Extracellular vesicles from Gram-positive and Gram-negative probiotics remediate stress-induced depressive behavior in mice

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Abstract

Chronic stress causes maladaptive changes in the brain that lead to depressive behavior. In the present study, we investigate whether chronic stress alters gut microbiota and whether specific mediators of probiotics remedies stress-induced maladaptive changes in the brain. Mice treated with daily 2-h restraint for 14 days (CRST) exhibit depressive-like behavior. Sequence readings of 16S rRNA genes prepared from fecal samples taken from CRST-treated mice suggest that chronic stress induces gut microbiota changes that are pronounced 14 days after the last restraint, relative to those that occur in the 14-day stress phase. The genus *Lactobacillus* is one such microbiota substantially changed following chronic stress. In contrast, post-stress treatment with extracellular vesicles (EVs) derived from the Gram-positive probiotic *Lactobacillus plantarum* are sufficient to ameliorate stress-induced depressive-like behavior. Interestingly, EVs from the Gram-positive probiotic *Bacillus subtilis* and EVs from the Gram-negative probiotic *Akkermansia muciniphila* also produce anti-depressive-like effects. While chronic stress decreases expression of MeCP2, Sirt1, and/or neurotrophic factors in the hippocampus, EVs from the selected probiotics differentially restore stress-induced changes of these factors. These results suggest that chronic stress produces persistent changes in gut microbiota composition, whereas EVs of certain probiotics can be used for treatment of stress-induced maladaptive changes.

Introduction

Chronic stress is a potent environmental risk factor for depressive behavior. Stress responses proceed with the activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in glucocorticoid (GC) release into blood. When stress-induced GC release is excessive or prolonged, GC-dependent maladaptive changes occur in the brain, such as diminished sensitivity to GC in the limbic system and abnormal HPA axis activation [1, 2]. Furthermore, chronic stress and stress-induced excesses of GC induce profound genomic responses that exceed the homeostatic range in the brain. Those changes can cause dendritic and spine atrophy in the limbic system, and produce neural circuit activity changes [3]. These stress-induced maladaptive changes in the limbic system are responsible for behavioral disturbances such as cognitive impairment and mood disorders [3, 4].

Recent studies have reported that the gut microbiota are closely associated with the pathophysiology of stress-related mood disorders [5, 6, 7, 8]. Patients with depression have altered gut microbiota composition [9], whereas supplementation with probiotics results in anti-depressant effects in depression patients [10]. Recently, several laboratories have reported that gut microbiota changes are involved in depressive-like behaviors in animal models of depression. Mice exposed to chronic unpredictable mild stress (CUMS) had decreased levels of *Lactobacillus*, whereas administration of *Lactobacillus reuteri* (ATCC 23272) during CUMS treatment attenuated despair-like behavior [11]. Transferring fecal microbiota prepared from the mice subjected to 7 weeks of CUMS to healthy recipients produced despair-like behavior, decreased neurogenesis in the hippocampus, and impaired the anti-depressant effects of fluoxetine [12]. Supplementation with *Lactobacillus casei* also produced anti-depressant-like effects in CUMS-treated rats [13]. These studies support the idea that gut microbiota changes could affect

depressive-like behavior, and certain probiotics like *Lactobacillus* could be beneficial for treatment of stress-induced depressive behavior. However, the mechanisms by which probiotics affect brain functions and produce anti-depressant effects are not clearly understood.

Several mechanisms have been proposed to explain how gut microbiota communicate with the brain [7, 14]. The neural mechanism involving vagus nerve innervation of gut epithelial cells has been suggested, and immune responses stimulated by cytokines and peptide hormones, such as ghrelin, somatostatin, cholecystokinin, gastrin, GLP-1, and peptide YY, released from gut microbiota-stimulated enteroendocrine cells have been implicated. Neuronal effects of bacterial metabolites including dopamine, GABA, tryptophan or 5-HT precursors have been demonstrated in other studies. Microbial by-products including short-chain fatty acids, carbohydrates, and bile acids, and/or bacteria-derived extracellular vesicles (EVs) have been proposed as mediators of gut microbiota [7, 15, 16, 17]. Of those proposed divergent mechanisms, the EV-mediated mechanism has been recently the focus of several studies [17]. Both Gram-negative and Gram-positive bacteria secrete EVs, which contain bacterial genomic DNA, RNA, proteins including various enzymes, and other metabolites. Bacteria are believed to employ EVs to communicate with host organisms or other organisms [17]. For example, EVs from *Akkermansia muciniphila* contain cargo contents that increase phosphorylation of AMPK and protect against LPS-induced intestinal permeability changes [18], and decrease colitis-induced inflammation [19]. EVs from *Lactobacillus plantarum* increase BDNF expression in cultured hippocampal cells [20]. Thus, EVs derived from specific bacteria could change cellular function in the brain and exert physiological effects on the host body, although the detailed mechanisms by which EVs from different bacteria and associated key components act on the host body remain to be investigated.

In the present study, we investigated the temporal profiles of changes to the gut microbiota composition during and after chronic stress, and we sought to understand whether administration of EVs derived from Gram-negative and Gram-positive probiotics could affect stress-induced maladaptive changes in the brain. We also examined the ability of specific EVs produce anti-depressant-like effects.

Materials And Methods

Animals

Seven-week old male C57BL6 mice were purchased from Daehan BioLink (Eumsung, Chungbuk, Republic of Korea). Upon arrival, mice were grouped and housed in pairs in standard clear plastic cages in a temperature (23–24°C)- and humidity (50–60%)-controlled environment under a 12 h light/dark cycle (lights on at 07:00–19:00 h), with ad libitum access to water and food. Animals were handled in accordance with the animal care guidelines of Ewha Womans University. Restraint procedures and EV treatment in this study were approved by the Ewha Womans University Animal Care and Use Committee (IACUC 15 – 012).

Chronic restraint stress and fecal collection

Mice were exposed to restraints as described previously [21, 22]. In brief, mice were individually placed in a well-ventilated, 50-ml polypropylene conical tube, with the head of the animal orienting toward the conical side by pushing the backside of the animal with a cut piece of a 15-ml conical tube. Thereafter, animals were restrained in this manner for 2-h daily starting at 10 a.m. After each session of restraint, they were returned to their home cages and housed with their cage mate with free access to food and water. This procedure was repeated each day for 14 days or other indicated time. Control mice housed in pairs were maintained in home cages without disturbance.

To collect fecal samples, mice were placed in an empty autoclaved cage with no bedding. The first one to two fecal pellets per animal were collected in a 1.5 mL microcentrifuge tube using a sterile toothpick, and were immediately stored at -80°C until shipping to MD Healthcare Inc. for analysis.

