Biological Psychiatry

Archival Report

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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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 underinvestigated microbiota manipulation. Here we tested whether chronic prebiotic treatment modifies behavior across domains relevant to anxiety, depression, cognition, stress response, and social behavior.

METHODS: C57BL/6J 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 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 short-chain fatty acid concentrations, prebiotic administration increased cecal acetate and propionate and reduced isobutyrate concentrations, changes that correlated significantly with the positive effects seen on behavior. Moreover, FOS+GOS reduced chronic stress-induced elevations in corticosterone and proinflammatory cytokine levels and depression-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: Animal behavior, Anxiety, Microbiota-gut-brain axis, Prebiotics, SCFAs, Stress

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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 procognitive effects in both rodents (7–10) and humans (11–14). Although single- or multistrain 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 underinvestigated 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, thereby conferring benefits on 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-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are soluble fibers extensively used as prebiotics that

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are 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 expression and *N*-methyl-D-aspartate 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 central nervous system (CNS) effects of prebiotic administration have not been extensively explored, and the links to a behavioral repertoire require extensive elaboration.

In the current study, we investigated whether administration of the prebiotics FOS and GOS, alone or in combination, affects behavior—specifically anxiety, depression-like behavior, 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, hypothalamic-pituitary-adrenal axis, immune system, and microbiota.

METHODS AND MATERIALS

Animals

In this study male C57BL/6J mice (n = 69; Harlan, Cambridgeshire, UK; 7 weeks of age on arrival) were used. (More details can be found in the Supplement.) All experiments were conducted in accordance with European Directive 86/609/EEC, 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, Dublin, Ireland) FOS, GOS, a combination of FOS and GOS (dissolved in drinking water for 0.3–0.4 g/mouse/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 2 to 3 weeks of treatment with prebiotics (21,22,24,25).

Anxiety-like Behavior

Anxiety-like 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 the Supplement. The experimental design is presented in Figure 1.

Depression-Related Behavior

Anhedonia was assessed using the female urine sniffing test (26), and antidepressant sensitive behaviors were assessed with the tail suspension and forced swim tests as previously detailed (7,27) (see Supplement).

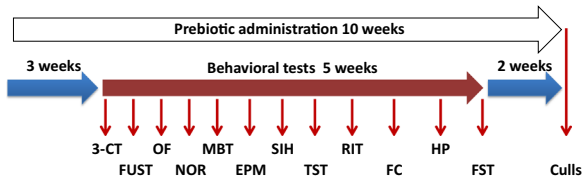

Figure 1. Experimental schedule of study 1 during the 10 weeks. Behavioral testing was conducted starting with the least stressful test to the most stressful test. Except for stress-induced hyperthermia, animals were brought to the experimental room 30 minutes prior to testing, which occurred between 8 AM and 4 PM (between 8 AM and 12 noon for the forced swim test). Briefly, 40 adult male mice (n = 10 per group) had a battery of different behavioral tests during 5 weeks. Week 4: 3-CT, three-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.

Social Behavior

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

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 (see Supplement).

Corticosterone, Tryptophan, and Neurotransmitter Levels

Plasma corticosterone and tryptophan levels, as well as brain neurotransmitter, were measured as previously described (32) and detailed in the Supplement.

Social Defeat/Overcrowding Procedure Followed by Social Interaction Test

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

Spleen Cytokine Assay

Spleens were collected immediately following sacrifice and cultured as previously described (33) (see Supplement).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the *mir*Vana microRNA Isolation Kit (Ambion/Life Technologies, Paisley, UK) and DNase treated (see Supplement).

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) (see Supplement).

Quantitative Polymerase Chain Reaction Analysis for Bacteria

Absolute quantification of *Lactobacillus* spp., *Bifidobacterium* spp., and total bacteria numbers in cecum was carried out by quantitative polymerase chain reaction (qPCR) as previously described (34) (see Supplement).

SCFA Concentration Analysis From Cecal Content

The analysis of SCFAs was carried out as previously described (35) (see Supplement).

Bioinformatic and Statistical Analysis

Bioinformatics sequence analysis is outlined in the Supplement. Statistical analyses were conducted using SPSS software, version 22 (IBM Corp., Armonk, NY). Bacterial compositional and behavioral nonparametric data were analyzed using the nonparametric 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 analysis of variance (ANOVA). For all other data, a one-way ANOVA was conducted, followed by Fisher's least significant difference post hoc test. Correlation analyses were performed using a Pearson correlation coefficient. Statistical significance was set at $p < .05$.

RESULTS

Detailed results and statistical analysis can be found in the figure legends and detailed in the Supplement.

Study 1: General Effects of Prebiotic Administration

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

Study 1: 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 the control mice group from that of groups fed with

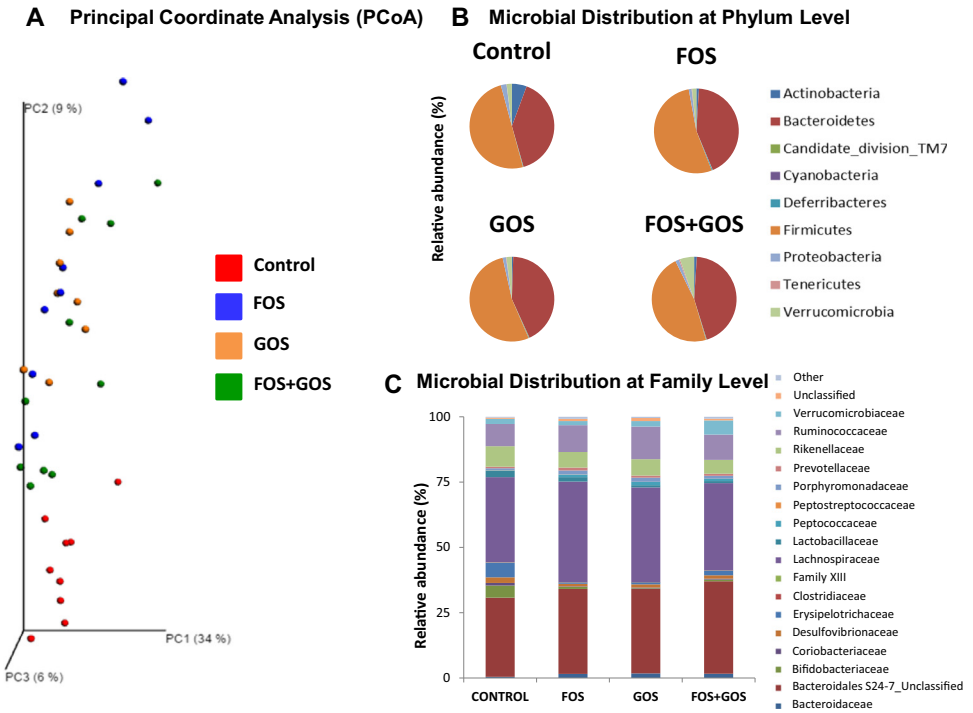

Figure 2. **(A)** Principal coordinate analysis (PCoA). PCoA based on unweighted UniFrac distances of cecum microbiota from the four mice groups of study is shown. Mice groups color coding: red, control group; blue, mice with fructo-oligosaccharides (FOS) administration; orange, mice with galacto-oligosaccharides (GOS) administration; green, mice with FOS+GOS administration. **(B)** Microbial distribution at phylum level. Relative abundances of phylum-level distributions of cecum microbiota are shown. *Proteobacteria* and *Actinobacteria* were significantly decreased in the prebiotic groups compared with the control group ($p < .05$), and FOS+GOS supplementation was associated with significantly increased *Verrucomicrobia* levels compared with the other prebiotics and control groups ($p < .05$ and $p < .01$, respectively). **(C)** Microbial distribution at family level. The proportions of *Bifidobacteriaceae, Coriobacteriaceae, Clostridiaceae, Desulfovibrionaceae, Erysipelotrichaceae, Lactobacillaceae,* and *Family XIII* were significantly decreased in the prebiotics groups compared with the control group. However, *Bacteroidaceae* 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 mouse groups of study are shown. All families comprising less than 1% of the total abundance were combined into the "Other" category.

