoid receptor type 1 (Cnr1) (one-way ANOVA $F_{3,39}=0.126$, $p=n.s.$) (Figure S7D), GABA receptor Aα2 subunit (Gabra2) (one-way ANOVA $F_{3,31}=0.902$, $p=n.s.$) (Figure S7E) and metabotropic glutamate receptor 4 (mGluR4; Grm4) (one-way ANOVA $F_{3,31}=0.422$, $p=n.s.$) (Figure S7F).

Hypothalamic Gene Expression
Prebiotic administration had a significant effect on expression of several genes in the hypothalamus. FOS+GOS administration significantly reduced mRNA levels of glucocorticoid receptor (Nr3c1) in hypothalamus ($F_{3,31}=3.574$, $p<0.05$) (Figure 10B), but not corticotropin releasing factor receptor 1 (Ctfr1) ($F_{3,31}=0.493$, $p=n.s.$) (Figure 10A) or mineralocorticoid receptor (Nr3c2) ($F_{3,31}=0.825$, $p = n.s.$) (Figure 10C).

Tryptophan and Tryptophan Metabolites
GOS and FOS+GOS administration reduced L-tryptophan levels in the plasma (one-way ANOVA $F_{3,39}=5.199$, $p<0.01$) (Table 1).

Brain Monoamines
FOS and FOS+GOS administration increased serotonin levels in the prefrontal cortex ($F_{3,39}=3.013$, $p<0.05$. FOS+GOS administration decreased dihydroxyphenylacetic acid
SCFAs Levels Correlate with Behavior and Gene Expression

The altered concentrations of SCFAs in cecum correlates with observed behaviors and gene expression data. A correlation analysis revealed a significantly positive association of acetate concentration and sniffing time in female urine test to measure anhedonic behavior \((r=0.42, \ p<0.01)\) (Figure 11). For propionate, a negative association was revealed with immobility time in forced swim test \((r=-0.61, \ p<0.001)\) and tail suspension test \((r=-0.39, \ p<0.05)\), buried marbles \((r=-0.3, \ p=0.059)\), rectal temperature increase in stress-induced hyperthermia \((r=-0.34, \ p<0.05)\), corticosterone elevation 45 min after stress \((r=-0.33, \ p<0.05)\) or overall corticosterone response (AUC) \((r=-0.41, \ p<0.01)\). The same effect was also revealed for mRNA levels of mineralocorticoid receptor \((r=-0.38, \ p<0.05)\), NMDA receptor 2A subunit \((r=-0.37, \ p<0.05)\), GABA receptor Aα2 subunit \((r=-0.3, \ p<0.01)\) and a tendency on corticotropin releasing factor receptor 1 \((r=-0.32, \ p=0.076)\) in hippocampus. A significantly positive association of propionate concentration was revealed with social behavior in resident-intruder test \((r=0.33, \ p<0.05)\) and sniffing time in female urine test \((r=0.37, \ p<0.05)\) (Figure 11). Reduced concentrations of iso-butyrate after prebiotic administration had significantly positive association with reduced immobility time in forced swim test \((r=0.32, \ p<0.05)\), latency to enter into the center of open field test \((r=0.34, \ p<0.05)\), corticosterone levels 45 min after stress \((r=0.35, \ p<0.05)\) and mRNA levels of mineralocorticoid receptor in the hypothalamus \((r=0.46, \ p<0.01)\).

In contrast, significantly negative association of iso-butyrate was revealed with sociability (preference for mouse vs object in 3-Chamber test) \((r=-0.41, \ p<0.01)\), sniffing time in female urine test \((r=-0.32, \ p<0.05)\), percentage of entrance into open arms \((r=-0.4, \ p<0.01)\), number of enters into the center \((r=-0.33, \ p<0.05)\), time in the center in open field test \((r=-0.32, \ p<0.05)\) and mRNA levels of NMDA receptor 2B subunit in hippocampus \((r=-0.44, \ p<0.05)\) (Figure 11). n-Butyrate levels had a significantly positive association with anhedonic behavior in female sniffing urine test \((r=0.32, \ p<0.05)\), corticosterone levels 90 min after stress \((r=0.41, \ p<0.01)\) and a
negative association with the latency to enter into the center of open field test ($r=-0.3$, $p=0.056$) (Figure 11).