Analysis of 16S ribosomal RNAs and taxonomic assignment

On-bead emulsion-based PCR amplification of isolated bacterial DNA

Bacterial DNA isolation and emulsion-based PCR (emPCR) were carried out as described previously [23, 24]. Bacterial DNAs were extracted from fecal samples using a PowerWater® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). PCR products were used to construct a single-stranded DNA library with adaptors for each sample using the 454 sequencing library preparation process (Roche, Branford, CT, USA). Constructed libraries were quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA), and were amplified using the 454 GS-FLX system and emulsion-based PCR (emPCR) Kit (Roche). In brief, single-stranded DNA libraries with adaptors were immobilized onto DNA capture beads, which were added to a mixture of amplification mix and oil, which were then vigorously shaken on a TissueLyser II (Qiagen, Valencia, CA, USA) to create "micro-reactors" containing both DNA capture beads and PCR amplification reagents in water-in-oil emulsions. The emulsion was dispensed into a 96-well plate and PCR amplification was carried out according to the manufacturer's instructions. Each PCR reaction contained 20 ng of DNA in 50 μl of PCR reaction volume. The universal primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 518R (5'-WTTACCGCGGCTGCTGG-3') were used to amplify the variable regions 1 to 3 (V1-V3) of 16S rRNA genes. PCR was carried out using the FastStart High Fidelity PCR System (Roche) under the following conditions: 94°C for 3 min followed by 35 cycles at 94°C for 15 sec, 55°C for 45 sec and 72°C for 1 min, followed by a final elongation step at 72°C for 8 min. After emPCR, PCR products were purified using an Agencourt AMPure Bead kit (Beckman Coulter Inc., Brea, CA), and DNA concentration and quality was quantified using the Picogreen method (Invitrogen, Carlsbad, CA, USA).

DNA Sequencing of variable regions of 16S ribosomal DNA

DNA sequencing was carried out by Macrogen Inc. (Seoul, South Korea) using a Genome Sequencer FLX + System (Roche, Basel, Switzerland), as described previously [23]. The emulsion containing emPCR

products was chemically lysed and the beads carrying amplified DNA libraries were recovered and washed by filtration. Biotinylated primer A (complementary to adaptor A) was used to purify streptavidin-coated magnetic beads. The amplified single-stranded DNAs were separated from magnetic beads by melting double-stranded amplification products. The sequencing primer was then annealed to the amplified single-stranded DNA. The beads carrying amplified single-stranded DNA were counted with a Particle Counter (Beckman Coulter). Each sample was loaded on the 75 mm PicoTiter plate (Roche Diagnostics) fitted with an 8-lane gasket.

Analysis of sequencing reads of 16S rRNAs and taxonomic assignment

Analysis of sequencing reads of 16S rRNAs and taxonomic assignment were carried out as described previously [23]. Sequencing reads with the lengths greater than > 300 bp and average Phred scores > 20 were selected. Operational Taxonomy Units (OUTs) were assigned using the sequence clustering algorithm UCLUST, and taxonomy assignment was achieved using QIIME by searching the 16sRNA sequence database of GreenGenes 8.15.13 using the following similarity cut-offs: species, > 97% similarity; genus, > 94% similarity; family, > 90% similarity; order, > 85% similarity; class, > 80% similarity; and phylum, > 75% similarity.

Preparation of EVs from Lactobacillus plantarum, Bacillus subtilis, **and** Akkermansia muciniphila.

Bacterial culture and EV isolation were carried out as described previously [20, 25, 26]. In brief, *Lactobacillus plantarum* (KCTC 11401BP) was cultured in MRS broth (MB Cell, CA, USA) for 18 h at 37°C with gentle shaking (150 rpm). When the optical density of the cultures at 600 nm reached 1.0, bacteria were pelleted by centrifugation at 10,000 ×g for 20 min. The supernatant was passed through a 0.22-μm bottle-top filter (Corning, NY, USA) to remove remaining cells or cell debris. The filtrate was concentrated using a MasterFlex pump system (Cole-Parmer, IL, USA) and a 100-KDa Pellicon 2 Cassette filter membrane (Merck Millipore, MA, USA), and passed through a 0.22-μm bottle-top filter again. EVs were pelleted from the resulting filtrate by ultracentrifugation at 150,000 x g for 3 h at 4°C. Pellets were washed and resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) and resuspended EV fractions of *Lactobacillus plantarum (Lac-EV)* were stored at − 80°C until use.

Bacillus subtilis (KCTC 3135) was grown in brain heart infusion (BHI) media (Becton–Dickinson, Franklin Lakes, NJ) for 12 h at 37°C as described previously [27]. Microscopic analysis indicated that sporulation had begun at 17 h, but not at 12 h. *Bacillus subtilis* cells cultured for 12 h, which had not undergone sporulation, were pelleted by centrifugation at 10,000 x g for 20 min and the supernatant was retained. *Bacillus subtilis* EVs (*Bac*-EV) were collected from the supernatants as described above, and collected EVs were stored at − 80°C until use.

Akkermansia muciniphila (KCTC #15667) was cultured under anaerobic conditions (99% N_2 at 37°C) until the optical density at 600 nm reached 1.5, as described previously [28, 29]. Bacterial cultures were

pelleted at 10,000 ×g for 20 min and the supernatant was collected. EVs were isolated from the supernatant as described above, and isolated *Akkermansia muciniphila* EVs (*Akk*-EV) were stored at −80°C until use.

Administration of EVs to mice.

EVs were administered to mice via the intraperitoneal route as described previously [20]. *Lac*-EV, *Bac*-EV or *Akk*-EV were intraperitoneally injected, each with 6 μg in 100 μl of injection volume, for the indicated days.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was carried out as described previously [20, 30]. Briefly, hippocampal tissues were homogenized using pellet pestles (Z359971, Sigma-Aldrich) in TRI-zol reagent (15596-018, Invitrogen), and total RNA was isolated from the homogenates. Two μg of total RNA was converted to cDNA using a reverse transcriptase system (Promega, Madison, WI, USA).

qPCR reaction contained 4 μl of 1/8 diluted cDNA, 10 μl of 2X iQTM SYBR Green Supermix (Bio-Rad Laboratories, Foster City, CA, USA), and 1 μl each of 5 pmol/μl forward and reverse primers in 20 μl of volume. The qPCR reaction was carried out using the CFX 96 Real-Time PCR System Detector (Bio-Rad Laboratories). Transcript levels were normalized relative to *Gapdh* and *L32* levels.

Each primers used in this study were: t*Bdnf* (total form), forward 5’-TGGCTGACACTTTTGAGCAC-3’ and reverse 5’-GTTTGCGGCATCCAGGTAAT-3’; *Bdnf1*, forward 5’-CCTGCATCTGTTGGGGAGAC-3’ and reverse 5’-GCCTTGTCCGTGGACGTTTA-3’; *Bdnf4*, forward 5’-CAGAGCAGCTGCCTTGATGTT-3’ and reverse 5’-GCCTTGTCCGTGGACGTTTA-3’; *Nt3*, forward 5’-TACTACGGCAACAGAGACG-3’ and reverse 5’-GTTGCCCACATAATCCTCC-3’; *Nt4/5*, forward 5’-AGCGTTGCCTAGGAATACAGC-3’ and reverse 5’-GGTCATGTTGGATGGGAGGTATC-3’; *Ngf*, forward 5’-AGCATTCCCTTGACACAG-3’ and reverse 5’-GGTCTACAGTGATGTTGC-3’; *Hdac2*, forward 5’-GGGACAGGCTTGGTTGTTTC-3’ and reverse 5’-GAGCATCAGCAATGGCAAGT-3’; *MeCP2*, forward 5’-ACAGCGGCGCTCCATTATC-3’ and reverse 5’-CCCAGTTACCGTGAAGTCAAAA-3’; *Sirt1*, forward 5’-GATCCTTCAGTGTCATGGTTC-3’ and reverse 5’-ATGGCAAGTGGCTCATCA-3’; *Gapdh*, forward 5’-AGAAGGTGGTGAAGCAGGCATC-3’ and reverse 5’-CGAAGGTGGAAGA GTGGGAGTTG-3’; *L32*, forward 5'-GCTGCCATCTGTTTTACGG-3' and reverse 5'-TGACTGGTGCCTGATGAACT-3'.