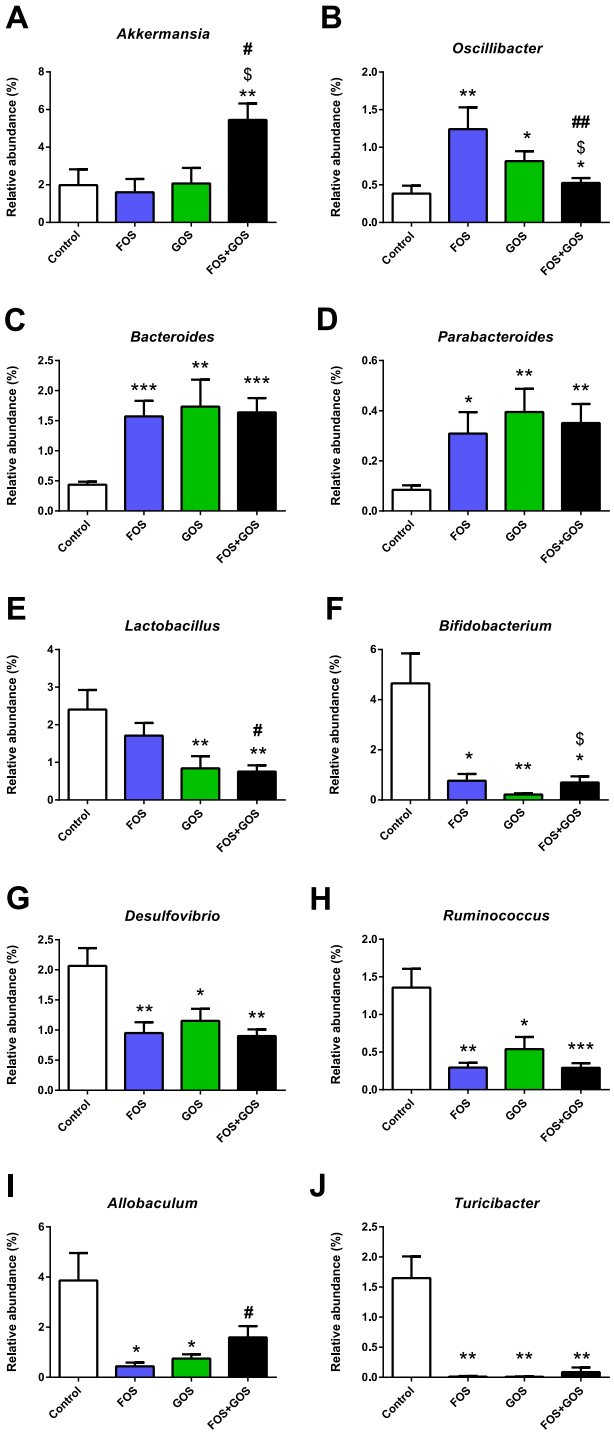

Figure 3. Relative abundance of selected genera with significant differences among the four mice groups of study. Relative abundance of *Akkermansia* **(A)**, *Oscillibacter* **(B)**, *Bacteroides* **(C)**, *Parabacteroides* **(D)**, *Lactobacillus* **(E)**, *Bifidobacterium* **(F)**, *Desulfovibrio* **(G)**, *Ruminococcus* **(H)**, *Allobaculum* **(I)**, and *Turicibacter* **(J)** are shown. The nonparametric Kruskal–Wallis test was used to analyze the differences among the mice groups, and the Mann-Whitney test was used in case of pairwise comparison. Statistical significance was accepted at $p < .05$. Superscript symbols indicate statistically significant differences between the following: *, each group with respect to the control group; $, fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) vs. GOS mice groups; #, FOS+GOS vs. FOS mice groups; $n = 10$; data represent mean ± SEM.

prebiotics (Figure 2A), suggesting that the cecal microbiota composition was altered following dietary supplementation with prebiotics. No statistical differences were shown in alpha diversity (Supplemental Figure S4 and Supplement).

Taxonomic shifts were also investigated, and at the phylum level the murine cecal microbiota was dominated by *Firmicutes* and *Bacteroidetes,* showing slight changes among the mice groups (Figure 2B). At the family level, the murine cecal microbiota was dominated by *Lachnospiraceae* and the group *S24-7_Unclassified,* both of which were higher in prebiotics groups than in the control group (Figure 2C).

In accordance with these results, at the genus level *Lachnospiraceae_Unclassified* and *S24-7_Unclassified* were the dominant microbial groups (Supplemental Table S1). The significant increase in the *Verrucomicrobiaceae* family was attributed to a significant increase in relative abundance of *Akkermansia* in the FOS/GOS group compared with the control group ($p < .01$) and the other two prebiotic groups ($p < .05$) (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, in some cases reaching significance compared with the control group (Figure 3). The qPCR results showed that prebiotic administration produced a significant increase in total bacteria numbers (Supplemental Figure S5), while no significant differences in *Lactobacillus* and *Bifidobacterium* levels were found among the four groups in the study. This suggests that a 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.

Study 1: Short-Chain Fatty Acids

Prebiotic administration had a significant effect on cecum SCFA production, as shown in Figure 4 and detailed in the Supplement.

Study 1: Behavior

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–C).

There was no effect of prebiotic administration on percentage time spent in open arms in the 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 the defensive marble burying test (Figure 5F).

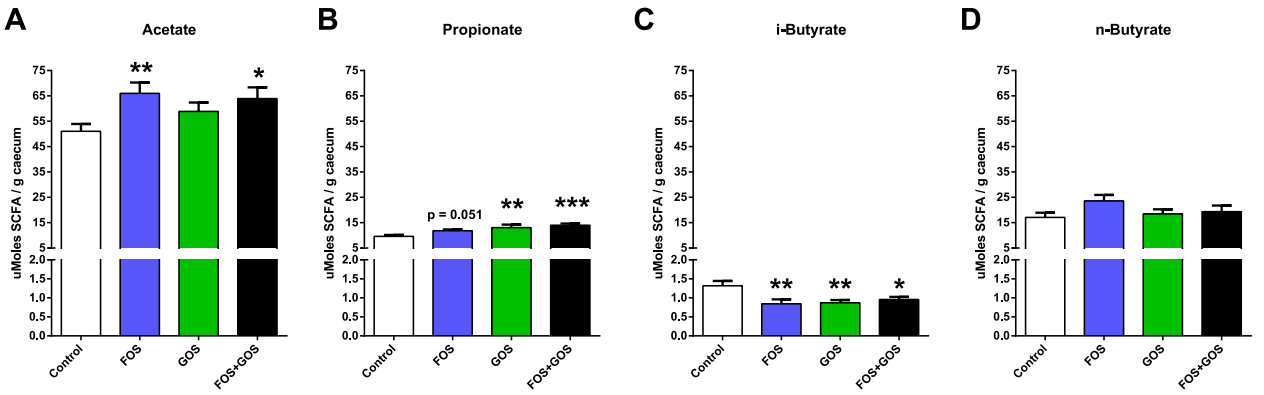

Figure 4. Short-chain fatty acid (SCFA) concentrations in cecum. **(A)** Fructo-oligosaccharides (FOS) and FOS + galacto-oligosaccharides (GOS) administrations increased acetate levels in cecum ($p < .05$). **(B–D)** All administrations increased propionate levels **(B)** but decreased isobutyrate levels **(C)**, whereas *n*-butyrate was not affected by any of the administrations **(D)**. *$p < .05$; **$p < .01$; ***$p < .001$; one-way analysis of variance followed by least significant difference post hoc test; $n = 8–10$; data represent mean ± SEM.