**Study 2: The Impact of FOS/GOS on Psychosocial Stress-induced Changes**

**Organs**

One-way ANOVA revealed that cecum was significantly heavier ($F_{2,26}=68.98$, $p<0.001$) (Figure S8A) and colon length was increased ($F_{2,26}=11.13$, $p<0.001$) (Figure S8B) only of animals with FOS+GOS administration.

**Behavior**

Three weeks of chronic social stress significantly reduced social interaction ($F_{2,25}=4.318$, $p<0.05$) (Figure 12B). Stress significantly impaired long-term memory by decreasing the DI in the novel object recognition test ($F_{2,22}=19.110$, $p<0.001$) (Figure 12C) whereas prebiotics had a tendency to improve it. The total object exploration time was not affected ($F_{2,22}=0.743$, $p=n.s.$) (Figure S8C). Stress also influenced anhedonia-like behavior where the time for sniffing female urine was reduced but an effect was attenuated in mice treated with the prebiotics ($F_{2,26}=17.06$, $p<0.001$) (Figure 12D). The number of buried marbles was increased by stress ($F_{2,26}=6.749$, $p<0.01$) but not in those treated with prebiotics (Figure 12E). There was a significant effect of treatment on anxiety-like behavior in the elevated plus maze test as characterized by reduced number of entries in open arms ($F_{2,26}=7.562$, $p<0.01$) (Figure 12F) and time spent there ($F_{2,26}=14.16$, $p<0.001$) (Figure 12G). However, following post-hoc analysis revealed that animals with prebiotics spent more time in open arm than stressed ones (Figure 12G). Number of entries to the center of open field was also reduced by stress but was not reversed by prebiotic co-treatment ($F_{2,26}=6.13$, $p<0.01$) (Figure 12H). No differences were observed in time spent in the center ($F_{2,25}=0.587$, $p=n.s.$) (Figure S8D) and latency to enter into the center ($F_{2,25}=1.345$, $p=n.s.$) of the open field test (Figure S8E).

Stress significantly increased immobility time in the tail suspension test ($F_{2,25}=7.244$, $p<0.01$) where FOS+GOS administration attenuated the effects of stress (Figure 13A). Similarly, stress significantly increased immobility time in the forced swim test ($F_{2,26}=21.37$, $p<0.001$), but animals with FOS+GOS had an attenuated response (Figure...
13B). Stress also increased defecation in the forced swim test ($F_{2,26}=3.444, p<0.05$) but not in the group with prebiotics (Figure 13C).

**Acute Stress & Endocrine Response**

Animals administrated with FOS+GOS had lower stress-induced hyperthermia than control or only stressed animals ($F_{2,26}=5.432, p<0.05$) (Figure 13D). One-way ANOVA revealed that only stressed animals significantly increased basal corticosterone levels ($F_{2,26}=10.39, p<0.001$) (Figure 13E). Similarly, stress also lead to higher levels of corticosterone 45min after beginning of forced swim test ($F_{2,26}=15.29, p<0.001$), this was attenuated by prebiotic treatment which had lower levels than only stressed animals (Figure 13F).