Behavioral tests

The behavioral tests were performed as described previously [21, 22]. Mice were allowed to adapt to the behavior testing room for a minimum of 30 min prior to the start of the test. All behavioral tests were monitored with a video tracking system (SMART; Panlab, Barcelona, Spain) and/or a webcam recording system (HD Webcam C210, Logitech, Newark, CA, USA).

Sociability test

The sociability test was carried out as described previously [21, 22]. Briefly, an open field (40 × 40 cm^2) was partitioned by a wall (20-cm wide and 20-cm high) at the center point to prepare a U-shaped two-choice field. Circular grid cages (12 cm in diameter × 33 cm height) were placed on each side of the U-shaped two-choice field. A subject mouse was allowed to freely explore this space placed with an empty circular grid cage on each side for 5 min and was then returned to the home cage. After 10 min, a social target was loaded into a circular grid cage on one side of the field and the habituated subject mouse was placed in the center of the U-shaped two-choice field. The subject mouse was allowed to explore both fields for 10 min while the trajectory of the mouse's movements and time spent in each field was recorded by a video tracking system. Social targets were the same age and sex as the subject mice. The position of the field placed with the circular grid cage containing a social target and the position of the field containing an empty grid cage were defined as the target field and non-target field, respectively.

Tail suspension test

The tail suspension test (TST) was carried out as described previously [21, 22]. Mice were suspended individually by fixing their tails with adhesive tape to the ceiling of a shelf 50 cm above a bottom floor, and were recorded with a webcam recording system for 6 min. Cumulative immobility time was measured. Immobility was defined as the time the animal spent suspended with all limbs motionless.

Forced swim test

The forced swim test (FST) was performed as described previously [21, 22]. Mice were placed in a Plexiglas cylinder (15 cm in diameter × 27 cm height) containing water at a temperature of 24°C and a depth of 15 cm. Mice were placed in the cylinder for 6 min and the cumulative immobility time was measured for the last 5 min. Immobility was defined as time of the animal spent floating with all limbs motionless. The performance during the test was recorded using a webcam recording system and then analyzed.

Statistical analysis

Two sample comparison was carried out using the Student t-test. Multiple comparisons were performed by one-way ANOVA followed by the Newman-Keuls post hoc test or two-way ANOVA followed by the Bonferroni post hoc test. All data are represented as mean ± SEM, and statistical significance was accepted at the 5% level.

Results

Metagenome analysis revealed that gut microbiota composition was markedly changed during and after repeated exposure to stressors.

Mice treated with daily 2-h restraint for 14 days, called chronic restraint stress (CRST), exhibit depressive-like behaviors that last longer than 3 months [31, 32]. CRST-induced behavioral deficits are produced by stress-induced maladaptive changes in the hippocampus and neuroendocrine systems [33]. In the present

study, we expanded upon previous investigations into the method by which CRST produces changes in gut microbiota.

After they were purchased from a local vendor and habituated in our animal room for 5 days, C57BL6/J mice were randomly divided into two groups: control and stress groups. Mice assigned to stress group were treated with daily 2-h restraint for 14 days and stools were collected at day 1, day 14, and post-stress day 14. Stools were collected from control mice in parallel (Fig. 1A). Bacterial DNA was isolated from fecal samples and used to obtain DNA sequence readings of variable regions of 16S ribosomal RNA (rRNA) genes. This led to the identification of 21,811 and 19,148 operational taxonomic units (OTUs) in the control and CRST groups, respectively. Expected sample taxonomic richness increased with the number of DNA sequence reads in both groups (Fig. 1B). The microbiota identified in CRST and control groups consisted of 22 OTUs at the phylum level, 43 OTUs at the class level, 79 OTUs at the order level, 176 OTUs at the family level, and 376 OTUs at the genus level. Analysis of the relative occupancy of the most abundant 15 phyla with an occupancy $\geq 0.1\%$, which counted for $> 95\%$ of identified OTUs, indicated that the CRST group exhibited significant changes in their % composition relative to the control group over the test period, with a particularly dramatic change at post-stress day 14 (Fig. 1C).

The relative occupancy of the top 15 phyla within a group at each time point was calculated and converted to % composition of the mean value and the mean difference between control group and CRST group, expressed as a standard deviation unit (z score) at each time point. On post-stress day 14, the occupancy of p_Bacteroidetes decreased from 36.90 to 15.33% (-1.95 x z-score), the occupancy of p_Tenericutes decreased from 0.17 to 0.00% (-3.38 x z), and the occupancy of an unassigned phylum decreased from 7.95 to 3.32% (-5.28 x z) (Fig. 1D). In contrast, the occupancy of p_Actinobacteria increased from 4.11 to 12.76% (6.14 x z), the occupancy of p_TM7 increased from 0.07 to 1.50% (56.63 x z), and the occupancy of p_Cyanobacteria increased from 0.11 to 1.09% (18.6 x z) (Fig. 1D).

Chronic stress caused dramatic changes in gut microbiota composition at the genus level.

We next analyzed stress-induced changes in gut microbiota at the genus level. The identified OTUs contained 104 genus members with a relative occupancy of $\geq 0.1\%$ in control or CRST groups at any of the three time points. The most abundant top 10, 20 and 30 genus members in the control group comprised total 88.7, 96.8 and 98.6%, respectively, of those identified OTUs at stress day 1; 91.8, 96.7 and 98.5%, respectively, at stress day 14; and 91.7, 97.2 and 98.7%, respectively, at post-stress day 14 (Fig. 2; Supplemental Table 1). In contrast, those top 10, 20 and 30 genus members in the CRST group comprised 80.8, 92.8 and 96.1%, respectively, of those identified OTUs at stress day 1; 88.0, 92.8 and 96.0%, respectively, at stress day 14; and 22.4, 39.4 and 41.1%, respectively, at post-stress day 14 (Fig. 2; Supplemental Table 1). Thus, the relative abundance of the most abundant top 10–30 genus members in the control group was not changed within a month of the test period, whereas the relative abundance of the same top 10–30 genus members in the CRST group were reduced slightly during the stress period and severely in the post-stress period.

Analysis of the relative occupancy of identified OTUs indicated that 34 genus members had a relative occupancy of ≥ 0.1% in control or CRST groups at any of the three time points. Of those genus members, 6 members were significantly upregulated and 6 members were downregulated in the CRST group over time (time factor, two-way ANOVA), whereas the remaining 22 members were statistically insignificantly changed or unchanged over the test period (Fig. 3; Supplemental Table 1).