Depression-Related Behavior. 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 or on interaction between mouse and novel mouse (Figure 7A, B). Animals did not present aggressive behavior in the resident-intruder test. However, prebiotic administration significantly increased bouts of prosocial behavior in the resident-intruder test (Figure 7C).

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

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

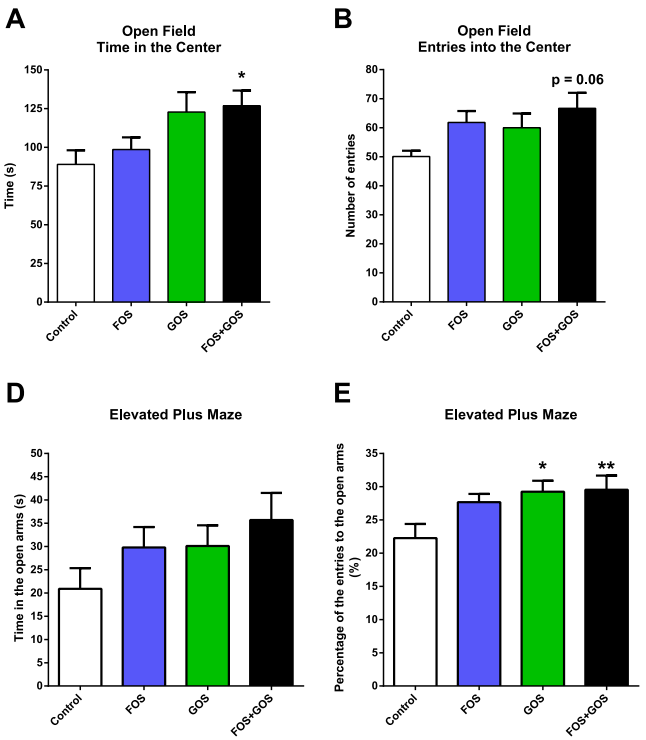

Figure 5. Anxiety-like behavior. **(A, B)** Fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) administration increased time spent in the center of the open field **(A)** and had a tendency to increase the number of entries into the center **(B)**. **(C)** The latency to enter into the center was not affected by any of the administrations. **(D, E)** Percentage of time spent in the open arms was not affected by any of the administrations in the elevated plus maze test **(D)**, but they increased the percentage of the entries into the open arms **(E)**. **(F)** The numbers of buried marbles in the defensive marble burying test are shown. *$p < .05$; **$p < .01$; one-way analysis of variance followed by least significant difference post hoc test [Mann-Whitney test in **(F)**]; $n = 10$; data represent mean ± SEM [median in **(F)**].

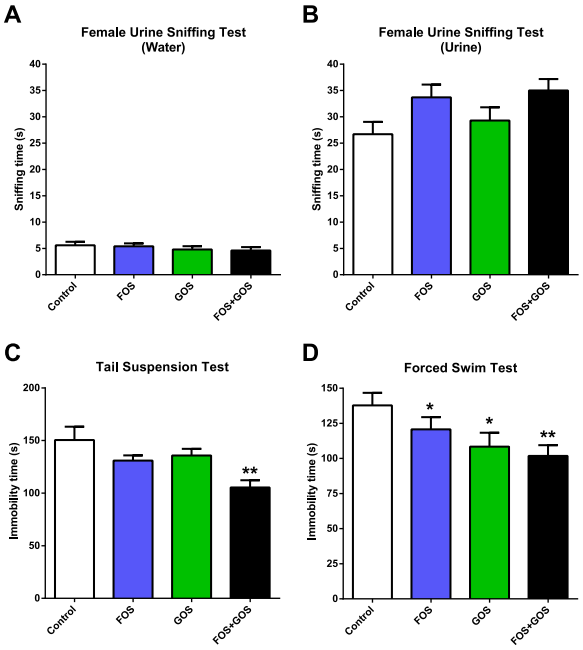

Figure 6. Depression-like behavior. **(A, B)** There was no effect of prebiotic administration on anhedonia in the female urine sniffing test: no effect on water sniffing time **(A)** or on female urine sniffing time **(B)**. **(C)** Fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) administration decreased immobility time in the tail suspension test. **(D)** All prebiotic administrations decreased immobility time in the forced swim test. $^{*}p < .05$; $^{**}p < .01$; one-way analysis of variance followed by least significant difference post hoc test; $n = 10$; data represent mean ± SEM.

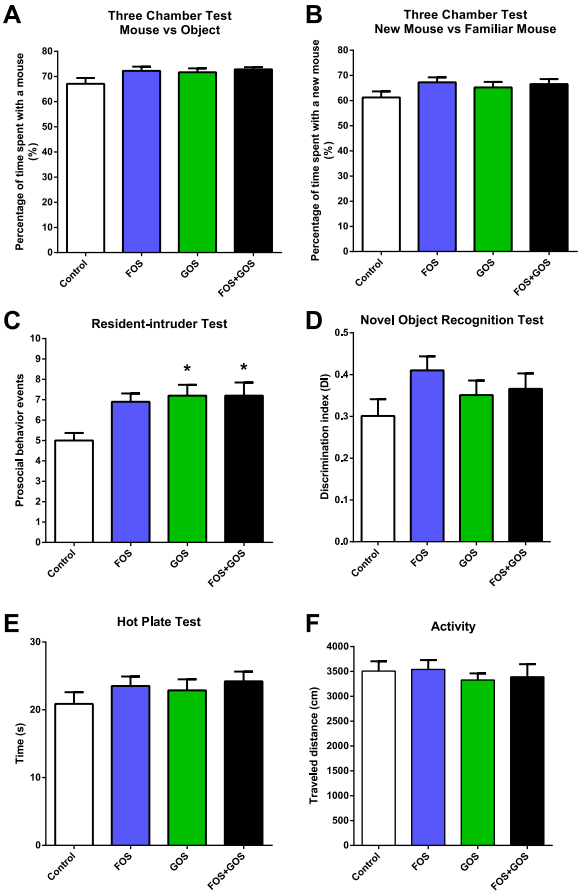

Figure 7. Social behavior and cognition. **(A, B)** Prebiotic administration had no effect on interaction between mouse and object in the three-chamber test **(A)** and on interaction between mouse and novel mouse **(B)**. **(C)** Prebiotic administrations increased the number of prosocial behavior events in the resident-intruder test. **(D)** Prebiotic administration had no effect on the discrimination index for memory in the novel object recognition test. **(E, F)** The pain response was not modified by prebiotics in the hot plate test **(E)** or total animal activity measured for 10 min **(F)**. $^{*}p < .05$; one-way analysis of variance followed by least significant difference post hoc test; $n = 10$; data represent mean ± SEM. FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.

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

Study 1: 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 minutes 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).

Study 1: Hippocampal and 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), gamma-aminobutyric acid B1 ($GABA_{B1}$) receptor gene (Figure 9C) and $GABA_{B2}$ receptor gene (Figure 9D). GOS and FOS+GOS administrations reduced messenger RNA (mRNA) levels of *Crfr1* (Figure 9B). FOS administration increased, and FOS+GOS administration decreased, *N*-methyl-D-aspartate receptor 2A subunit (Figure 9E) but had no effect on the 2B subunit (Figure 9F). No changes were observed on *N*-methyl-D-aspartate subunit 1, cannabinoid type 1, $GABA_A\alpha 2$ receptor, metabotropic glutamate receptor 4, glucocorticoid, and mineralocorticoid receptor mRNA levels after prebiotic administration (Supplemental Figure S7). FOS+GOS administration significantly reduced mRNA levels of glucocorticoid receptor in hypothalamus but not *Crfr1* or mineralocorticoid receptor (Figure 10).