**Spleen Cytokine Production After Stimulation with ConA and LPS**

Two-way ANOVA revealed significant main effect of cytokine stimulation ($F_{2,83}=70.348, p<0.001$) but not of experimental condition ($F_{2,83}=2.409, p=n.s.$) and a significant interaction between the factors ($F_{4,83}=3.665, p<0.05$). Further, Tukey HSD post hoc showed that only stress group had a higher concentration of Interleukin 6 (IL-6) after stimulation with Concanavalin A (ConA) and animals with prebiotics had similar levels like controls (Figure 13G). Analyzing TNF-α, two-way ANOVA revealed main effect of stimulation ($F_{2,83}=105.468, p<0.001$), experimental condition ($F_{2,83}=4.032, p<0.05$) and significant interaction between the factors ($F_{4,83}=3.883, p<0.01$). Following Tukey HSD post hoc analyses revealed that stress induced an increased concentration in TNF-α after ConA stimulation and in animals with prebiotics this had normalized to control levels (Figure 13H). Analyzing IL-10, two-way ANOVA revealed main effect of stimulation ($F_{2,84}=65.821, p<0.001$) but not of experimental condition ($F_{2,84}=0.424, p=n.s.$) or an interaction between the factors ($F_{4,84}=0.206, p=n.s.$). Following Tukey HSD post hoc analyses did not revealed differences among groups on concentration of IL-10 (Figure S8F). Two-way ANOVA revealed significant main effect of cytokine stimulation ($F_{2,83}=29.214, p<0.001$) but not of experimental condition ($F_{2,83}=1.42, p=n.s.$) or an interaction between the factors ($F_{4,83}=1.039, p=n.s.$) where further Tukey HSD post hoc also did not show differences among groups on concentration of Interleukin 1β (IL-1β). However, there was a tendency to increase IL-1β levels only in a stress group after ConA stimulation and animals with prebiotics had similar levels like controls (Figure S8G).
16S Compositional Analysis of Cecal Microbiota (study 2)

Alpha diversity of the cecal microbiota in mice was not affected by stress condition and prebiotic treatment, no differences were observed in richness and diversity estimators (data not shown).

At family level, group S24-7 and Lachnospiraceae were dominant, followed by Ruminococcaceae (Figure S9). Stressed animals had lower proportions of S24-7, Coriobacteriaceae, Erysipelotrichaceae and Bifidobacteriaceae than control group; however, the stress/FOS+GOS group showed a recovery of those families proportions being higher than stress mice. Conversely, stress group of mice exhibited higher abundance of Lachnospiraceae, Prevotellaceae and Family XII than control group, being those microbial families decreased in stressed animals with prebiotic administration. This suggests that prebiotic administration counteracts the effects of stress on the gut microbiota. Desulfovibrionaceae abundance were significantly (p<0.01) lower in both stressed groups compared to control group.

At genus level, the most interesting result is a decrease in relative abundance of Bifidobacterium (p<0.01) and this effect was abolished by treatment with prebiotics (p<0.001) (Figure 15A). Bifidobacteria and Allobaculum were only detected in 2 and 4 stressed mice respectively, in very low proportions; in contrast, both microbial groups were detected in higher proportions in all mice in the control group and mice with prebiotic administration. This fact suggests that prebiotics administered to stressed animals prevents the detrimental effects of stress in those microbial genera.

A similar opposite effects were observed in relative abundance of Alloprevotella, Peptococcus, Anaerotruncus, Blautia where stress increased but stress/FOS+GOS group were similar to control, or sometimes lower (Figure 15B, C, D, F). Moreover, only stress reduced relative abundance of Allobaculum (p<0.01) (Figure 15E). Low abundances of Prevotella and Enterorhabdus were observed in both stress groups compared with control group (Figure 15G, H). On the other hand, only stress/FOS+GOS group showed a decrease in vadinBB60 uncultured bacterium, Defluviitaleaceae_Incertae_Sedis and Ruminococcaceae_Incertae_Sedis (Figure 15I, J,
M) and an increase in Parabacteroides (p<0.01) (Figure 15L). S24-7_uncultured made up 46% of relative abundance in stress/FOS+GOS group, whereas only stressed animals displayed 34%, which was significantly lower (p<0.05) (Figure 15K). Similar to the results of the study 1, FOS+GOS administration even under the stress conditions had a tendency to increase relative abundance Akkermansia and decrease of Desulfovibrio (p<0.01) (Figure 15N, O).

