Title: Heat-sterilized *Bifidobacterium breve* prevents depression-like behavior and interleukin-1β expression in mice exposed to chronic social defeat stress.

Running title: *B. breve* prevents depression-like behavior and inflammation.

Aika Kosuge[a, †], Kazuo Kunisawa[a, †], Satoshi Arai[b], Yumika Sugawara[a], Katsuki Shinohara[a], Tsubasa Iida[a], Bolati Wulaer[c,d], Tomoki Kawai[a], Hidetsugu Fujigaki[d], Yasuko Yamamoto[d], Kuniaki Saito[c,d,e], Toshitaka Nabeshima[c,e], Akihiro Mouri[a,e].

Affiliations

[a]Department of Regulatory Science for Evaluation & Development of Pharmaceuticals & Devices, Fujita Health University Graduate School of Health Sciences, Aichi, Japan.

[b]Morinaga Milk Industry Co., Ltd., R&D Division, Food Ingredients & Technology Institute, Kanagawa, Japan.

[c]Advanced Diagnostic System Research Laboratory, Fujita Health University Graduate School of Health Science, Aichi, Japan.

[d]Department of Disease Control and Prevention, Fujita Health University Graduate School of Health Sciences, Aichi, Japan.

[e]Japanese Drug Organization of Appropriate Use and Research, Aichi, Japan.

[†]Aika Kosuge and Kazuo Kunisawa contributed equally to this work.

***Corresponding author**: Akihiro Mouri

Address: Department of Regulatory Science for Evaluation and Development of Pharmaceuticals and Devices, Fujita Health University, Graduate School of Health Sciences, Aichi, 470-1192, Japan.

Tel.: +81-562-93-2520

Fax: +81-562-93-2521

E-mail: mouri@fujita-hu.ac.jp

Abbreviations: Arg, Arginase; *B. breve* M-16V, *Bifidobacterium breve* M-16V; CSDS, chronic social defeat stress; CCR2, chemokine receptor 2; HIP; hippocampus, IL-1β, interleukin-1β; IL-6, interleukin-6; LEfSe, Linear discriminant analysis effect size; LDA, Linear discriminant analysis; MDD, major depressive disorder; PFC, prefrontal cortex; PCoA, principal coordinate analysis; SIT, social interaction test; TNF-α, tumor necrosis factor-α.

Keywords: heat-sterilized *Bifidobacterium*, CSDS, depression, gut-brain axis, interleukin-1β

Main text

Abstract

Major depressive disorder (MDD) is a common and serious psychiatric disease that involves brain inflammation. *Bifidobacterium breve* is commonly used as a probiotic and was shown to improve colitis and allergic diseases by suppressing the inflammatory response. Heat-sterilized *B. breve* has beneficial effects on inflammation. We hypothesize, therefore, that this probiotic might reduce depression symptoms. We tested this is a mouse model of social defeat stress. C57BL/6J mice exposed to chronic social defeat stress (CSDS) for five consecutive days developed a mild depression-like behavior characterized by a social interaction impairment. CSDS also altered the gut microbiota composition, such as increased abundance of Bacilli, Bacteroidia, Mollicutes, and Verrucomicrobiae classes and decreased Erysipelotrichi class. The prophylactic effect of heat-sterilized *B. breve* as a functional food ingredient was evaluated on the depression-like behavior in mice. The supplementation started two weeks before and lasted two weeks after the last exposure to CSDS. Two weeks after CSDS, the mice showed deficits in social interaction and increased levels of inflammatory cytokines, including interleukin-1β (IL-1β) in the prefrontal cortex (PFC) and hippocampus (HIP). Heat-sterilized *B. breve* supplementation significantly prevented social interaction impairment, suppressed IL-1β increase in the PFC and HIP, and modulated the alteration of the gut microbiota composition induced by CSDS. These findings suggest that heat-sterilized *B. breve* prevents depression-like behavior and IL-1β expression induced by CSDS through modulation of the gut microbiota composition in mice. Therefore, heat-sterilized *B. breve* used as an ingredient of functional food might prevent MDD.

Introduction

Major depressive disorder (MDD) is a common and serious psychiatric disease characterized by fatigue, diminished interest and/or pleasure, and despair (Cryan and Holmes, 2005). MDD affects 350 million individuals worldwide and is responsible for a million of suicide deaths each year (Altaf et al., 2015; Wang et al., 2016). Approximately one-third of patients with MDD do not respond to currently available treatments (De Berardis et al., 2020; McHugh et al., 2013). Thus, a better understanding of MDD pathophysiology is crucial for developing more effective therapeutic agents and functional foods.