Of those altered genus members, the relative occupancy of an unclassified member of *f__S24-7 (f__S24-7;g__)*, an unassigned bacterium, an unclassified member of *o_Clostridiales;f_;g_, g__Adlercreutzia* and *g__Desulfovibrio* decreased in the CRST group at post-stress day 14, and the relative occupancy of *g__Lactobacillus* decreased in the CRST group at all three time points examined. In contrast, the relative occupancy of *g__Bacteroides, f__Enterobacteriaceae;g__, f__Comamonadaceae;g__, g__Rhodococcus, g__Pseudomonas*, and *g__Enhydrobacter* increased in the CRST group at post-stress day 14 (Fig. 3; Supplemental Table 1). The relative occupancy of *g__Akkermansia*, *g__Lactococcus, f__Aerococcaceae;g_, g__Ruminococcus* and other unclassified member of *g__Ruminococcus, g__Faecalibacterium, g__Acinetobacter, o__Streptophyta;f__;g__, g__Parabacteroides, g__Propionibacterium*, and *g__Blautia* appeared to be changed in the CRST group at post-stress day 14, although these differences were not statistically significant (Fig. 3; Supplemental Table 1).

Administration of EVs from three different probiotics to CRST mice induced expression of neurotrophic factors in the hippocampus.

Amongst the taxonomic members of microbiota whose relative abundance was changed by chronic stress, the stress-dependent decrease of *Lactobacillus* was particularly remarkable (Fig. 3; Supplemental Table 1). Recently, we reported that EVs derived from *Lactobacillus plantarum* increased Sirt1-dependent Bdnf expression in HT22 cells and in the hippocampus [20]. Consistent with this report, post-stress treatment with EVs from *Lactobacillus plantarum* rescued the stress-induced decrease in expression of *Bdnf* and *Sirt1* in the hippocampus (Fig. 4A and B). This treatment also reversed stress-induced downregulation of *MeCP2* (Fig. 4B), an epigenetic factor which also regulates *Bdnf* expression [30].

Next, we investigated whether EVs from *Bacillus subtilis* and *Akkermansia muciniphila*, which are taxonomically remote from *Lactobacillus*, could induce similar genomic responses. Post-stress treatment of CRST-treated mice with EVs of *Bacillus subtilis* and *Akkermansia muciniphila* also rescued stress-induced expression reduction of *Bdnf, Nt3, or Nt4/5*. The post-stress treatment with EVs from *Bacillus subtilis* and *Akkermansia muciniphila* also increased stress-induced reduced expression of *Sirt1*, although their changes were not statistically significant (Fig. 4C-F). However, EVs from *Bacillus subtilis* did not restore stress-induced reduction of *MeCP2* expression (Fig. 4D).

EVs from three different types of probiotics all exerted anti-depressant effects.

We next asked whether administration of EVs from those Gram-positive and Gram-negative probiotics could produce behavioral changes in a stress-induced model of depression. EVs from each of the three

probiotics described above were administered during the CRST treatment phase via the intraperitoneal route, which permits systemic circulation [34].

Mice exposed to chronic restraint stress (CRST) using daily 2-h restraint for 14 days exhibited increased immobility in the TST and FST (Fig. 5A-C). In contrast, post-stress treatment of CRST-treated mice with EVs of *Lactobacillus plantarum* for 14 days reversed stress-induced increases in immobility in the TST and FST (Fig. 5A-C).

Post-stress treatment of CRST-treated mice with EVs of *Bacillus subtilis* and *Akkermansia muciniphila* for 14 days also rescued stress-induced increases in immobility in the FST, which were comparable to those induced by EVs of *Lactobacillus* (Fig. 5A-C). However, post-stress treatment of CRST-treated mice with EVs of *Bacillus subtilis* and *Akkermansia muciniphila* did not significantly reverse stress-induced increases in immobility in the TST (Fig. 5A-C).

These results suggest that EVs from *Lactobacillus plantarum, Bacillus subtilis* and *Akkermansia muciniphila* confer anti-depressant-like effects in CRST-treated mice, although the effects of EVs of *Bacillus subtilis* and *Akkermansia muciniphila* are relatively weak.

Discussion

Chronic restraint changed gut microbiota composition particularly in the post-stress period.

In the present study, we demonstrated that chronic stress evoked by daily 2-h restraint for 14 days (CRST) changed gut microbiota composition at multiple levels, from phylum to genus. Interestingly, stress-induced changes were more dramatic in the post-stress period than those observed in the stress phase. This finding raises several important interrelated issues. First, repeated restraint stress treated with daily 2-h restraint for 14 days produced gut microflora changes in mice that were housed in a relatively constant physical environment and with a regular food supply. Furthermore, these changes occurred in a period of a month. Although underlying mechanisms need to be studied further, our results suggest that repeated stress imposed by our experimental model not only produce maladaptive changes in the brain and depressive-like behaviors, but also significantly impact the relative composition of gut microbiota. Our results are partly consistent with previous reports; five-weeks of chronic unpredictable mild stress (CUMS) caused changes in gut microbiota composition [11]. In an independent study, nine weeks of CUMS also changed gut microbiota composition, whereas transferring gut microbiota prepared from stools of CUMS-treated mice to normal recipient mice decreased hippocampal neurogenesis and induced depressive-like behaviors in the recipients [12]. Thus, chronic stress causes maladaptive changes in the brain and also gut microbiota composition. Second, the relative abundance of gut microbiota was changed more drastically in the post-stress period compared to the period of time of the termination of repeated restraint protocol. It remains to be determined whether these changes represent a state of imbalance that might occur following the removal of stressors, or if stress-induced changes in gut microbiota were preceded in a protracted manner that affected our observations of gut flora abundance and composition. Third, concerning the detailed mechanisms by which repeated stress causes intestinal

microbiota changes, it might be possible that stress-induced adaptive changes in the brain not only produce HPA axis dysregulation, but also influence gut microbiota composition. Neuronal dysfunction that involves the autonomic nervous system was associated with altered cellular function of gut epithelial cells [35] (Carabotti et al, 2015). This possibility supports the notion that brain dysfunction can be linked to changes in gut microbiota, but more extensive future work will be required to establish a definitive causal relationship.

Administration of EVs from Gram-positive (Lactobacillus and Bacillus) and Gram-negative (Akkermansia) probiotics produced anti-depressant effects.

Administration of EVs from *Lactobacillus plantarum, Bacillus subtilis* and *Akkermansia muciniphila* all produced anti-depressant-like effects in CRST-treated mice (Fig. 5), although these three bacterial families are taxonomically distinct. *Lactobacillus plantarum* is catalase-negative, facultatively heterofermentative, non-spore-forming, rod-shaped, Gram-positive bacterium belonging to the phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae and genus *Lactobacillus* [36]. *Bacillus subtilis* is a catalase-positive, spore-forming, rod-shaped, Gram-positive bacterium belonging to the phylum Firmicutes, class Bacilli, order Bacillales, family Bacillaceae, and genus *Bacillus* [37]. *Akkermansia muciniphila* is a strictly anaerobic, non-spore-forming, oval-shaped, Gram-negative, mucin-degrading bacterium belonging to the phylum Verrucomicrobia, class Verrucomicrobiae, order Verrucomicrobiales, family Akkermansiaceae, and genus *Akkermansia* [38]. Thus, *Lactobacillus* and *Bacillus* are Gram-positive bacteria that share distinctive prokaryotic features, whereas *Akkermansia* is a Gram-negative bacterium that is far remote from *Lactobacillus* and *Bacillus* at the taxonomic level. Nonetheless, EVs from those three probiotics induced similar genomic responses of MeCP2, Sirt1 and/or neurotrophic factors in the hippocampus (Fig. 4) and produce anti-depressant effects (Fig. 5), although there were some differences in detail. These results suggest that EVs from those three probiotics might contain the cargo components that directly and commonly activate the transcription of MeCP2, Sirt1 and/or neurotrophic factors in the brain and produce anti-depressant-like effects, although the specific contents of EVs that could induce those responses remains to be characterized in future work.

