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Authors	Burokas, Aurelijus;Arboleya, Silvia;Moloney, Rachel D.;Peterson, Veronica L.;Murphy, Kiera;Clarke, Gerard;Stanton, Catherine;Dinan, Timothy G.;Cryan, John F.
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Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects in Mice

Supplemental Information

Supplemental Methods and Materials

Animals

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

Open field

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

Defensive marble burying

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

Elevated plus maze

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

Female urine sniffing test

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

Tail suspension test

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

Forced swim test

Mice were individually placed in a clear glass cylinder (24 × 21 cm diameter), containing 15-cm-depth water (25 ± 0.5 °C). Water was changed between each animal

tested to remove odors. The test lasted 6 min and experiments were videotaped using a numeric tripod-fixed camera; data were further scored twice using the videos (Video Media Player software) and averaged by an experimenter blind to conditions. The latency to immobility was scored. The time of immobility (s) was measured for the last 4 min of the test, with immobility being defined as a total absence of movement except slight motions to maintain the head above the water.

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

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

Resident-intruder test

Intruder animals of a similar weight were housed four per cage. Each session consisted in placing an intruder mouse into the resident's home cage for a period of 4 minutes. The latency to first approach, aggressive (attack or menace like tail-beating) interactions, as well as non-aggressive (general sniffing, anogenital sniffing, grooming and rearing) interactions of the resident mouse with its counterpart were quantified.

Novel object recognition test

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

Fear conditioning

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

Stress-induced hyperthermia

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

Plasma collection

Mice were not restrained and the end of the tail was held with two fingers. Using a single edge razor blade a diagonal incision of 2-5 mm long was made from the end of the tail. Approximately 100 μl blood was collected in a collecting tube containing EDTA to avoid blood coagulation by increasing the pressure of the fingers on the tail above the incision. Blood was mixed with EDTA by gently inverting the tube and centrifuged at 3500 × g at 4 °C temperature for 15 min. Plasma was carefully aspirated and stored at −80 °C. The same animal was sampled at all time points.

Corticosterone assay

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

Tissue collection

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

Neurotransmitter concentrations

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

HPLC assay for tryptophan and kynurenine

HPLC analysis involved using a system comprising a Waters 510 pump (Waters Ireland, Dublin, Ireland), 717plus cooled autosampler, a 996 PDA detector, a Hewlett Packard 1046A Fluorescent Detector (Waters Ireland, Dublin, Ireland), a waters bus SAT/IN module and a croco-cil column oven. System components were used in conjunction with Waters Empower software (Waters Ireland, Dublin, Ireland). All samples were injected onto a reversed phase Luna 3µ C18(2) 150 × 2 mm column (Phenomenex, Macclesfield, UK), which was protected by Krudkatcher disposable precolumn filters and security guard cartridges (Phenomenex). HPLC grade acetonitrile, acetic acid, and perchloric acid were obtained from Fisher Scientific Ireland (Dublin, Ireland). The analysis method was based on that by Herve et al. (1996). The mobile phase consisted of 50 mmol L−1 acetic acid, 100 mmol L−1 Zinc Acetate with 3% (v/v)

acetonitrile and was filtered through a 0.45 μm Millipore filter (AGB, Dublin, Ireland) and vacuum degassed prior to use. Separations were achieved by isocratic elution at 0.3 mL min−1. The fluorescent detector was set to an excitation wavelength of 254 nm and an emission wavelength of 404 nm. The PDA detector start wavelength was 210 nm and the end wavelength was 400 nm with chromatogram extraction at 330 nm. Working standard dilutions were prepared from millimolar stock solutions of each standard and stored at −80°C until required for analysis. Samples were deproteinized by the addition of 20 μL of 4 mol L−1 perchloric acid to 200 μL of plasma spiked with 3-nitro-L-tyrosine as internal standard. Twenty microliters of either sample or standard was injected onto the HPLC system and chromatograms generated were processed using Waters Empower software. Analytes were identified based on their characteristic retention time and their concentrations determined using Analyte:Internal standard peak height ratios; these were measured and compared with standard injections which were run at regular intervals during the sample analysis. Results were expressed at ng analyte per mL of supernatant/plasma.

Social defeat/overcrowding procedure

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

Social interaction test

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

Spleen cytokine assay

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

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

Total RNA was extracted using the *mir*Vana™ miRNA Isolation kit (Ambion/Llife technologies, Paisley, UK) and DNase treated (Turbo DNA-free, Ambion/life

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

DNA extraction from cecal contents and amplicon sequencing

Total DNA was extracted from the cecal content of all samples using the QIAamp DNA Stool Mini Kit (Qiagen, Sussex, UK) according to manufacturer's instructions, with the addition of an initial bead-beating step. Isolated DNA was frozen at -20°C until analysis. The V3-V4 hypervariable region of the 16S rRNA gene was amplified as outlined in the Illumina 16S Metagenomic Sequencing Library preparation guide (Illumina). Briefly, DNA was amplified using the primers F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') and products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Takeley, UK). Dual indices and Illumina sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA), according to manufacturer's instructions and a second clean-up step using

the Agencourt AMPure XP system was performed. Library quantification, normalization, pooling and denaturation were performed as per manufacturer's protocol (Illumina). Samples were sequenced at Clinical-Microbiomics, Denmark on the Illumina MiSeq platform using a 2 x 300 bp kit.

Bioinformatics sequence analysis

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

qRT-PCR analysis for bacteria

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

Short chain fatty acids concentration analysis from cecum content

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

Supplemental Results

Study 1

General Effects of Prebiotic Administration

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

16S Compositional Analysis of Cecal Microbiota

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

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

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

Short Chain Fatty Acids (SCFAs)

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

Anxiety-like Behavior

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

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

Only data from Marble burying test (no other behavioral tests) failed normality testing. There was a tendency of prebiotic administration to reduce the number of buried marbles in defensive marble burying test ($\chi^2_{(3)}$=6.951, p=0.073) (Figure 5F).