Although the pathophysiology of MDD is not fully understood, a link between neuroinflammation and MDD has been established (Hodes et al., 2015; Wohleb et al., 2016; Yirmiya et al., 2015). Indeed, microglial activation was observed in patients with MDD and animal models (Bayer et al., 1999; de Pablos et al., 2014; Pan et al., 2014). High levels of inflammatory cytokines, such as interleukin-1β (IL-1β), were found in the postmortem brains of patients with MDD (Raison et al., 2006; Schiepers et al., 2005). In animal models, chronic social defeat stress (CSDS) is a psychosocial stress paradigm widely used to study MDD (Berton et al., 2006). CSDS induces neuroinflammation as evidenced by the increase of microglial activation and inflammatory cytokines (Hodes et al., 2014; McKim et al., 2018; Wohleb et al., 2014b). Thus, using CSDS to investigate the mechanisms involved in MDD induced by inflammation might provide valuable insight for the development of novel therapeutic agents and functional foods.

Probiotics have various health-promoting benefits including modulation of the immune response. *Bifidobacterium* is one of the most widely used and studied probiotic bacteria. *Bifidobacterium* inhibits harmful bacteria multiplication, improves the function of the gastrointestinal barrier, and is protective against pathogens (Xue et al., 2017). It also prevents various intestinal diseases, including inflammatory bowel disease and allergies (Fu et al., 2017; Izumi et al., 2015; Srutkova et al., 2015). The *Bifidobacterium breve* M-16V (*B. breve* M-16V) strain is predominant in

the intestine of healthy infants and is one of the most frequently isolated fecal *Bifidobacterium* species (Matsuki et al., 1999; Mikami et al., 2012). Previous studies have shown that live *B. breve* M-16V suppresses the inflammatory response, prevents allergic responses, and promotes normal gut microbiota (Hougee et al., 2010; Inoue et al., 2009; Izumi et al., 2015; Li et al., 2004; Satoh et al., 2016). Pretreatment with living *B. breve* M-16V prevented dextran sulfate sodium-induced colitis by altering the systemic immune function and suppressing the inflammatory response (Izumi et al., 2015). In addition, supplementation of milk with living *B. breve* M-16V significantly suppressed the increase of inflammatory cytokines in the neonatal necrotizing enterocolitis rat model (Satoh et al., 2016). However, the safety of using living probiotics is still a matter of debate. Use of living bacteria as probiotics is associated with risks of 1) developing systemic infections due to translocation, 2) acquiring antibiotic resistance genes, and 3) interfering with gut colonization in neonates (Boyle et al., 2006). To circumvent these risks, a growing interest for heat-sterilized probiotic bacteria has emerged. Heat-sterilized *B. breve* M-16V has been shown to modulate immunity and suppress the production of inflammatory cytokines (Sugahara et al., 2017). Therefore, heat-sterilized *B. breve* M-16V might constitute a potential functional food to prevent inflammation-associated diseases, including MDD.

In this study, we aimed to determine whether heat-sterilized *B. breve* M-16V suppressed the inflammatory response and improved depression-like symptoms induced by CSDS to assess its prophylactic use in alleviating MDD.

Material and methods

Animals

Male C57BL/6J and ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). Only male mice were used to exclude any potential estrous cycle effects. Male C57BL/6J mice (7 weeks old) were exposed to CSDS. Aggressive, male ICR mice (> 10 weeks old), were used to induce CSDS. ICR mice that attacked C57BL/6J mice for > 1 min were used as aggressors. Unfamiliar target male ICR mice (8-9 weeks old) were used for the social interaction test. All mice were housed in a plastic cage and maintained on a 12 h light/dark cycle (lights on at 8:00 A.M.) with food and water ad libitum. All experiments were carried out in accordance with the guidelines established by the Japanese Pharmacological Society and the Institute for Experimental Animals at Fujita Health University. The protocols were approved by the Ethics Committee of Animal Experiments at the Institute for Experimental Animals at Fujita Health University in April 2017 (Permit Number: AP16044). Animal experiments were carried out from April 2018 to March 2020.

Chronic social defeat stress

Mice were exposed to CSDS according to the method outlined in our previous report (Mouri et al., 2018). Prior to CSDS, an aggressive ICR mouse was habituated to CSDS cages (28 × 45 × 20 cm high) for 10 min. C57BL/6J mice were exposed to a different aggressive ICR mouse for 10 min each day for 5 consecutive days. After each stress exposure, the mice were returned to their home cages. The pairing of CSDS and aggressive mice was randomized daily to minimize the effects of variability in the aggression that the mice were exposed to. Control mice were exposed to an anesthetized, aggressive ICR mouse. Defeat was defined as the display of defensive behaviors by C57BL/6J mice, such as escape or submissive postures during physical attacks by an aggressive mouse. Submissive posture was defined as standing upright with the belly exposed to the aggressor. The duration of defensive behaviors was recorded according to our previous report (Mouri et al.,

2018). Mice injured, by CSDS, with open would exceeding 2 cm or weight loss exceeding 15% were excluded from this experiment.

The prophylactic effects of heat-sterilized B. breve M-16V were assessed in a model of mild depression-like behavior induced by CSDS for 5 consecutive days. In this paradigm, mice develop sustained social impairment for 2 weeks, but recover to baseline social behavior 4 weeks after the stress (Mouri et al., 2018).