Recently, it was reported that in rats, exposure to 7 weeks of CUMS produced changes intestinal microbiota composition and depressive-like behaviors. In contrast, administration of *Lactobacillus casei* from 4 weeks to the end of the 7 weeks of the CUMS regimen improved depression-like behavior and reversed stress-induced reduction of BDNF expression and TrkB signaling in the frontal cortex [13], although the mechanism whereby *Lactobacillus casei* increased BDNF expression was not explored in their study. Administration of *Lactobacillus reuteri* from 4 weeks to 7 weeks in mice similarly exposed to 7 weeks of the CUMS regimen improved depression-like behavior in the FST by inhibiting the expression of indoleamine-pyrrole 2,3-dioxygenase, a key enzyme in the kynurenine pathway in the intestine, and lowering circulating kynurenine levels [11]. The tryptophan-kynurenine pathway is functional not only in intestinal cells, but also in the liver [40]. A close relationship between the tryptophan-kynurenine pathway and BDNF-TrkB signaling in the limbic system has been proposed [39]. According to these studies, *Lactobacillus casei* and *Lactobacillus reuteri* can induce BDNF-TrkB signaling in the limbic system via the

tryptophan-kynurenine pathway, as EVs of *Lactobacillus plantarum, Bacillus subtilis* and *Akkermansia muciniphila* induce expression of neurotrophic factors in the hippocampus (Fig. 4). Considering these results, it might be important to address whether anti-depressant effects of *Lactobacillus casei* and *Lactobacillus reuteri* observed in those studies are produced by mechanisms that depend on EVs, or if they act through other, independent mechanisms.

Abbreviations

Bdnf	Brain derived neurotrophic factor
CREB	cAMP response element binding protein
CRST	Chronic restraint stress
EVs	Extracellular vesicles
GC	Glucocorticoid
HDAC2	Histone Deacetylase 2
IMI	Imipramine
MeCP2	Mitogen-activated protein kinase phosphatase-1
Ngf	*Nerve growth factor*
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
OTUs	Operational taxonomic units
rRNA	Ribosomal RNA
Sirt1	Sirtuin 1
TrkB	*Tropomyosin receptor kinase B*

Declarations

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Conflicts of interest/Competing interests

JC, HK and PLH have no competing financial interests; YKK belongs to MD Healthcare Inc.

Code availability

Not applicable

Authors' contributions

JC and HK carried out the experiments; YKK provided EVs; JC, HK and PLH designed the experiments, performed the statistical analysis, and wrote the manuscript.

Consent for publication

All authors consent to the publication of the manuscript in Mol Neurobiol, should the article be accepted by the Editor-in-chief.

Ethics approval and consent to participate

All animals were handled in accordance with the animal care guidelines of Ewha Womans University (IACUC 15-012).

Availability of Data and Materials

Data and materials will be made available on reasonable request.

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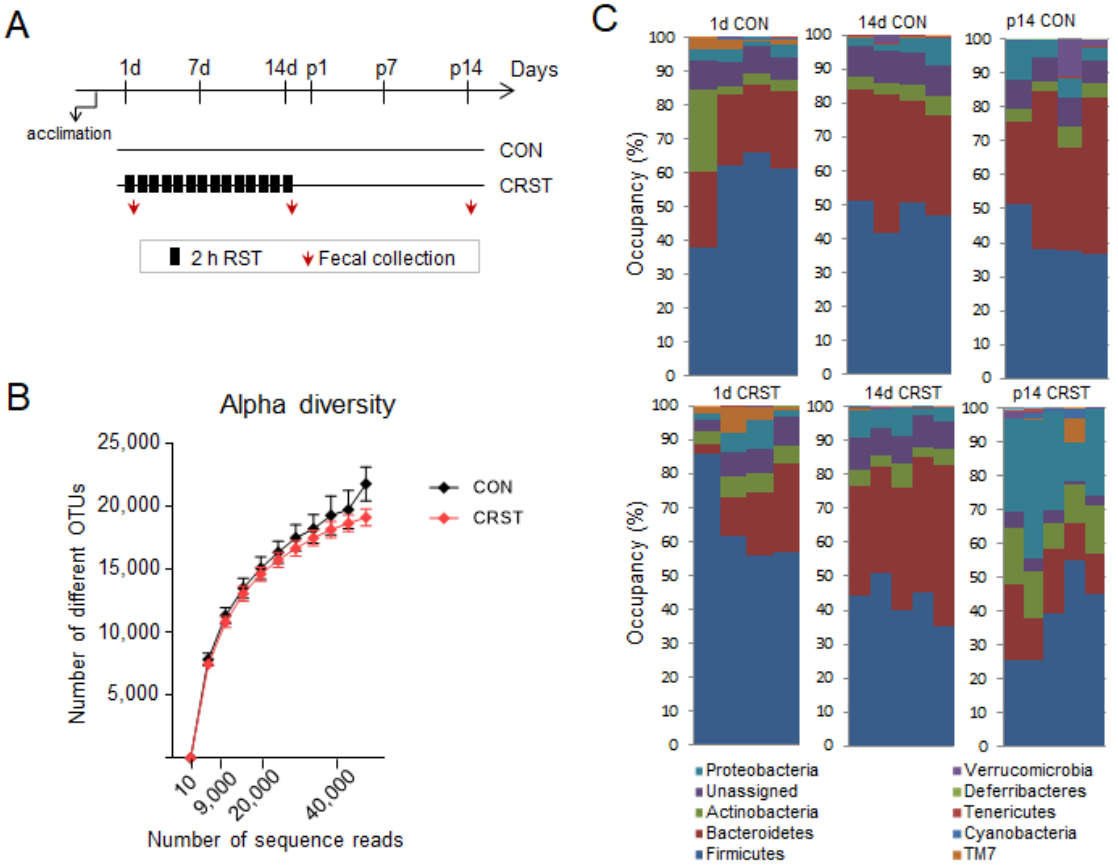
Supplementary Figure

