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PII: S0006-3223(17)30042-2
DOI: http://dx.doi.org/10.1016/j.biopsych.2016.12.031
Reference: BPS13098

To appear in: Biological Psychiatry

Cite this article as: Aurelijus Burokas, Silvia Arboleya, Rachel D. Moloney, Veronica L. Peterson, Kiera Murphy, Gerard Clarke, Catherine Stanton, Timothy G. Dinan and John F. Cryan, Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice “Prebiotics for Stress-Related Disorders”, Biological Psychiatry, http://dx.doi.org/10.1016/j.biopsych.2016.12.031

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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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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 (*CRFR1*) (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 *CRFR1* 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 treated 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	extsubscript{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	extsubscript{A} and GABA	extsubscript{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
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 Actinobacteria:Proteobacteria was decreased after stress an effect that was normalized by prebiotic treatment. Moreover, the decreased Actinobacteria:Proteobacteria ratio was also observed in patients with major depressive disorder (56). Similarly to our results, previous studies showed an increase in Anaerotruncus and 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 Actinobacteria was decreased at phylum level, 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 parasympathetic 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.

Acknowledgements and Financial Disclosures

We thank Dr. S. El Aidy, Dr. R. Stilling, Dr. M. Ida, Dr. C. Torres-Fuentes, Dr. M. Pusceddu, C. Manley, P. Fitzgerald and A. Hoban for invaluable technical assistance, Dr. G. Moloney and Dr. K. Rea for critical interpretation of the data.

The APC Microbiome Institute is a research institute funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan. The authors and their work were supported by SFI (grant numbers SFI/12/RC/2273, 02/CE/B124 and 07/CE/B1368).

JFC & TGD have research support from Mead Johnson, Cremo, 4D Pharma, Suntory Wellness, and Nutricia. The authors have spoken at meetings sponsored by food and pharmaceutical companies. All other authors report no biomedical financial interests or potential conflicts of interest.
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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 *Verrucomicrobia* 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-butryate 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. TST; tail suspension test, FST; forced swim test, FUST; female urine sniffing test, EPM; elevated plus maze test, RIT; resident-intruder test, SIH; stress-induced hyperthermia. OF; open field, CORT; corticosterone.

**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 (TNFα) (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.
Table 1.
Concentrations of L-kynurenine, L-tryptophan, kynurenic acid (ng/ml) and the tryptophan:kynurenine and kynurenic acid:kynurenine ratios in plasma.

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<th>L-Kynurenine (kyn)</th>
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<th>Kynurenic Acid (KA)</th>
<th>Kyn:Try ratio</th>
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Data are expressed as means ±SEM. ** p < 0.01 vs. control. Statistically significant values are highlighted in grey.
Table 2. Concentrations (ng/mg of tissue) of noradrenaline (NA), dopamine (DA), serotonin (5-HT), and their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindole acetic acid (5-HIAA) and their ratios.

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| FOS | ME | 49 | 1357, 417, 272, 783. | AN | 6.0 | 7 | 1 | 9 | - | 4* | 3.58 | 0.36 |
| | SE | ±41 | ±124, ±73, ±25, ±36. | M | .3 | 2 | 4 | 1 | 9 | ±0.47 | ±0.04 |

| GOS | ME | 42 | 1348, 612, 284, 783, | AN | 9.2 | 4 | 5 | 9 | - | 7 | 3.33 | 0.36 |
| | SE | ±79 | ±140, ±19, ±30, ±65. | M | .2 | 0 | 4.5 | 9 | 3 | ±0.60 | ±0.01 |

| FOS+ | ME | 35 | 1344, 456, 296, 773. | AN | 6.7 | 0 | 3 | 9 | - | 4* | 3.06 | 0.37 |
| | SE | ±20 | ±43, ±19, ±23. | M | .7 | ±41.7 | 4 | 0 | 3 | ±0.24 | ±0.02 |

Data are expressed as means ±SEM. * p < 0.05; ** p < 0.01 vs. control. Statistically significant values are highlighted in grey.
**Figure 2**

A **Principal Coordinate Analysis (PCoA)**

B **Microbial Distribution at Phylum Level**

C **Microbial Distribution at Family Level**

**Control**
- Actinobacteria
- Bacteroidetes
- Candidate_division_TM7
- Cyanobacteria
- Deferribacteres
- Firmicutes
- Proteobacteria
- Tenericutes
- Verrucomicrobia