Depressive-related Behaviors

FOS+GOS administration significantly decreased immobility time in the tail suspension test ($F_{3,39}$=5.249, p<0.01) (Figure 6C). All prebiotic administrations significantly decreased immobility time in the forced swim test ($F_{3,39}$=5.403, p<0.01) (Figure 6D). However, there was no significant effect of prebiotic administration on anhedonia in the female urine-sniffing test. ANOVA did not reveal significant differences between water sniffing time ($F_{3,39}$=0.588, p=n.s.) (Figure 6A) and female urine sniffing time ($F_{3,39}$=2.651, p=n.s.) (Figure 6B).

Social Behavior

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

Cognition

Novel object recognition test

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

Fear conditioning

Prebiotic administration had no effect on acquisition, recall and extinction in fear conditioning test (Figure S6). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252}$=126.877, p<0.001), but no effect of prebiotic administration ($F_{3,36}$=1.906, p=n.s.), or interaction between these two factors ($F_{21,252}$=0.736, p=n.s.) during acquisition depending on context (Figure S6A). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252}$=7.163, p<0.001), but no effect of prebiotic administration ($F_{3,36}$=2.787, p=n.s.), or interaction between these two factors ($F_{21,252}$=0.986, p=n.s.) during memory recall (day 2) depending on context (Figure S6B). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{7,252}$=7.738, p<0.001), but no effect of prebiotic administration ($F_{3,36}$=1.358, p=n.s.), or interaction between these two

factors ($F_{21,252}$=1.162, p=n.s.) during extinction (day 3) depending on context (Figure S6C). During acquisition, depending on cue, repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{5,175}$=69.390, p<0.001), but no effect of prebiotic administration ($F_{3,35}$=0.922, p=n.s.), or interaction between these two factors ($F_{15,180}$=0.573, p=n.s.) (Figure S6D). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{5,180}$=2.294, p<0.05), but no effect of prebiotic administration ($F_{3,36}$=1.490, p=n.s.), or interaction between these two factors ($F_{15,180}$=0.556, p=n.s.) during memory recall (day 2) depending on cue (Figure S6E). Repeated measures two-way ANOVA revealed that there was a significant effect of time ($F_{5,180}$=2.922, p<0.05), but no effect of prebiotic administration ($F_{3,36}$=2.314, p=n.s.), or interaction between these two factors ($F_{15,180}$=0.713, p=n.s.) during extinction (day 3) depending on cue (Figure S6F).

Nociception

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

Locomotor Activity

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

Endocrine Response

Repeated measures two-way ANOVA revealed that prebiotic administration significantly decreased corticosterone levels. There were significant effects of time ($F_{3,108}$=146.535, p<0.001) and prebiotic administration ($F_{3,36}$=3.906, p<0.05), but no interaction between these two factors ($F_{9,108}$=0.944, p=n.s.) (Figure 8A). Area under the curve for corticosterone levels was reduced in prebiotic administration groups ($F_{3,39}$=4.974, p< 0.05) (Figure 8B). Moreover, stress-induced corticosterone levels after 45 min were also reduced in prebiotic treated groups ($F_{3,39}$=3.148, p<0.05) (Figure 8C). Stress-induced hyperthermia was reduced by FOS+GOS administration ($F_{3,39}$=4.666, p<0.01) (Figure 8D) and stress-induced defecation was reduced by GOS and FOS+GOS administrations ($F_{3,39}$=3.729, p<0.05) (Figure 8E).

Hippocampal Gene Expression

Prebiotic administration had a significant effect on expression of several genes in the hippocampus. FOS+GOS administration significantly increased *Bdnf* gene expression in hippocampus ($F_{3,31}$=4.665, p<0.01) (Figure 9A), GABA B1 receptor gene (*Gabbr1*) ($F_{3,31}$=16.887, p<0.001) (Figure 9C) and GABA B2 receptor gene (*Gabbr2*) ($F_{3,31}$=3.606, p<0.05) (Figure 9D). GOS and FOS+GOS administrations reduced mRNA levels of corticotropin releasing factor receptor 1 (*Crfr1*) ($F_{3,31}$=6.917, p<0.01) (Figure 9B). FOS administration increased and FOS+GOS administration decreased NMDA receptor 2A subunit (*Grin2a*) ($F_{3,31}$=8.439, p<0.001) (Figure 9E) but no effect on 2B subunit (*Grin2b*) ($F_{3,31}$=1.743, p=n.s.) (Figure 9F). No changes were observed on several other receptors mRNA levels after prebiotic administration: glucocorticoid receptor (*Nr3c1*) (one-way ANOVA $F_{3,31}$=1.296, p=n.s.) (Figure S7A), mineralocorticoid receptor (*Nr3c2*) (one-way ANOVA $F_{3,31}$=2.004, p=n.s.) (Figure S7B), NMDA receptor subunit 1 (*Grin1*) (one-way ANOVA $F_{3,31}$ = 1.868, p = n.s.) (Figure S7C), cannabinoid receptor type 1 (*Cnr1*) (one-way ANOVA $F_{3,39}$=0.126, p=n.s.) (Figure S7D), GABA receptor Aα2 subunit (*Gabra2*) (one-way ANOVA $F_{3,31}$=0.902, p=n.s.) (Figure S7E) and metabotropic glutamate receptor 4 (mGluR4; *Grm4*) (one-way ANOVA $F_{3,31}$=0.422, p=n.s.) (Figure S7F).

Hypothalamic Gene Expression

Prebiotic administration had a significant effect on expression of several genes in the hypothalamus. FOS+GOS administration significantly reduced mRNA levels of glucocorticoid receptor (*Nr3c1*) in hypothalamus ($F_{3,31}$=3.574, p<0.05) (Figure 10B), but not corticotropin releasing factor receptor 1 (*Crfr1*) ($F_{3,31}$=0.493, p=n.s.) (Figure 10A) or mineralocorticoid receptor (*Nr3c2*) ($F_{3,31}$=0.825, p = n.s.) (Figure 10C).