Study 1: Tryptophan and Tryptophan Metabolites

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

Study 1: Brain Monoamines

FOS and FOS+GOS administration increased serotonin levels in the prefrontal cortex. FOS+GOS administration decreased

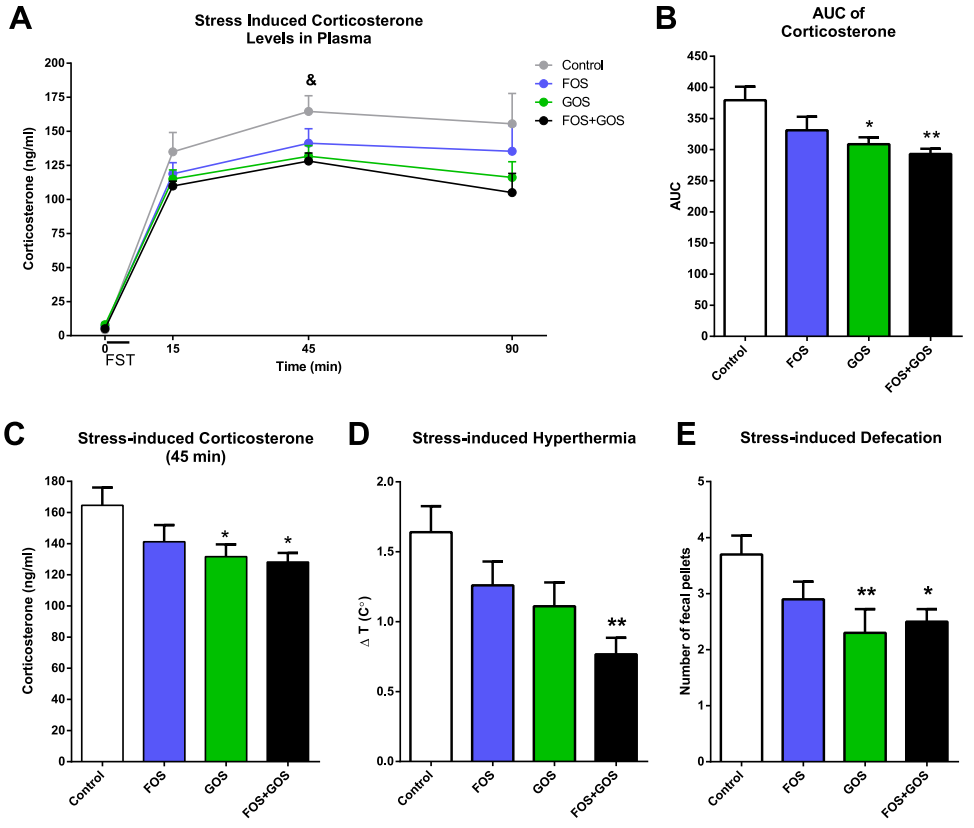

Figure 8. Endocrine response. **(A)** Prebiotic administration decreased corticosterone levels after a stressful event (forced swim test [FST]). **(B)** Area under the curve (AUC) for corticosterone levels was reduced in prebiotic administration groups. **(C)** Stress-induced corticosterone levels after 45 minutes were reduced in prebiotic-treated groups. **(D, E)** Stress-induced hyperthermia was reduced by fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) administration **(D)**, and stress-induced defecation was reduced by GOS and FOS+GOS administrations **(E)**. $^{*}p < .05$; $^{**}p < .01$; $^{\&}p < .05$ comparing control group with GOS and FOS+GOS groups; repeated measures or one-way analysis of variance followed by least significant difference post hoc test; $n = 10$; data represent mean ± SEM.

dihydroxyphenylacetic acid levels in the brainstem. Conversely, GOS and FOS+GOS administration increased dihydroxyphenylacetic acid levels in the frontal cortex (Table 2).

Study 1: SCFA Levels Correlate With Behavior and Gene Expression

The altered concentrations of SCFAs in cecum correlate 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), whereas FOS+GOS administration protected from this effect. Stress significantly impaired long-term memory by decreasing the discrimination index in the novel object recognition test (Figure 12C), whereas prebiotics had a tendency to protect 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 stress on anxiety-like behavior in the elevated plus maze test, as characterized by a reduced number of entries in open arms (Figure 12F) and time spent there (Figure 12G). However, following post hoc analysis revealed that animals receiving prebiotics spent more time in open arms than only stressed ones (Figure 12G). Number of entries to the center of open field was also reduced by stress but was not reversed by prebiotic cotreatment (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 and Endocrine Response. Animals administered 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 led to higher levels of corticosterone 45 minutes after the beginning of the forced swim test; this was attenuated by prebiotic treatment having lower levels than only stressed animals (Figure 13F).

Study 2: Spleen Cytokine Production After Stimulation With Concanavalin A and Lipopolysaccharide

The only stress group presented a higher concentration of interleukin 6 after stimulation with concanavalin A, and animals with prebiotics had similar levels to control animals (Figure 13G). Similarly, stress induced an increased concentration in tumor necrosis factor alpha after concanavalin

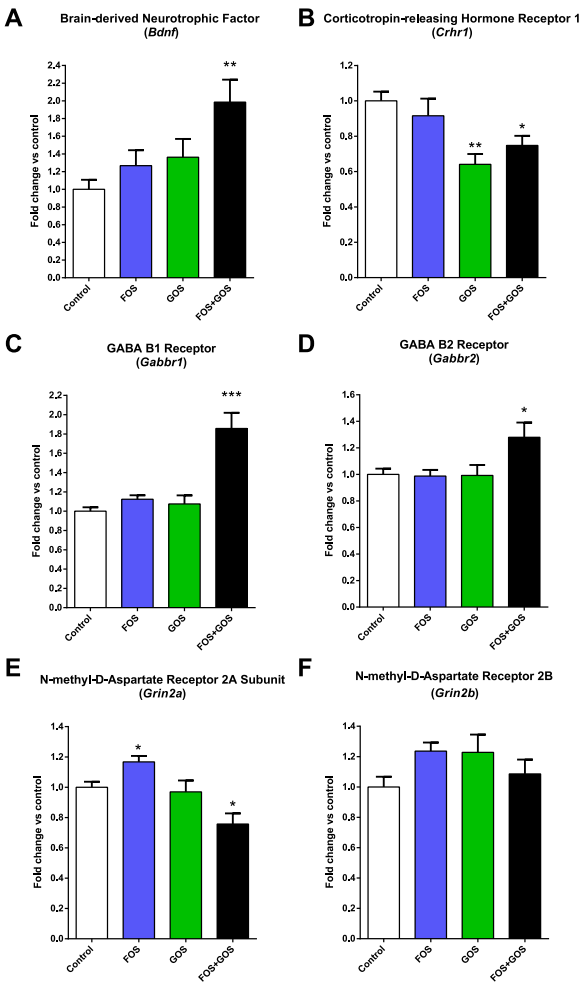

Figure 9. Hippocampal gene expression. **(A)** Fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) administration increased messenger RNA (mRNA) levels of brain-derived neurotrophic factor (*Bdnf*) not only compared with the control group but also compared with other administrations. **(B)** GOS and FOS+GOS administrations reduced mRNA of corticotropin-releasing hormone receptor 1 (*Crhr1*). **(C, D)** FOS+GOS administration increased mRNA levels of gamma-aminobutyric acid B1 ($GABA_{B1}$) receptor **(C)** and mRNA of $GABA_{B2}$ receptor **(D)** compared with all the groups. **(E, F)** FOS administration increased, whereas FOS+GOS administration decreased, mRNA levels of *N*-methyl-D-aspartate (NMDA) receptor 2A subunit **(E)**, but there were no changes of mRNA for NMDA receptor 2B subunit **(F)**. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; one-way analysis of variance followed by least significant difference post hoc test; $n = 8–10$; data represent mean ± SEM.