Supplementary Figure 1 is not available with this version

Figures



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Taxon	Occupancy (%; Mean±SD)						CON vs. CRST Z-score		
	CON			CRST					
	1d	14d	p14	1d	14d	p14	1d	14d	p14
Firmicutes	56.97 ± 12.82	47.61 ± 4.51	40.98 ± 6.91	65.06 ± 14.12	43.15 ± 5.84	38.21 ± 12.81	0.63	-0.99	-0.40
Bacteroidetes	21.59 ± 1.32	33.31 ± 5.46	36.90 ± 11.07	14.78 ± 9.93	37.37 ± 6.65	15.33 ± 4.99	-5.16	0.74	-1.95
Actinobacteria	8.21 ± 10.54	4.47 ± 1.03	4.11 ± 1.41	5.28 ± 1.25	4.74 ± 1.76	12.76 ± 3.51	-0.28	0.26	6.14
Unassigned	7.60 ± 0.90	9.18 ± 0.28	7.95 ± 0.88	6.55 ± 2.19	8.45 ± 0.69	3.32 ± 1.48	-1.17	-2.63	-5.28
Proteobacteria	3.07 ± 1.21	4.32 ± 2.74	6.40 ± 3.37	4.63 ± 3.14	5.50 ± 2.74	26.89 ± 10.48	1.30	0.43	6.07
TM7	2.12 ± 1.12	0.08 ± 0.02	0.07 ± 0.03	3.34 ± 2.94	0.15 ± 0.12	1.50 ± 2.98	1.09	2.76	56.63
Cyanobacteria	0.22 ± 0.18	0.09 ± 0.07	0.11 ± 0.05	0.08 ± 0.07	0.13 ± 0.07	1.09 ± 1.22	-0.81	0.56	18.60
Tenericutes	0.20 ± 0.07	0.35 ± 0.15	0.17 ± 0.05	0.25 ± 0.16	0.50 ± 0.24	0.00 ± 0.00	0.64	1.04	-3.38
Deferribacteres	0.01 ± 0.01	0.00 ± 0.01	0.03 ± 0.05	0.01 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00	0.79	-0.54
Verrucomicrobia	0.01 ± 0.00	0.58 ± 1.14	3.28 ± 5.27	0.00 ± 0.01	0.00 ± 0.00	0.48 ± 0.83	-0.65	-0.51	-0.53
Other	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	-0.14	0.21	-0.50
Planctomycetes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-0.17	-	-
Acidobacteria	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-0.04	0.40	-0.69
[Thermi]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.28 ± 0.29	4.65	-0.50	-
Synergistetes	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-	-	-

Figure 1

Taxonomic richness and percent composition of most abundant top 15 phyla of gut microbiota in mice exposed to chronic stress. (A) Experimental design for treatment with 2-h restraint for 14 days (CRST) and time points for fecal collection. (B) Rarefaction curves with mean OTUs for each group over the number of DNA sequence reads of 16S ribosomal RNA genes in control (blue) and CRST (red) groups (n= 12 for control and 14 for CRST group). Data points are mean occupancy (%) +/− SEM. (C) Clustered

stacked column bar graphs depicting the relative occupancy of the most abundant phyla of gut microbiota in control and CRST groups. Resolved OTUs with occupancies ≥0.1% are presented. Each column represents resolved OTUs from 4-5 independent fecal samples (CON, n=4 fecal samples for all time points; CRST, n =4 fecal samples for day 1; n =5 fecal samples for day 14, and n =5 fecal samples for post-stress day 14). (D) The relative occupancy of most abundant top 15 phyla in control and CSRT groups at the indicated time points and the difference (z-score) in the mean occupancy between control and CSRT groups (CON vs. CRST). SD, standard deviation. Data are presented as mean occupancy (%) +/− SD (CON, n=4 for all time points; CRST, n =4 - 5). The z-score is X − μ/σ, where μ=mean, X=individual score, and σ=standard deviation. << denotes increase in the mean occupancy over 100.

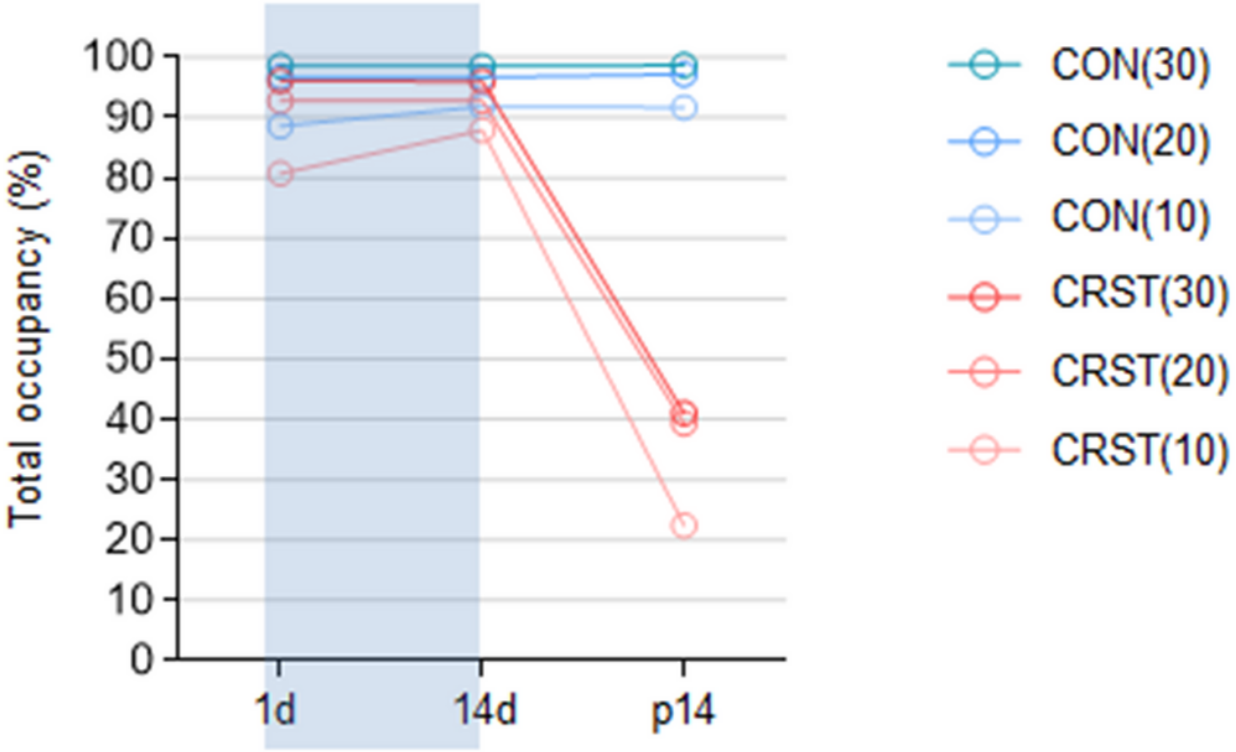

Figure 2

Temporal changes in summed occupancy of the most abundant top 10, 20, and 30 genus members in control and CRST groups during and after treatment with CRST. The summed occupancy (%) of the most abundant top 10, 20, and 30 genus members of the identified OTUs in control and CRST groups during and after CRST.