Tryptophan and Tryptophan Metabolites

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

Brain Monoamines

FOS and FOS+GOS administration increased serotonin levels in the prefrontal cortex ($F_{3,39}$=3.013, p<0.05. FOS+GOS administration decreased dihydroxyphenylacetic acid (DOPAC) levels in brainstem ($F_{3,39}$=4.370, p<0.01). Conversely, GOS and FOS+GOS

administration increased DOPAC levels in frontal cortex ($F_{3,39}$=3.683, p<0.05). (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. A correlation analysis revealed a significantly positive association of acetate concentration and sniffing time in female urine test to measure anhedonic behavior (r=0.42, p<0.01) (Figure 11). For propionate, a negative association was revealed with immobility time in forced swim test (r=-0.61, p<0.001) and tail suspension test (r=-0.39, p<0.05), buried marbles (r=-0.3, p=0.059), rectal temperature increase in stress-induced hyperthermia (r=-0.34, p<0.05), corticosterone elevation 45 min after stress (r=-0.33, p<0.05) or overall corticosterone response (AUC) (r=-0.41, p<0.01). The same effect was also revealed for mRNA levels of mineralocorticoid receptor (r=-0.38, p<0.05), NMDA receptor 2A subunit (r=-0.37, p<0.05), GABA receptor Aα2 subunit (r=-0.3, p<0.01) and a tendency on corticotropin releasing factor receptor 1 (r=-0.32, p=0.076) in hippocampus. A significantly positive association of propionate concentration was revealed with social behavior in resident-intruder test (r=0.33, p<0.05) and sniffing time in female urine test (r=0.37, p<0.05) (Figure 11). Reduced concentrations of iso-butyrate after prebiotic administration had significantly positive association with reduced immobility time in forced swim test (r=0.32, p<0.05), latency to enter into the center of open field test (r=0.34, p<0.05), corticosterone levels 45 min after stress (r=0.35, p<0.05) and mRNA levels of mineralocorticoid receptor in the hypothalamus (r=0.46, p<0.01).

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

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

Organs

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

Behavior

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

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

Acute Stress & Endocrine Response

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

Spleen Cytokine Production After Stimulation with ConA and LPS

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

16S Compositional Analysis of Cecal Microbiota (study 2)

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

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

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

A similar opposite effects were observed in relative abundance of *Alloprevotella*, *Peptococcus, Anaerotruncus, Blautia* where stress increased but stress/FOS+GOS group were similar to control, or sometimes lower (Figure 15B, C, D, F). Moreover, only stress reduced relative abundance of *Allobaculum* ($p<0.01$) (Figure 15E). Low abundances of *Prevotella* and *Enterorhabdus* were observed in both stress groups compared with control group (Figure 15G, H). On the other hand, only stress/FOS+GOS group showed a decrease in *vadinBB60* uncultured bacterium, *Defluviitaleaceae_Incertae Sedis* and *Ruminococcaceae_Incertae Sedis* (Figure 15I, J, M) and an increase in *Parabacteroides* ($p<0.01$) (Figure 15L). S24-7_uncultured made up 46% of relative abundance in stress/FOS+GOS group, whereas only stressed animals

displayed 34%, which was significantly lower ($p<0.05$) (Figure 15K). Similar to the results of the study 1, FOS+GOS administration even under the stress conditions had a tendency to increase relative abundance *Akkermansia* and decrease of *Desulfovibrio* ($p<0.01$) (Figure 15N, O).

Bacterial Concentration by q-PCR in Cecum

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

Supplemental Figures

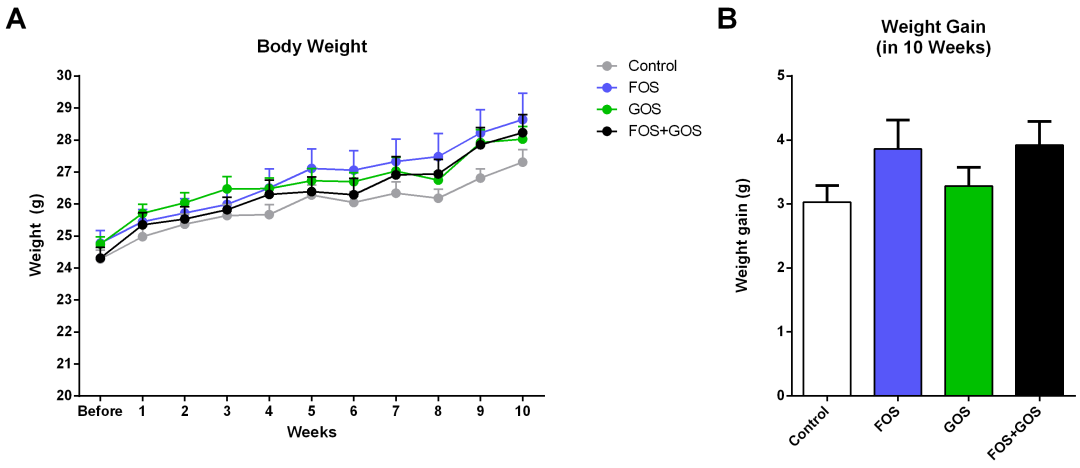

Figure S1. Animal weight during 10 weeks of the experiment. Prebiotics had no effect on animal weight **(A)** or their weight gain after 10 weeks **(B)**. n = 10; data represent mean ± SEM.

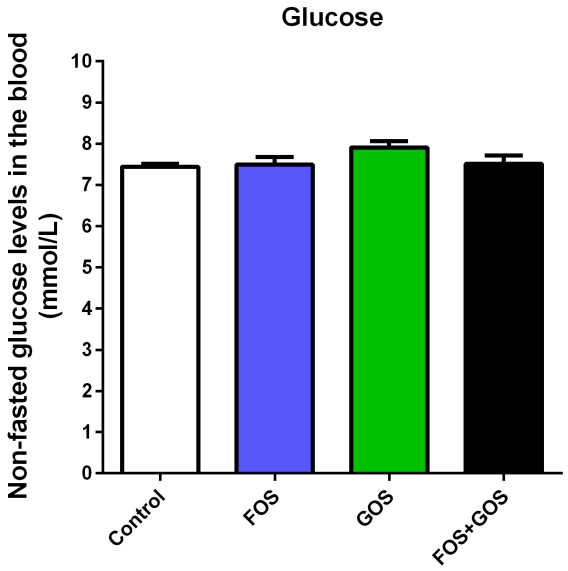

Figure S2. Glucose levels in the blood. Prebiotics had no effect on non-fasted glucose levels in the blood at the end of the experiment. n = 10; data represent mean ± SEM.