A stimulation, and in animals with prebiotics this had normalized to control levels (Figure 13H). There were no effects on interleukin 1β and interleukin 10 (see Supplement).

Study 2: 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 the control and stress/FOS+GOS groups, separated from the stress group (Figure 14A).

The different cecal microbiota composition was reflected in significant differences at multiple taxonomical levels (Figures 14 and 15, as detailed in the Supplement). At the genus level, the most interesting result is a decrease in relative abundance of *Bifidobacterium* ($p < .01$), and this effect was abolished by treatment with prebiotics ($p < .001$) (Figure 15A). In addition, qPCR results corroborate the higher concentration (cfu/g cecum) of not only *Bifidobacterium* but also *Lactobacillus* in the control and prebiotic administration groups than in stressed animals (Supplemental 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 CNS (22,24,25) and behavior (21). In this study, we report that prebiotics (i.e., FOS, GOS, and a combination of both) were able to markedly modify behavior and brain chemistry relevant to anxiety and depression in mice. In addition, we report that the 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 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 the 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), and probiotic administration (38). It is unclear whether the current alteration in tryptophan availability reflects increased bacterial use 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 that prebiotics have beneficial effects on stress responses, we

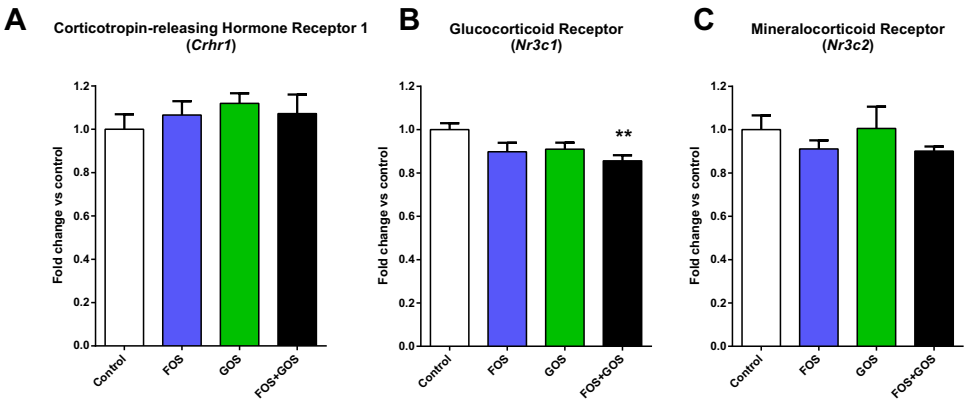

Figure 10. Hypothalamic gene expression. **(B)** Fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) administration decreased messenger RNA levels of glucocorticoid receptor (*Nr3c1*) compared with the control group. **(A, C)** Prebiotics had no effects on messenger RNA levels of corticotropin-releasing hormone receptor 1 (*Crhr1*) **(A)** or mineralocorticoid receptor (*Nr3c2*) **(C)** in hypothalamus. $^{**}p < .01$; one-way analysis of variance followed by least significant difference post hoc test; $n = 8–10$; data represent mean ± SEM.

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 depression-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 given that the 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* sp. has received a lot of attention for its beneficial role in the host-like protection from diet-induced obesity, insulin resistance, intestinal inflammation (44–46), and gut barrier impairment (47), and it 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 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 prebiotics coincided with gene expression and monoamine-level alterations. Mice administered the FOS+GOS combination presented high levels of *Bdnf* expression in the hippocampus. Previously, we showed that mice consistently exhibited heightened anxiety-like behavior and depression-like behavior that were associated with decreased hippocampal *Bdnf* (50). Hippocampal mRNA levels for a subunit of the $GABA_B$ receptor were also increased in animals administered the FOS+GOS combination. Interestingly, probiotic lactic acid bacteria *Lactobacillus rhamnosus* (JB-1) administration could

Table 1. Concentrations of L-kynurenine, L-tryptophan, Kynurenic Acid (ng/mL), and the Tryptophan:Kynurenine and Kynurenic Acid:Kynurenine Ratios in Plasma

	L-Kynurenine	L-Tryptophan	Kynurenic Acid	Kynurenine:Tryptophan Ratio	Kynurenic Acid:Kynurenine Ratio
Control	203.1 ± 23.1	19754.8 ± 1859.1	5.9 ± 1.5	0.011 ± 0.001	0.029 ± 0.005
FOS	166.4 ± 21.0	15252.4 ± 1392.6	3.7 ± 0.1	0.012 ± 0.002	0.031 ± 0.013
GOS	146.8 ± 23.3	13758.5 ± 531.6[a]	4.0 ± 0.4	0.010 ± 0.002	0.038 ± 0.001
FOS+GOS	146.4 ± 32.6	13762.9 ± 556.0[a]	3.5 ± 0.5	0.010 ± 0.002	0.031 ± 0.007

Data are expressed as mean ± SEM.
FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.
[a]$p < .01$ vs. control.

Table 2. Concentrations (ng/mg 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

		NA	DOPAC	DA	5-HIAA	HVA	5-HT	DOPAC/DA	HVA/DA	5-HIAA/5-HT
Brainstem	Control	324.6 ± 36.1	177.9 ± 21.9	288.1 ± 42.1	568.7 ± 28.7	866.0 ± 202.0	1030.2 ± 37.6	0.69 ± 0.09	2.85 ± 0.57	0.56 ± 0.03
	FOS	353.6 ± 29.3	187.2 ± 21.8	362.6 ± 34.9	582.5 ± 21.5	1214.8 ± 125.2	1043.3 ± 34.5	0.53 ± 0.08	3.54 ± 0.46	0.56 ± 0.01
	GOS	419.7 ± 53.3	152.0 ± 17.8	227.3 ± 50.9	593.7 ± 51.0	987.3 ± 335.0	1050.1 ± 63.0	0.75 ± 0.11	4.21 ± 1.08	0.56 ± 0.02
	FOS+GOS	420.3 ± 39.2	100.8 ± 12.4[b]	228.3 ± 46.8	606.4 ± 31.8	940.0 ± 259.4	1028.2 ± 42.8	0.55 ± 0.07	3.63 ± 0.62	0.59 ± 0.02
Frontal Cortex	Control	256.0 ± 14.2	1409.4 ± 65.3	2165.0 ± 232.1	198.0 ± 11.2	916.9 ± 52.4	689.6 ± 51.5	0.66 ± 0.08	0.47 ± 0.08	0.30 ± 0.01
	FOS	280.4 ± 62.0	1672.4 ± 135.7	3492.4 ± 496.5	233.5 ± 22.6	1089.5 ± 47.1	768.4 ± 73.6	0.63 ± 0.10	0.36 ± 0.08	0.30 ± 0.01
	GOS	351.4 ± 37.4	1837.1 ± 163.9[a]	2606.3 ± 526.3	230.8 ± 19.2	922.6 ± 308.8	764.4 ± 69.9	1.08 ± 0.24	0.37 ± 0.16	0.31 ± 0.01
	FOS+GOS	295.9 ± 84.8	1939.5 ± 102.8[b]	2689.2 ± 294.9	219.2 ± 11.0	931.8 ± 80.1	754.0 ± 26.4	0.83 ± 0.09	0.37 ± 0.06	0.29 ± 0.01
Prefrontal Cortex	Control	420.6 ± 41.0	1178.6 ± 123.9	501.3 ± 154.5	275.0 ± 18.3	–	658.9 ± 25.7	2.86 ± 0.33	–	0.42 ± 0.02
	FOS	496.0 ± 41.3	1357.7 ± 124.2	417.1 ± 73.4	272.9 ± 25.1	–	783.4 ± 36.9*	3.58 ± 0.47	–	0.36 ± 0.04
	GOS	429.2 ± 79.2	1348.4 ± 140.0	612.5 ± 194.5	284.9 ± 30.9	–	783.7 ± 65.3	3.33 ± 0.60	–	0.36 ± 0.01
	FOS+GOS	356.7 ± 20.7	1344.0 ± 41.7	456.3 ± 43.4	296.9 ± 19.0	–	773.4 ± 23.3*	3.06 ± 0.24	–	0.37 ± 0.02

Data are expressed as mean ± SEM.
FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides.
[a] $p < .05$.
[b] $p < .01$ vs. control.