Bacterial Concentration by q-PCR in Cecum
One-way ANOVA revealed that stress significantly reduced Bifidobacterium concentration in cecum ($F_{2,26}$=32.01, $p<0.001$) but not in stress/FOS+GOS group (Figure S10A). The same effect of stress was seen on concentration of Lactobacillus ($F_{2,26}$=10.88, $p<0.001$) where stress/FOS+GOS presented higher concentration than stress group (Figure S10B). On the other hand, both stress and stress/FOS+GOS groups presented lower concentration of total bacterium ($F_{2,26}$=11.58, $p<0.001$) (Figure S10C).
Supplemental Figures

Figure S1. Animal weight during 10 weeks of the experiment. Prebiotics had no effect on animal weight (A) or their weight gain after 10 weeks (B). n = 10; data represent mean ± SEM.

Figure S2. Glucose levels in the blood. Prebiotics had no effect on non-fasted glucose levels in the blood at the end of the experiment. n = 10; data represent mean ± SEM.
Figure S3. Cecum weights. All prebiotics increased cecum. ***p < 0.001; one-way ANOVA analysis followed by LSD post hoc test; n = 10; data represent mean ± SEM.
Figure S4. Alfa-diversity metrics of cecal microbiota. Prebiotics had no effect on Chao 1 index, estimator of species richness (A), Observed species, estimator of species richness (B), Simpson index, estimator of diversity and evenness (C) and Shannon index, estimator of diversity and evenness (D). n = 8-10 data; represent mean ± SEM.
**Figure S5. Bacterial concentration by q-PCR in cecum.** Absolute quantification of *Lactobacillus* spp. levels (A), *Bifidobacterium* spp. levels (B) and total bacteria levels (C) in cecum. One-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: ***p<0.001; n = 10; Mean ± SEM of CFU/g of cecum is depicted.
Figure S6. Fear conditioning. There was no any effect of prebiotic administration on fear-related behaviors. On day 1, analysis revealed no differences in the learning curves among groups towards context (A) or cues (D). On day 2 (memory testing), no differences were found on memory towards context (B) or cues (E). On day 3 (memory extinction), no differences were observed among the experimental groups towards context (C) or cues (F). Repeated measures two-way ANOVA analysis; n = 10; data represent mean ± SEM. The first electric foot shock is marked by the red line.
Figure S7. Hippocampal gene expression. Prebiotics had no effect on mRNA levels of glucocorticoid receptor (NR3C1) (A), mineralocorticoid receptor (NR3C2) (B), NMDA receptor subunit 1 (C), cannabinoid receptor type 1 (CNR1) (D), GABA receptor Aα2 subunit (GABRA2) (E) and metabotropic glutamate receptor 4 (GRM4) (F). One-way ANOVA analysis; n = 8-10; data represent mean ± SEM.
Figure S8. Study 2. Stress/FOS+GOS animals had heavier cecum (A) and longer colon (B) compared with other groups. There was no any difference in total exploration time in novel object recognition test (C), time in the center (D) or latency to the center (E) of open field test. Spleen cytokine production without stimulation (vehicle) or following stimulation with lipopolysaccharide (LPS) and concanavalin A (ConA). No differences among the group on concentrations of cytokine IL-10 (F). Stress group presented a tendency to increase levels of released IL-1b after ConA stimulation (G). One-way or two-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: ***p<0.001 comparing to the control group; ###p<0.001 comparing to the stress group; n = 9-10; Mean ± SEM.
Figure S9. Microbial distribution at family level in study 2. Relative abundances of family level distributions of cecum microbiota in the three mice groups of the study. All families comprising less than 1% of the total abundance were combined into the “Other” category.
Figure S10. Bacterial concentration by q-PCR in cecum in study 2. Absolute quantification of *Lactobacillus* spp. levels (A), *Bifidobacterium* spp. levels (B) and total bacteria levels (C) in cecum. One-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: *p<0.05; ***p<0.001 comparing to the control group; #p<0.05; ###p<0.001 comparing to the stress group; n = 9-10; Mean ± SEM of CFU/g of cecum is depicted.
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All genera comprising less than 1% of the total abundance in each group (whether classified or not) were combined into the “Other” category (except the genus with significant differences among groups). The non-parametric Kruskal-Wallis test was used to analyse the differences among the mice groups. Statistical significance was accepted at $p < 0.05$. 

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Supplemental References