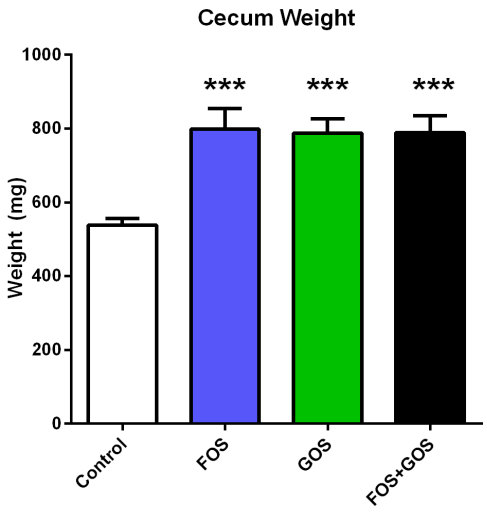

Figure S3. Cecum weights. All prebiotics increased cecum. ***p < 0.001; one-way ANOVA analysis followed by LSD post hoc test; n = 10; data represent mean ± SEM.

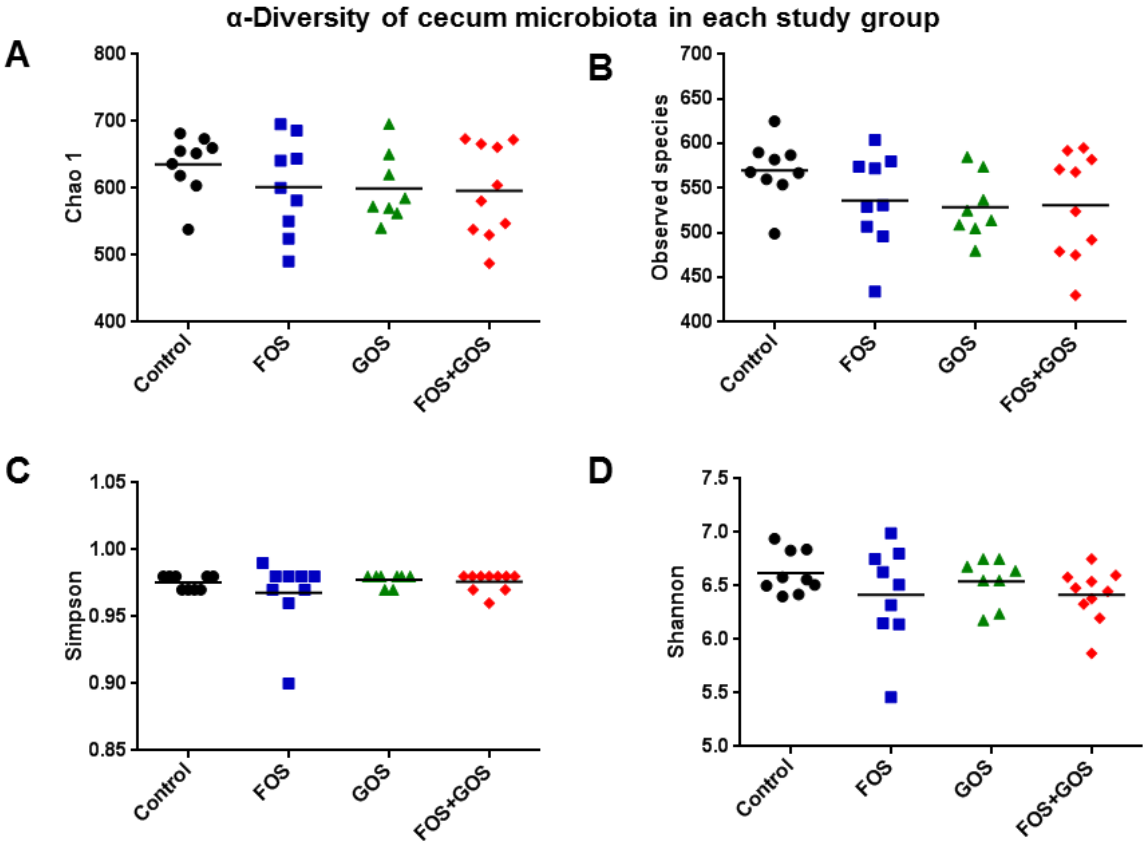

Figure S4. Alfa-diversity metrics of cecal microbiota. Prebiotics had no effect on Chao 1 index, estimator of species richness **(A)**, Observed species, estimator of species richness **(B)**, Simpson index, estimator of diversity and evenness **(C)** and Shannon index, estimator of diversity and evenness **(D)**. n = 8-10 data; represent mean ± SEM.