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 the 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 support 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 peptide YY and glucagon-like peptide 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. Because the FOS+GOS combination revealed the strongest effect, we also tested these prebiotics in animals subjected to chronic stress. Interestingly, animals receiving FOS+GOS had reduced anhedonia and anxiety- and depression-like behavior, compared 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 proinflammatory response that was normalized by FOS+GOS administration. A previous study showed that a specific bacterial strain, *Bifidobacterium infantis*, attenuated the exaggerated interleukin 6 response to concanavalin A stimulation in rats after early-life stress (55).

Intriguingly, FOS+GOS administration also protected from the impact of chronic stress on the microbiota. The *Actinobacteria:Proteobacteria* ratio 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). Similar to our results, previous studies showed an increase in *Anaerotruncus* and *Peptococcus* spp. after prenatal stress in rats (57). The microbiota of mice after chronic social stress was similar to that observed in a previous study in rats that received fecal microbiota transplantation from patients with depression (58); the relative abundance of *Actinobacteria* was decreased at the phylum level, the relative abundance(s) of *Bifidobacteriaceae* and *Coriobacteriaceae* were decreased and *Propionibacteriaceae* was increased at the family level, and the relative abundance(s) of *Bifidobacterium* and *Allobaculum* were decreased and *Peptococcus* was increased at the genus level. In addition, FOS+GOS administration

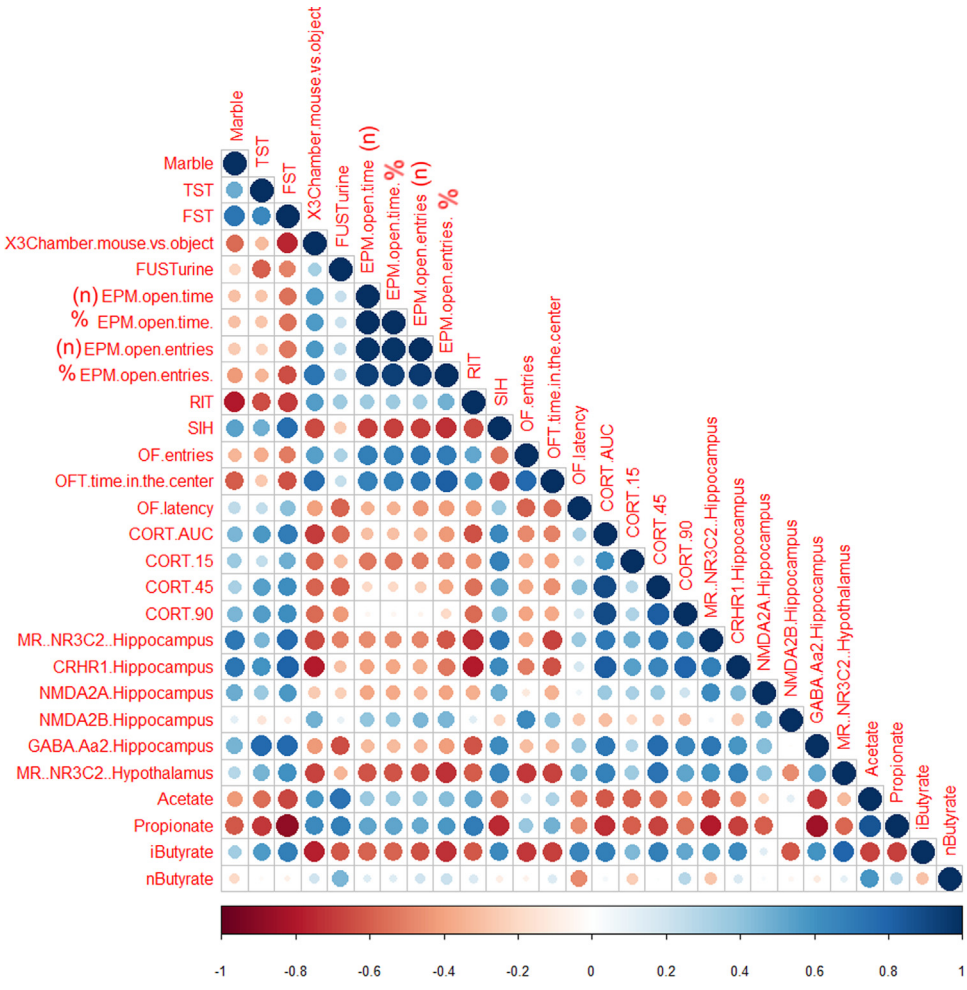

Figure 11. Short-chain fatty acid levels correlate with behavior and gene expression. The color and size of the circles in the matrix code for level of correlation; red represents negative correlation, and blue represents positive correlation. A correlation analysis revealed a significantly positive association of acetate concentration and sniffing time in the female urine test to measure anhedonic behavior. For propionate, a negative association was revealed with immobility time in the forced swim test and the tail suspension test, buried marbles, rectal temperature increase in stress-induced hyperthermia, corticosterone elevation 45 minutes after stress, or overall corticosterone response. The same effect was also revealed for messenger RNA levels of mineralocorticoid receptor, *N*-methyl-D-aspartate (NMDA) receptor 2A subunit, gamma-aminobutyric acid (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 the resident-intruder test and sniffing time in the female urine test. Reduced concentrations of isobutyrate after prebiotic administration had significantly positive association with reduced immobility time in the forced swim test, latency to enter into the center of the open field test, corticosterone levels 45 minutes after stress, and messenger RNA levels of mineralocorticoid receptor in the hypothalamus. In contrast, significantly negative association of isobutyrate was revealed with sociability (preference for mouse vs. object in the three-chamber test), sniffing time in the female urine test, percentage of entrance into open arms, number of entries into the center, time in the center in the open field test, and messenger RNA levels of NMDA receptor 2B subunit in hippocampus. *n*-Butyrate levels had a significantly positive association with anhedonic behavior in the female sniffing urine test, corticosterone levels 90 minutes after stress, and a negative association with the latency to enter into the center of the open field test. CORT, corticosterone; EPM, elevated plus maze test; FST, forced swim test; FUST, female urine sniffing test; OF, open field; RIT, resident-intruder test; SIH, stress-induced hyperthermia; TST, tail suspension test.

prevented the reduction of *Bifidobacterium* and *Lactobacillus* concentration caused by chronic stress. In agreement, lower *Bifidobacterium* and/or *Lactobacillus* counts are more common in patients with major depressive disorder compared with control subjects (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 modulate the ecology of the microbiota. There is still a lot needed to determine the role of the microbial composition and the vast quantity, diversity, and functional capabilities of all these gut microorganisms on the 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 and 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 possible new therapeutic targets in the field of nutritional neuropsychopharmacology.