**FOS**
- Other
- Unclassified
- Verrucomicrobiaceae
- Ruminococcaceae
- Rikenellaceae
- Prevotellaceae
- Porphyromonadaceae
- Peptostreptococcaceae
- Peptococcaceae
- Lactobacillaceae
- Lachnospiraceae
- Family XIII
- Clostridiaceae
- Erysipelotrichaceae
- Desulfovibrionaceae
- Coriobacteriaceae
- Bifidobacteriaceae
- Bacteroidales S24-7_Unclassified
- Bacteroidaceae

**GOS**

**FOS+GOS**

**Microbial Distribution at Family Level**

- Bacteroidaceae
- Bacteroidales S24-7_Unclassified
- Bifidobacteriaceae
- Coriobacteriaceae
- Erysipelotrichaceae
- Desulfovibrionaceae
- Lachnospiraceae
- Rikenellaceae
- Ruminococcaceae
- Peptococcaceae
- Peptostreptococcaceae
- Lactobacillaceae
- Verrucomicrobiaceae
- Family XIII
- Clostridiaceae
- Bacteroidales S24-7_Unclassified
- Bacteroidaceae
Figure 3

A. Akkermansia

B. Oscillibacter

C. Bacteroides

D. Parabacteroides

E. Lactobacillus

F. Bifidobacterium

G. Desulfovibrio

H. Ruminococcus

I. Allobaculum

J. Turicibacter
Figure 6

A  Female Urine Sniffing Test (Water)

B  Female Urine Sniffing Test (Urine)

C  Tail Suspension Test

D  Forced Swim Test
Figure 7

A. Three Chamber Test
   Mouse vs Object

B. Three Chamber Test
   New Mouse vs Familiar Mouse

C. Resident-intruder Test

D. Novel Object Recognition Test

E. Hot Plate Test

F. Activity

* Significance level
Figure 9

A  Brain-derived Neurotrophic Factor (BDNF)

B  Corticotropin-releasing Hormone Receptor 1 (CRHR1)

C  GABA B1 Receptor

D  GABA B2 Receptor

E  N-methyl-D-Aspartate Receptor 2A Subunit (NMDA 2A)

F  N-methyl-D-Aspartate Receptor 2B Subunit (NMDA 2B)
Figure 10

A  Corticotropin-releasing Hormone Receptor 1 (CRHR1)

B  Glucocorticoid Receptor (NR3C1)

C  Mineralocorticoid Receptor (NR3C2)
Figure 12

A) Behavioral tests

3 weeks 3 weeks

3 weeks Prebiotic treatment

6 weeks Social STRESS

B) Social Interaction Test

Interaction ratio

Control Stress Stress/FOS+GOS

C) Novel Object Recognition Test

Discrimination index (DI)

Control Stress Stress/FOS+GOS

p = 0.07

D) Female Urine Sniffing Test (Urine)

Sniffing time (s)

Control Stress Stress/FOS+GOS

E) Marble Burying Test

Buried marbles

Control Stress Stress/FOS+GOS

F) Elevated Plus Maze

Entries into the Open Arms

Number of entries

Control Stress Stress/FOS+GOS

G) Elevated Plus Maze

Time in the Open Arms

Time (s)

Control Stress Stress/FOS+GOS

H) Open Field

Entries into the Center

Number of entries

Control Stress Stress/FOS+GOS
Figure 13

A. Tail Suspension Test

B. Forced Swim Test

C. Stress-induced Defecation in Forced Swim Test

D. Stress-induced Hyperthermia

E. Corticosterone Levels Basal

F. Corticosterone Levels 45 min After Stress

G. Cytokines IL-6

H. Cytokines TNF-α
Figure 14

A Principal Coordinate Analysis (PCoA)

B Actinobacteria:Proteobacteria Ratio

C Microbial Distribution at Phylum Level

- Actinobacteria
- Bacteroidetes
- Candidate division TM7
- Cyanobacteria
- Deferribacteres
- Firmicutes
- Proteobacteria
- Tenericutes

Legend:
- Red: Control
- Blue: Stress
- Orange: Stress/FOS+GOS

Stress
Stress/FOS+GOS
Control