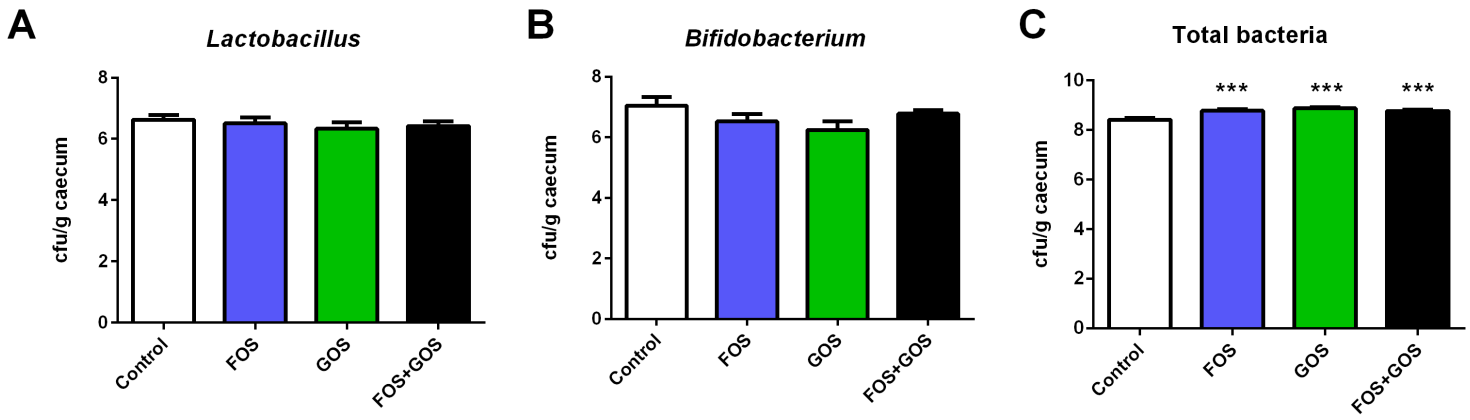

Figure S5. Bacterial concentration by q-PCR in cecum. Absolute quantification of *Lactobacillus* spp. levels **(A)**, *Bifidobacterium* spp. levels **(B)** and total bacteria levels **(C)** in cecum. One-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: ***$p<0.001$; n = 10; Mean ± SEM of CFU/g of cecum is depicted.

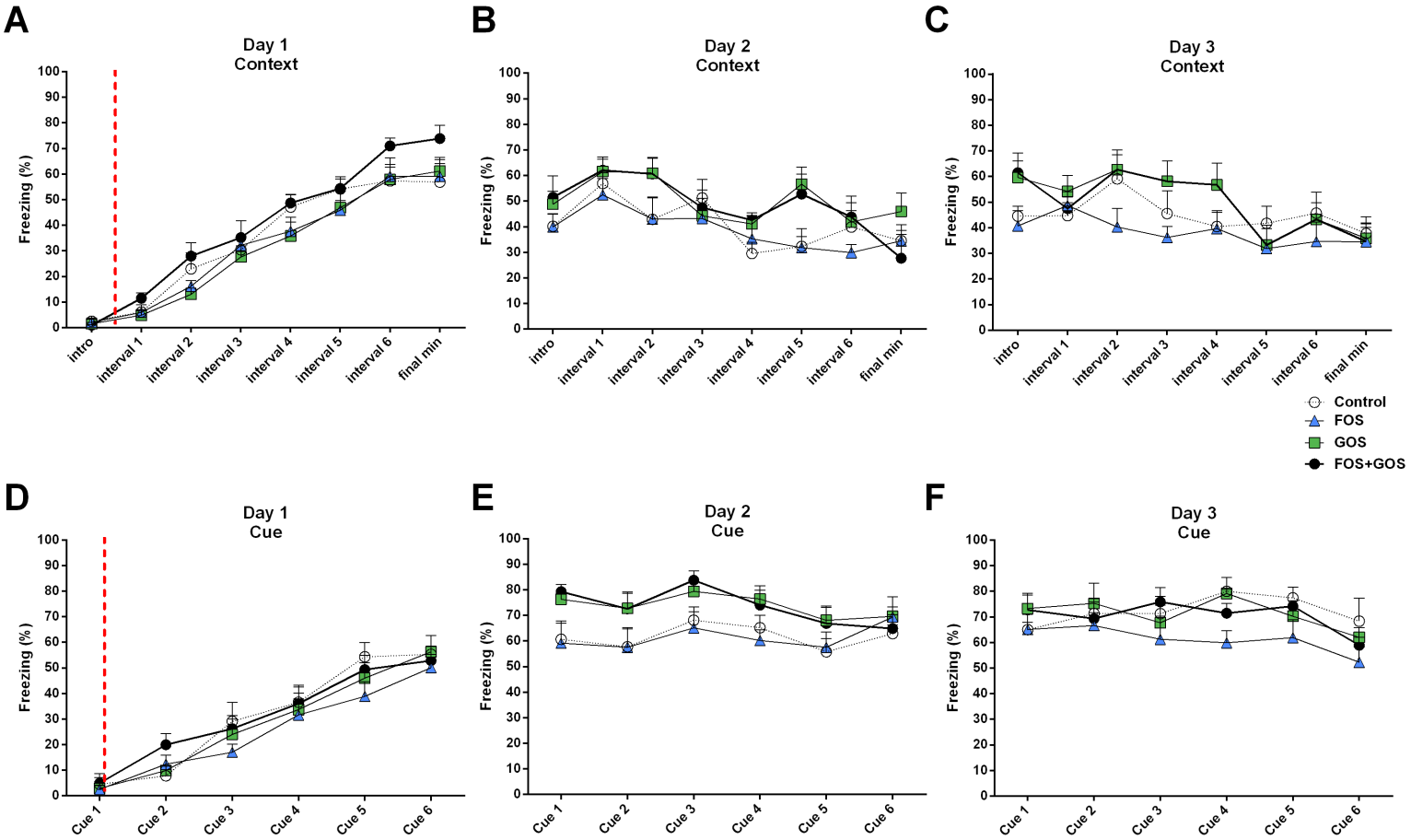

Figure S6. Fear conditioning. There was no any effect of prebiotic administration on fear-related behaviors. On day 1, analysis revealed no differences in the learning curves among groups towards context **(A)** or cues **(D)**. On day 2 (memory testing), no differences were found on memory towards context **(B)** or cues **(E)**. On day 3 (memory extinction), no differences were observed among the experimental groups towards context **(C)** or cues **(F)**. Repeated measures two-way ANOVA analysis; n = 10; data represent mean ± SEM. The first electric foot shock is marked by the red line.