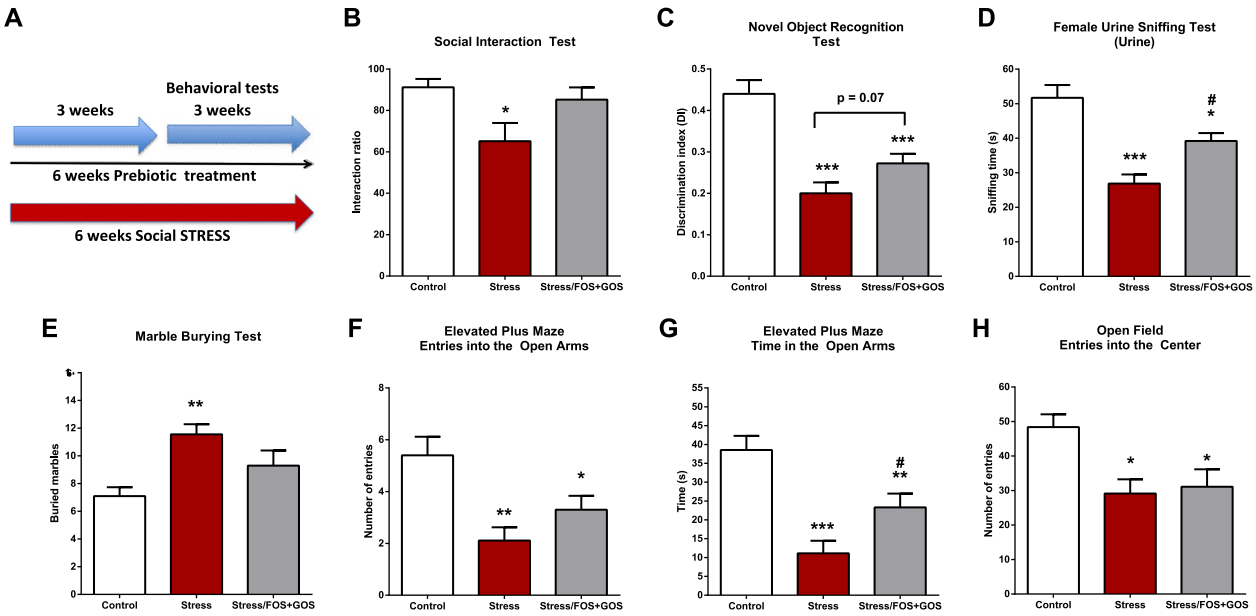

Figure 12. **(A)** Experimental schedule of study 2. Briefly, 29 adult mice were used (n = 9–10 per group). Behavioral testing was conducted in the same way as in the first study but with fewer tests. Chronic social unpredictable stress was applied during all 6 weeks, and the group with prebiotics received fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) throughout the experiment. Behavioral tests were conducted during the last 3 weeks of the study. **(B)** The stress group showed a reduced interaction ratio in the social interaction test, but the stress/FOS+GOS group did not. **(C)** The stress and stress/FOS+GOS groups presented a lower discrimination index for memory in the novel object recognition test, but the stress/FOS+GOS group showed a tendency to increase the discrimination index compared with the stress-only group. **(D)** In addition, the stress and stress/FOS+GOS groups reduced female urine sniffing time, although the group with FOS+GOS showed higher time than the stress-only group. **(E)** The numbers of buried marbles in the defensive marble burying test were increased only in the stress group. **(F, G)** Animals from the stress and stress/FOS+GOS groups reduced entries to the open arms **(F)** and time spent there **(G)**; however, the group administered prebiotics spent more time in open arms compared with the stress-only group **(G). (H)** The number of entries into the center was reduced in both stress groups compared with the control group; $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ comparing with the control group; $^{\#}p < .05$ comparing with the stress group. one-way analysis of variance followed by least significant difference post hoc test; n = 9–10; data represent mean ± SEM.

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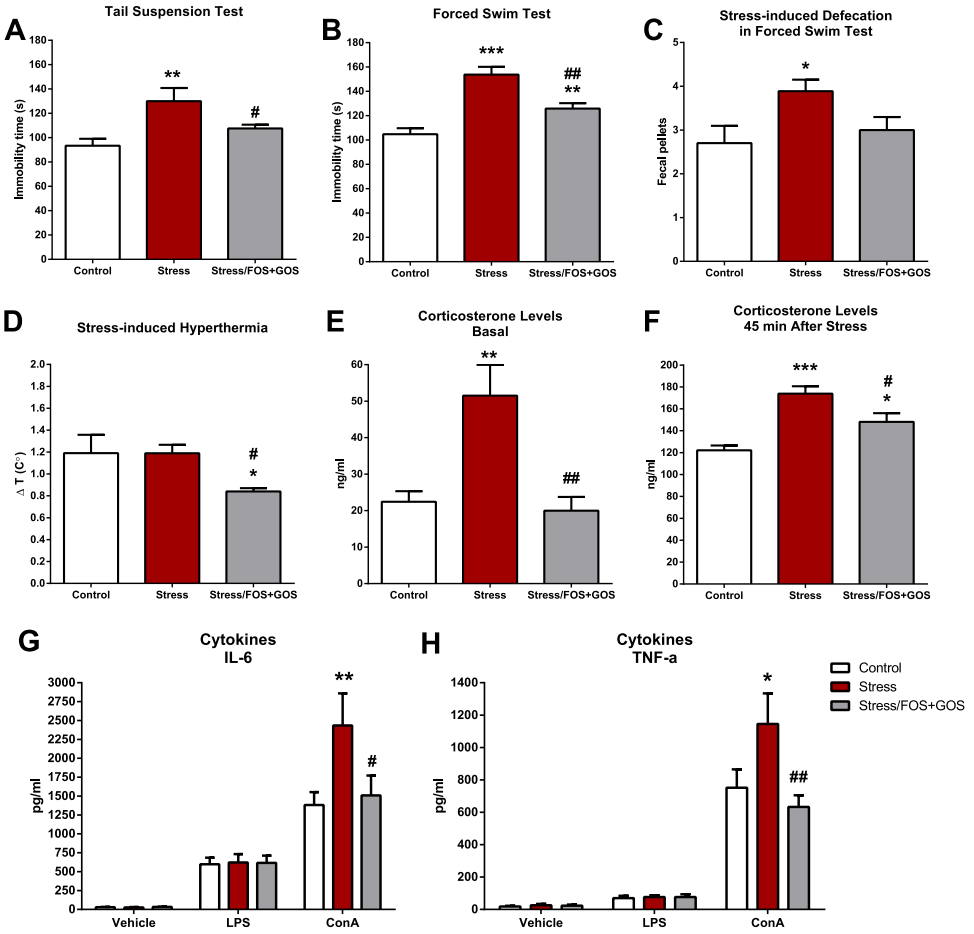

Figure 13. **(A, B)** The stress group presented increased immobility time in the tail suspension test **(A)** and in the forced swim test **(B)**, whereas the stress group with prebiotics presented lower increment in immobility time compared with the stress-only group. **(C)** Stress-induced defecation in the forced swim test was increased only in the stress group. **(D)** Stress-induced hyperthermia was reduced only in the stress/fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) group. **(E, F)** Chronic stress increased basal corticosterone levels **(E)** and corticosterone levels 45 minutes after a stressful event (forced swim test) **(F)**. The stress group with prebiotics presented lower corticosterone levels at 45 minutes after a stressful event **(F)**. **(G)** Spleen cytokine production without stimulation (vehicle) or following stimulation with lipopolysaccharide (LPS) and concanavalin A (ConA) is shown. The stress group presented increased levels of released interleukin 6 (IL-6) **(G)** and tumor necrosis factor alpha (TNF-a) **(H)** after ConA stimulation. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ comparing with the control group; $^{\#}p < .05$; $^{\#\#}p < .01$ comparing with the stress group; one-way analysis of variance followed by least significant difference post hoc test; $n = 9–10$; data represent mean ± SEM. T, temperature.