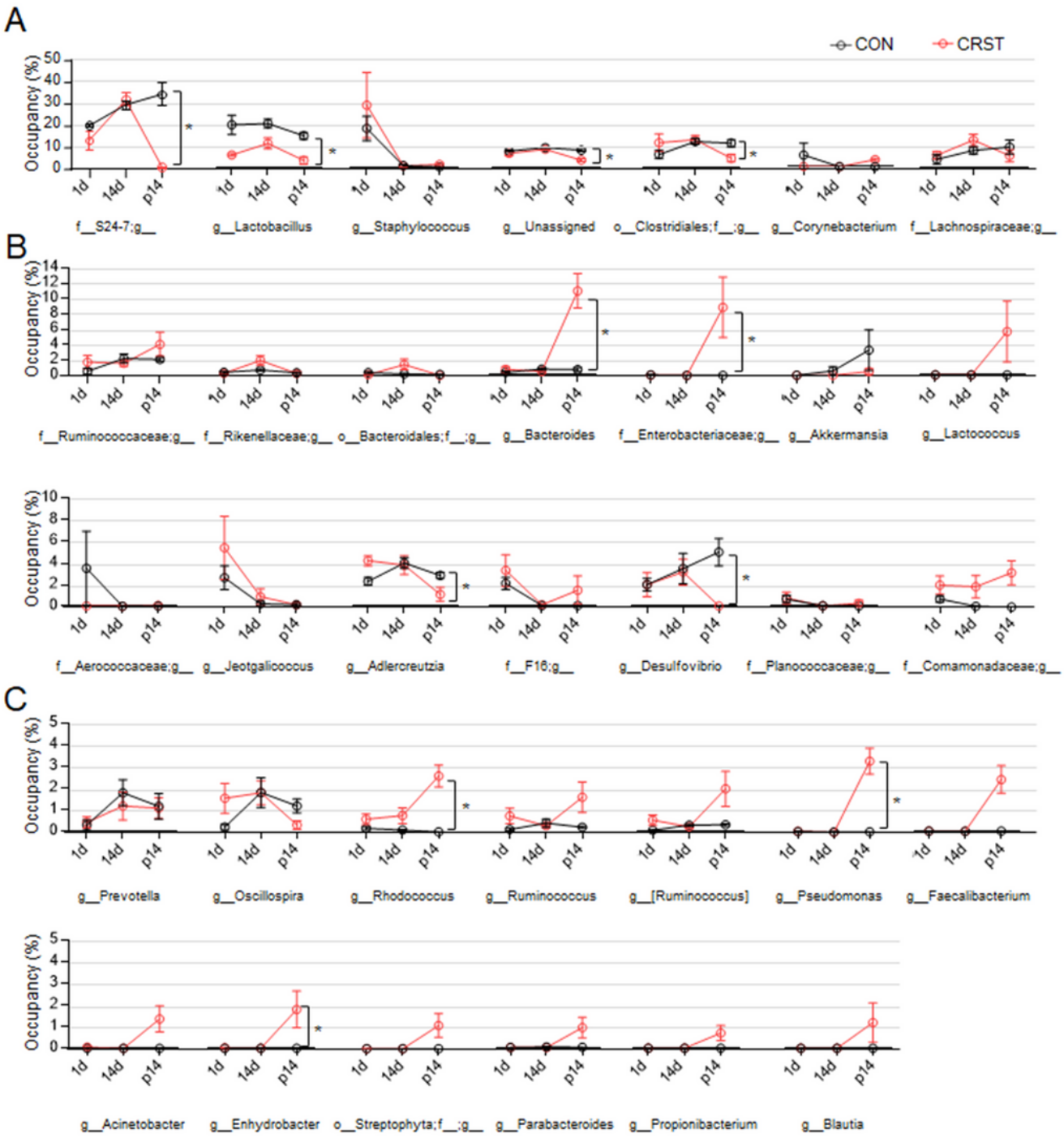

Figure 3

The relative occupancy of the most abundant top 34 individual genus members changed by chronic stress. (A-C) The percent occupancy of 34 individual genus members in control and CRST groups at stress day 1, stress day 14, and post-stress day 14. The 34 genus members were selected as those having a relative occupancy ≥0.1% in control or CRST groups at any of the three time points. (A) The percent occupancy of f__S24-7;g__, g__Lactobacillus, g__Staphylococcus, an unassigned member of bacterium,

an unclassified member of o_Clostridiales;f_;g_, g__Corynebacterium, and f__Lachnospiraceae;g_ in controls and CRST groups at stress day 1, stress day 14, and post-stress day 14. (B) The percent occupancy of f__Ruminococcaceae;g_, f__Rikenellaceae;g_, o__Bacteroidales;f_;g_, g__Bacteroides, f__Enterobacteriaceae;g_, *g__Akkermansia, g__Lactococcus, f__Aerococcaceae;g_, g__Jeotgalicoccus, g__Adlercreutzia, f__F16;g_, g__Desulfovibrio, f__Planococcaceae;g_, and f__Comamonadaceae;g_ in controls and CRST groups at stress day 1, stress day 14, and post-stress day 14. (C) The percent occupancy of g__Prevotella, g__Oscillospira, g__Rhodococcus, g__Ruminococcus, g__[Ruminococcus], g__Pseudomonas, g__Faecalibacterium, g__Acinetobacter, g__Adlercreutzia, o__Streptophyta;f__;g__, g__Parabacteroides, g__Propionibacterium, and g__Blautia in controls and CRST groups at stress day 1, stress day 14, and post-stress day 14. Data are presented as mean occupancy (%) +/− SEM. * denotes the difference between control and CRST groups (main effect of stress) at post-stress day 14 at $p<0.05$ (two-way ANOVA, followed by Bonferroni post hoc test).