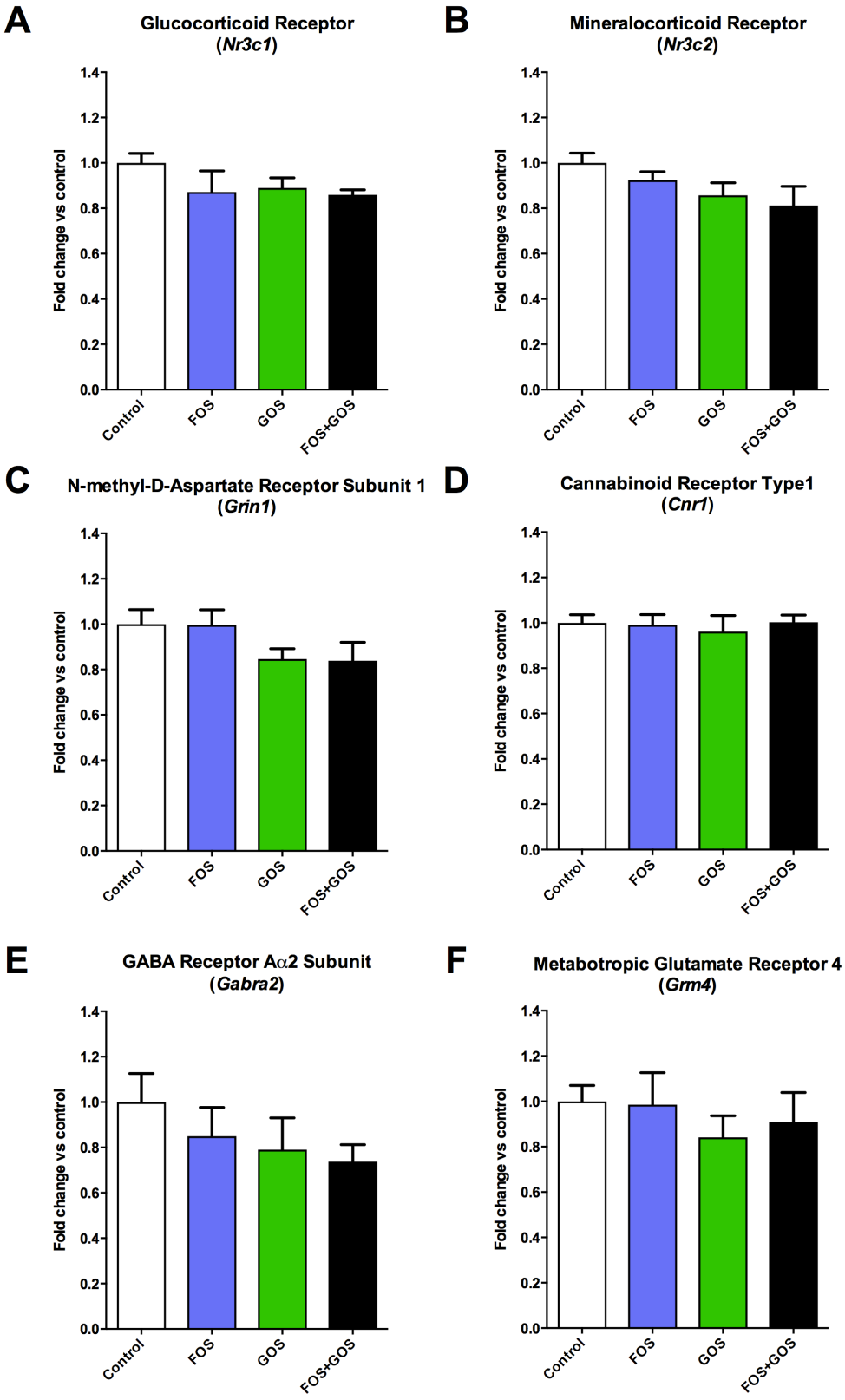

Figure S7. Hippocampal gene expression. Prebiotics had no effect on mRNA levels of glucocorticoid receptor (*NR3C1*) **(A)**, mineralocorticoid receptor (*NR3C2*) **(B)**, NMDA receptor subunit 1 **(C)**, cannabinoid receptor type 1 (*CNR1*) **(D)**, GABA receptor Aα2 subunit (*GABRA2*) **(E)** and metabotropic glutamate receptor 4 (*GRM4*) **(F)**. One-way ANOVA analysis; n = 8-10; data represent mean ± SEM.