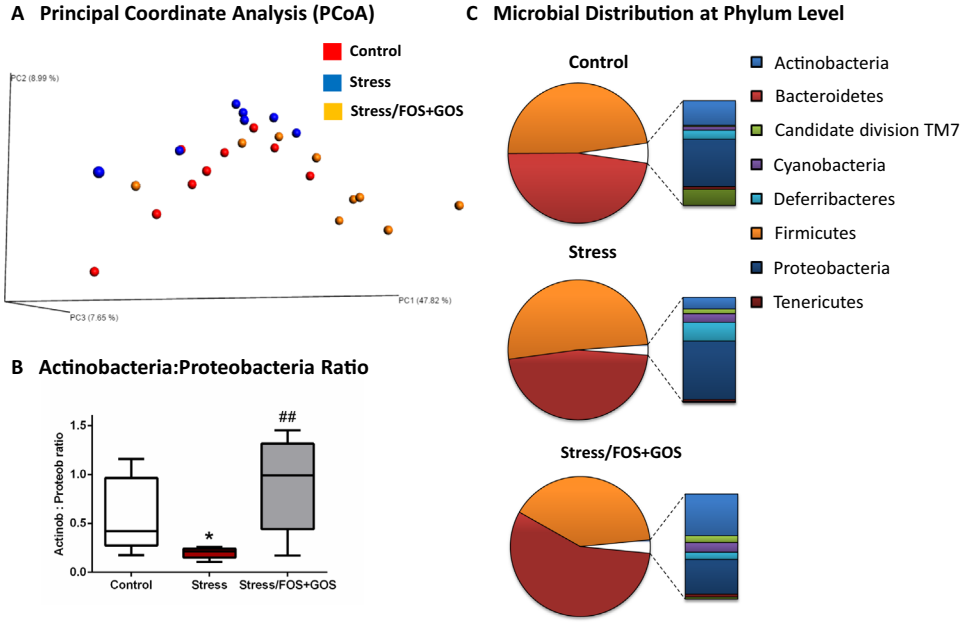

Figure 14. **(A)** Principal coordinate analysis (PCoA) in study 2. PCoA based on weighted UniFrac distances of cecum microbiota from the three mice groups of the study is shown. Mice groups color coding: red, control group; blue, mice from stress group; yellow, stress/fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) group. **(B)** *Actinobacteria:Proteobacteria* ratio. **(C)** Microbial distribution at phylum level. Relative abundances of phylum level distributions of cecum microbiota in the three mice groups of the study are shown.

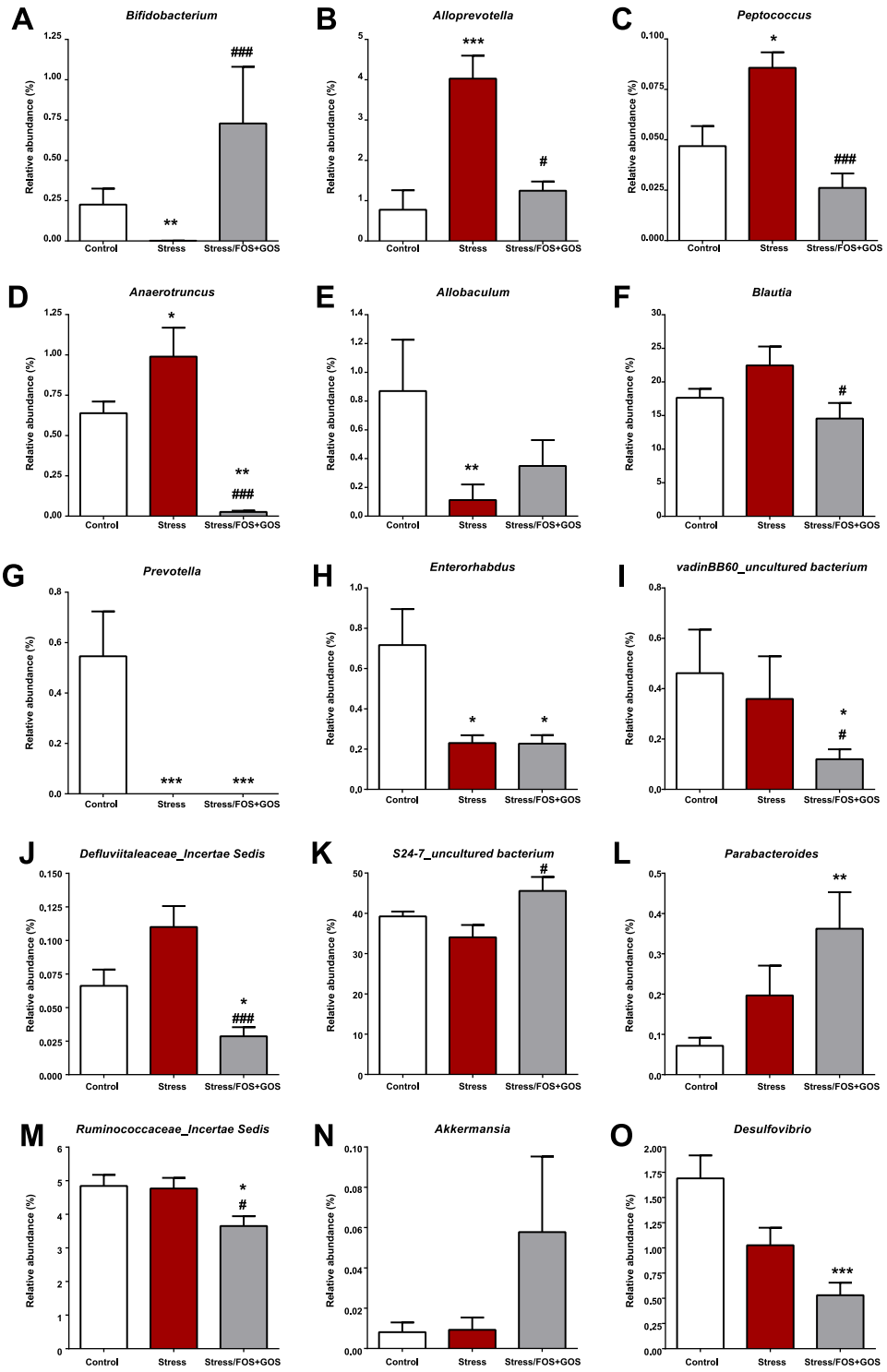

Figure 15. Relative abundances of selected genera in study 2. **(A)** At the genus level, relative abundance of *Bifidobacterium* is decreased in the stressed mice and the abolition of the effect by treatment with prebiotics ($p < .001$). **(B–D, F)** Similar opposite effects were observed in relative abundances of *Alloprevotella* **(B)**, *Peptococcus* **(C)**, *Anaerotruncus* **(D)**, and *Blautia* **(F)** where stress increased but the stress/fructo-oligosaccharides (FOS) + galacto-oligosaccharides (GOS) group presented similar to the control group or sometimes with lower relative abundance. **(E)** Only stress reduced the relative abundance of *Allobaculum* ($p < .01$). **(G, H)** Low abundances of *Prevotella* **(G)** and *Enterorhabdus* **(H)** were observed in both stress groups compared with the control group. **(I, J, L, M)** On the other hand, only the stress/FOS+GOS group showed a decrease in *vadinBB60_uncultured bacterium* **(I)**, *Defluviitaleaceae_Incertae Sedis* **(J)**, and *Ruminococcaceae_Incertae Sedis* **(M)** and an increase in *Parabacteroides* ($p < .01$) **(L)**. **(K)** *S24-7_uncultured bacterium* made up 46% of relative abundance in the stress/FOS+GOS group, whereas only stressed animals displayed 34%, which was significantly lower ($p < .05$). **(N, O)** Similar to the results of study 1, FOS+GOS administration even under the stress conditions had a tendency to increase relative abundance of *Akkermansia* **(N)** and to decrease that of *Desulfovibrio* **(O)** ($p < .01$). The nonparametric Kruskal–Wallis test was used to analyze the differences among the mice groups, and Dunn's test was used in case of pairwise multiple comparisons. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$ comparing with the control group: $^{\#}p < .05$ comparing with the stress group; $^{\#\#\#}p < .001$. n = 8–10; data represent mean ± SEM.

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