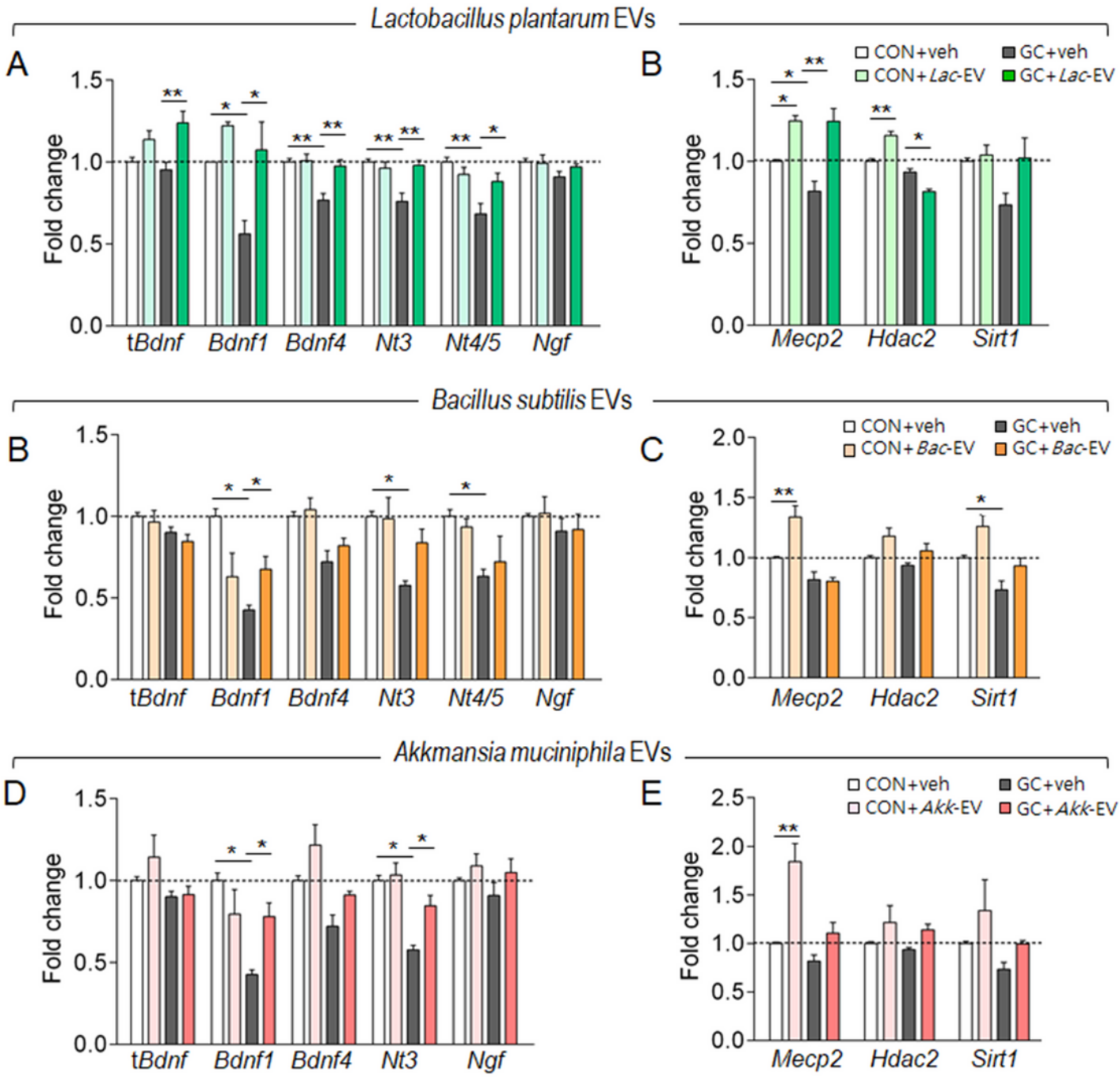

Figure 4

Treatment with EVs from Lactobacillus plantarum, Bacillus subtilis, and Akkermansia muciniphila increased the expression of MeCP2, Sirt1 and neurotrophic factors in the hippocampus. (A, B) Expression levels of total Bdnf (tBdnf), Bdnf1, Bdnf4, Nt3, Nt4/5, Ngf (A), MeCP2, Hdac2, and Sirt1 (B) in the hippocampus of mice treated with EVs from Lactobacillus plantarum. Veh, vehicle (n= 6 -12 qPCR repeats). (C,D) Expression levels of total Bdnf (tBdnf), Bdnf1, Bdnf4, Nt3, Nt4/5, Ngf (E), MeCP2, Hdac2,

and Sirt1 (F) in the hippocampus of mice treated with EVs from Bacillus subtilis. Veh, vehicle (n= 6 -10 qPCR repeats). (E,F) Expression levels of total Bdnf (tBdnf), Bdnf1, Bdnf4, Nt3, Nt4/5, Ngf (G), MeCP2, Hdac2, and Sirt1 (H) in the hippocampus of mice treated with EVs from Akkermansia muciniphila. Veh, vehicle (n= 6 -10 qPCR repeats). Data are presented as mean ± SEM. *p < .05; **p < .01 (Two-way ANOVA followed by Bonferroni post hoc test).

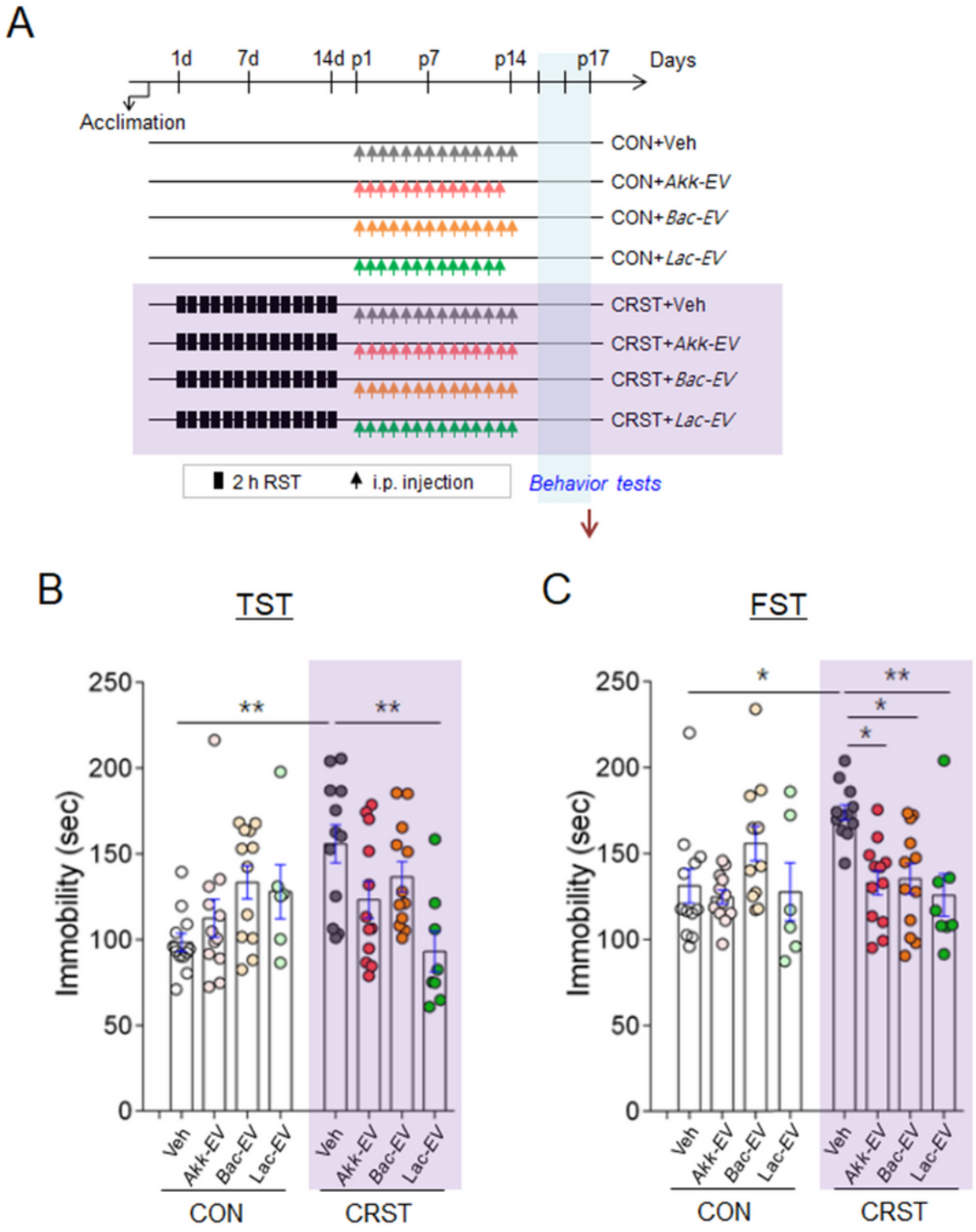

Figure 5

Administration of EVs from Lactobacillus plantarum, Bacillus subtilis and Akkermansia muciniphila conferred anti-depressant-like effects. (A) Experimental design for treatment with 2-h restraint for 14 days (CRST) followed by treatment with EVs from Lactobacillus plantarum (Lac-EV), Bacillus subtilis (Bac-EV), and Akkermansia muciniphila (Akk-EV). Behavior tests were performed on post-stress days 15-16 (p15~p16). Blue arrow, time point for tissue prep. EVs, 2 ug/100 ul/mouse/day (i.p.). Control mice injected with saline (CON+veh) or EVs (CON+Lac-EV, CON+Bac-EV, and CON+Akk-EV); CRST-treated mice injected with saline (CRST+veh) or EVs (CRST+Lac-EV, CRST+Bac-EV, and CRST+Akk-EV). (B,C) Immobility time in the TST (B) and FST (C) of CON+veh, CON+EVs, CRST+veh, and CRST+EVs (n=6-12 animals). Data are presented as mean ± SEM. *p < .05; **p < .01 (One-way ANOVA followed by Newman-Keuls post hoc test among control group or CRST group; Two-way ANOVA followed by Bonferroni post hoc test among CON+veh, CON+EVs, CRST+veh, and CRST+EVs groups).

Supplementary Files

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