Figure S8

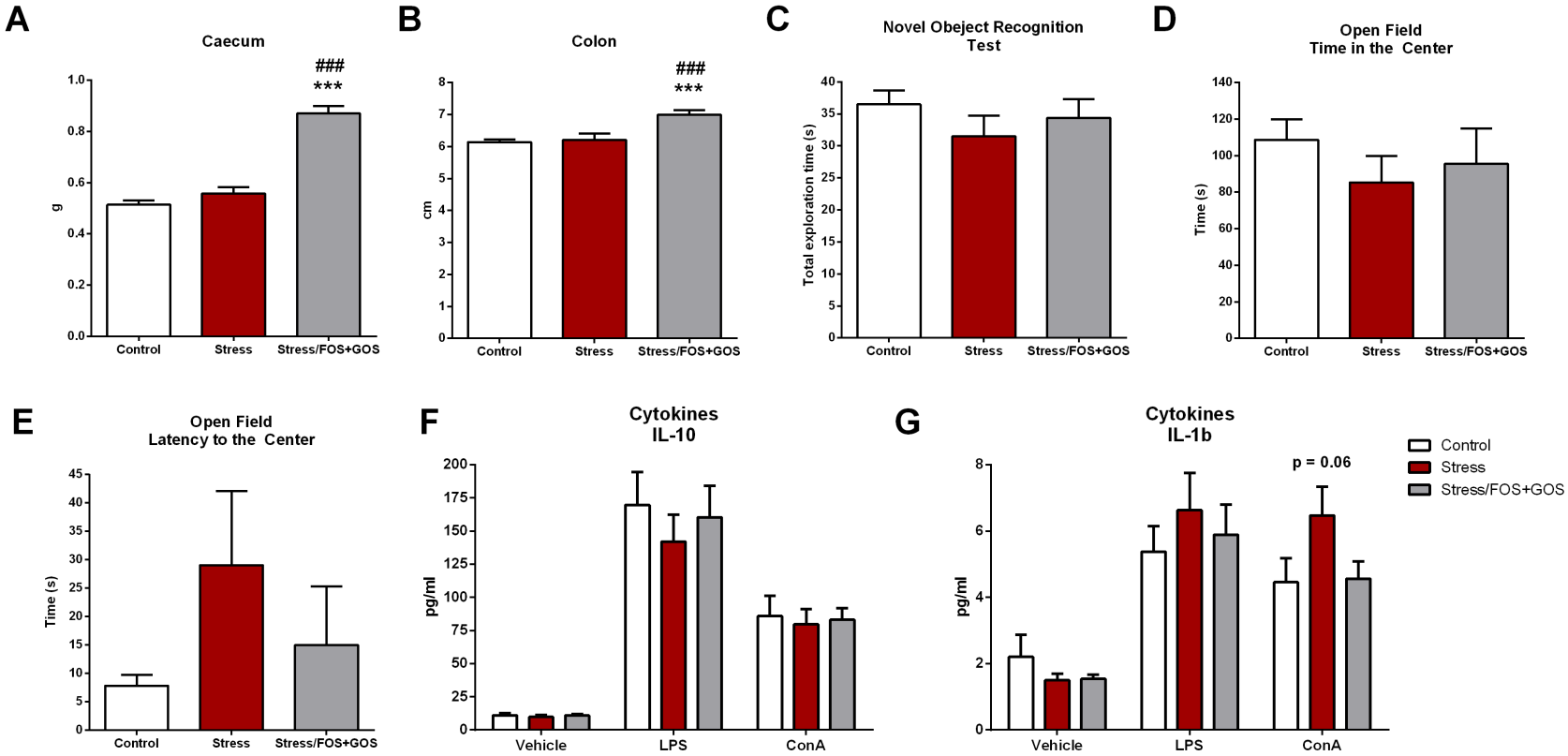

Figure S8. Study 2. Stress/FOS+GOS animals had heavier cecum **(A)** and longer colon **(B)** compared with other groups. There was no any difference in total exploration time in novel object recognition test **(C)**, time in the center **(D)** or latency to the center **(E)** of open field test. Spleen cytokine production without stimulation (vehicle) or following stimulation with lipopolysaccharide (LPS) and concanavalin A (ConA). No differences among the group on concentrations of cytokine IL-10 **(F)**. Stress group presented a tendency to increase levels of released IL-1b after ConA stimulation **(G)**. One-way or two-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: ***p<0.001 comparing to the control group; ###p<0.001 comparing to the stress group; n = 9-10; Mean ± SEM.

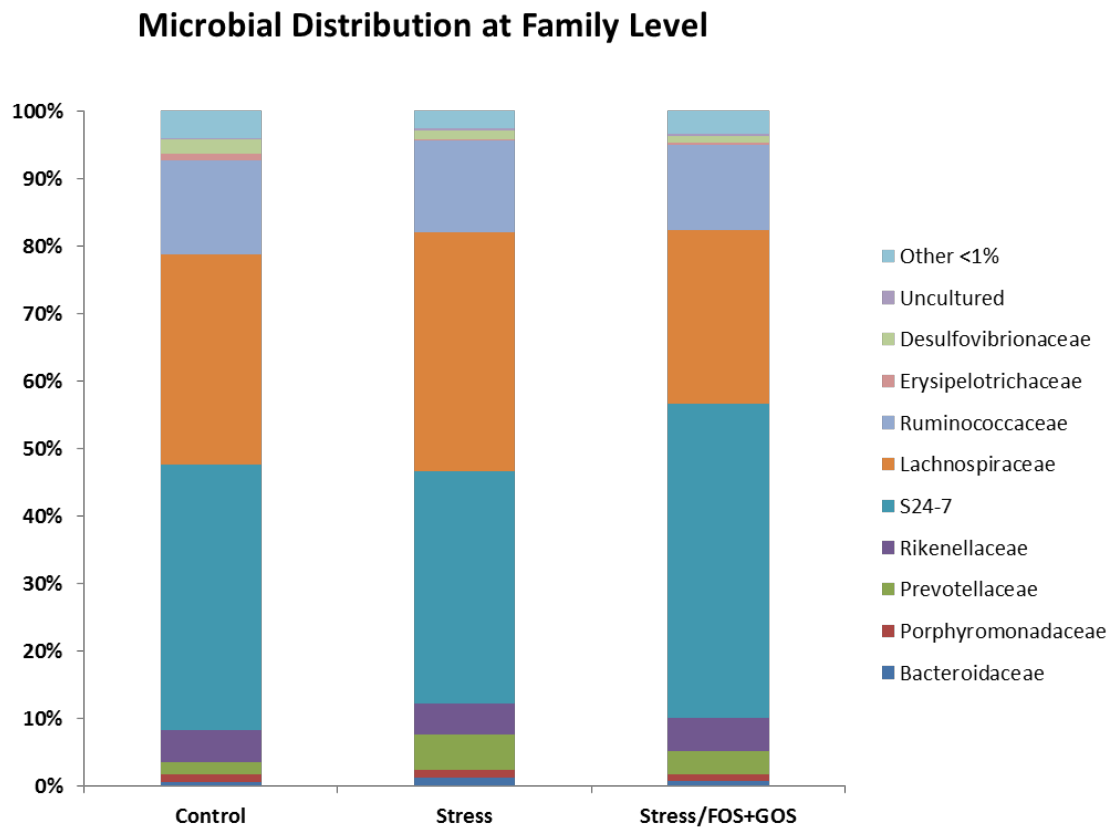

Figure S9. Microbial distribution at family level in study 2. Relative abundances of family level distributions of cecum microbiota in the three mice groups of the study. All families comprising less than 1% of the total abundance were combined into the "Other" category.

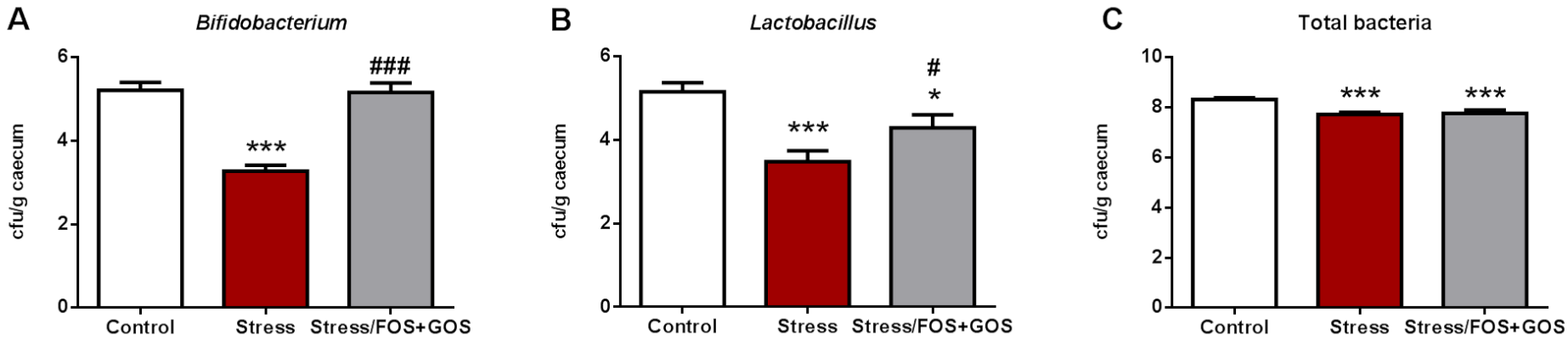

Figure S10. Bacterial concentration by q-PCR in cecum in study 2. Absolute quantification of *Lactobacillus* spp. levels **(A)**, *Bifidobacterium* spp. levels **(B)** and total bacteria levels **(C)** in cecum. One-way ANOVA analysis followed by LSD post hoc test was done. Statistical significances showed: **p*<0.05; ****p*<0.001 comparing to the control group; #p<0.05; ###p<0.001 comparing to the stress group; n = 9-10; Mean ± SEM of CFU/g of cecum is depicted.

Table S1. Relative abundance at genus level in cecal microbiota.

		CONTROL		FOS		GOS		FOS+GOS	
	p value	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Acetitomaculum	0.000	0.08	0.06±0.11	0.00	0±0	0.00	0.00±0.01	0.00	0.00±0.01
Peptococcaceae_Unclassified	0.000	0.20	0.17±0.26	0.80	0.59±1.46	1.23	0.93±1.58	0.99	0.67±1.09
Enterorhabdus	0.000	0.63	0.48±0.75	0.09	0.07±0.14	0.14	0.09±0.17	0.11	0.06±0.16
Ruminococcaceae_Unclassified	0.000	3.55	2.69±4.14	5.29	5.05±5.34	7.32	6.25±7.84	5.55	4.03±6.90
Bacteroides	0.000	0.44	0.31±0.58	1.54	1.05±1.74	1.30	0.68±2.83	1.65	0.98±2.21
Oscillibacter	0.001	0.28	0.20±0.40	0.87	0.62±1.87	0.76	0.53±0.99	0.49	0.39±0.66
Turicibacter	0.001	1.52	0.76±2.34	0.00	0.00±0.02	0.01	0.00±0.02	0.00	0.00±0.025
Ruminococcus	0.001	1.10	0.69±2.12	0.24	0.17±0.39	0.43	0.13±1.03	0.31	0.06±0.47
Family XIII_Unclassified	0.001	0.04	0.03±0.06	0.04	0.03±0.05	0.02	0.02±0.02	0.04	0.04±0.04
Coprococcus	0.003	0.17	0.12±0.26	0.04	0.03±0.08	0.07	0.04±0.08	0.08	0.04±0.10
Bifidobacterium	0.006	4.57	1.27±7.11	0.47	0.21±1.31	0.21	0.09±0.32	0.41	0.24±0.83
Lactobacillus	0.006	1.91	1.41±3.61	1.54	0.87±2.43	0.63	0.10±1.31	0.59	0.29±1.33
Desulfovibrio	0.007	1.88	1.45±2.33	0.83	0.49±1.23	1.28	0.53±1.63	0.93	0.63±1.27
Parabacteroides	0.012	0.09	0.04±0.14	0.16	0.12±0.46	0.42	0.13±0.64	0.33	0.19±0.46
Erysipelotrichaceae_Unclassified	0.018	0.02	0.01±0.03	0.02	0.01±0.03	0.02	0.01±0.05	0.05	0.03±0.10
Akkermansia	0.019	0.11	0.01±4.47	0.53	0.33±2.13	1.06	0.60±4.71	6.09	3.65±7.77
Allobaculum	0.020	3.53	0.77±6.00	0.41	0.09±0.53	0.71	0.39±1.00	1.27	0.44±2.41
Other	0.032	4.65	4.31±5.76	4.97	4.44±6.23	6.41	6.17±6.84	5.19	4.19±6.51
Christensenella	0.037	0.01	0.00±0.01	0.00	0.00±0.01	0.01	0.00±0.01	0.01	0.01±0.01
Rikenella	0.047	0.19	0.16±0.31	0.11	0.07±0.14	0.07	0.01±0.19	0.12	0.06±0.25
Lachnospiraceae_Unclassified	0.054	28.95	24.90±33.74	36.46	33.00±43.25	33.75	32.60±39.73	31.20	29.96±35.94
Roseburia	0.061	1.42	1.04±2.45	0.56	0.25±1.61	0.63	0.10±1.08	0.35	0.16±1.44
Prevotella	0.294	0.57	0.29±0.82	0.87	0.60±1.70	0.72	0.41±1.23	0.81	0.38±1.36
S24-7_Unclassified	0.306	29.16	23.66±36.99	31.97	28.17±36.90	31.33	29.49±35.27	36.64	32.02±37.94

		CONTROL		FOS		GOS		FOS+GOS	
	p value	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Odoribacter	0.412	0.56	0.23±1.14	0.70	0.41±0.95	0.81	0.52±1.95	0.70	0.32±0.84
Alistipes	0.472	5.84	2.93±9.33	3.36	2.60±7.17	5.31	3.23±7.50	3.68	3.20±5.69
RC9_gut_group	0.677	1.19	0.66±1.78	0.72	0.35±1.63	0.68	0.18±1.07	0.74	0.28±1.52

All genera comprising less than 1% of the total abundance in each group (whether classified or not) were combined into the “Other” category (except the genus with significant differences among groups). The non-parametric Kruskal-Wallis test was used to analyse the differences among the mice groups. Statistical significance was accepted at $p < 0.05$.

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