Social interaction test

Social interaction test (SIT) was performed according to the method outlined in previous reports (Berton et al., 2006; Krishnan et al., 2007; Nie et al., 2018; Tanaka et al., 2012; Venzala et al., 2012; Wook Koo et al., 2016; Zhang et al., 2019). CSDS mice were subjected to SIT 1 day and 2 weeks after the last stress exposure. The SIT was performed between 10:00 A.M. and 6:00 P.M., and carried out in a sound-attenuated and air-regulated experimental room, to which the mice were habituated for more than 3 hours before SIT (Nie et al., 2018; Tanaka et al., 2012). The apparatus consisted of an open, gray, non-reflecting acrylic box (42 × 42 × 30-cm high) and a transparent Plexiglas enclosure (10 × 6.5 × 30 cm high) with 30 holes (10 mm in diameter). A light bulb (54 W), which was not directly seen by the mouse, was attached to the upper part of the apparatus and provided constant illumination of approximately 20 lux. The SIT consisted of two sessions: in the first session (no target), the mouse was allowed to explore freely and habituated to the test environment for 30 min, in the absence of an unfamiliar target ICR mouse. This was carried out to reduce the time spent exploring the apparatus itself during the second session. The second session (target) commenced 1 min after the first session, and the mouse was returned to the apparatus for 5 min in the presence of an unfamiliar target ICR mouse (Berton et al., 2006; Wook Koo et al., 2016; Zhang et al., 2019). During the test, the time spent in the interaction zone (light grey zone) and corner zones (grey zone) were recorded for the last 5 min of the first (no target) and the second

(target) sessions (Figure 1B), using the ANY-maze video tracking system (Stoelting Co., Ltd., Wood Dale, IL, USA).

Preparation and supplementation of *Bifidobacterium breve* M-16V (*B. breve* M-16V)

Heat-sterilized *B. breve* M-16V was obtained from the Morinaga Culture Collection (Morinaga Milk Industry Co. Ltd., Kanagawa, Japan). The cells were anaerobically cultivated in MRS broth (Difco Laboratories, Franklin Lakes, NJ, USA) containing 0.05% L-cysteine-HCl for 16 h at 37°C. The cells were harvested, washed twice with saline, and then washed with sterile distilled water. The cells were suspended in sterile distilled water and killed by heating at 100°C for 30 min.

C57BL/6J mice were randomly divided into four groups: control, control with M-16V, CSDS, and CSDS with M-16V. Control and CSDS with the M-16V-treated groups were fed the AIN-93G diet (Oriental Yeast Co., Tokyo, Japan) which containing 5.0×10^9 nonviable cells / 0.5 g. To evaluate the preventive effect, each group was fed an AIN-93G diet with or without the cells separately from their usual diet from 2 weeks before the stress exposure until the end of the experiments (Figure 4A).

Microbiota profiling

The fecal samples were collected 1 day before and after the exposure to CSDS according to previous reports (Bastiaanssen et al., 2020; Werbner et al., 2019). The samples were placed in 1.5 ml tubes, snap-frozen on dry ice and stored at -80 °C. DNA was extracted using the bead-beating method described in a previous report (Odamaki et al., 2007). Briefly, after centrifugation at 14,000 × g for 5 min, 400 μl of the supernatant was extracted with phenol-chloroform, and 250 μl of the supernatant was precipitated with isopropanol. Purified DNA was suspended in 2,000 μl of Tris-EDTA buffer (pH 8.0). Subsequently, the V3-V4 region of the bacterial 16S rRNA gene was sequenced by Illumina Miseq (Illumina, Inc., San Diego, CA, USA) as described previously

(Odamaki et al., 2018). After removing sequences consistent with data from the Genome Reference Consortium human build 38 and phiX reads from the raw Illumina paired end reads, the sequences were analyzed using the QIIME2 software package version 2017.10 (https://qiime2.org/). Potential chimeric sequences were removed using DADA2 (Callahan et al., 2016), followed by trimming 30 and 90 bases of the 3' region of the forward and the reverse reads, respectively. Taxonomical classification was performed using Naive Bayes classifier trained on the Greengenes13.8 with a 99 % threshold of OTU full-length sequences. Weighted and unweighted UniFrac distance was calculated using QIIME2 software.

The diversity of gut microbiota was evaluated by Bray-Curtis and Jaccard-based principal coordinate analysis (PCoA) and analysed by permutational multivariate analysis of variance (PERMANOVA) with *adonis* function in the "vegan" R-package. Linear discriminant analysis (LDA) effect size (LEfSe) was performed with default parameters to identify microbial taxa that were differentially abundant among groups (Segata et al., 2011).

Sample collection

The mice were deeply anesthetized with isoflurane (8.131 mol/L; Fuji Film Wako Pure Chemical Co., Osaka, Japan) and transcardially perfused with ice-cold PBS 2 weeks after the last stress exposure. The intestine was dissected by excising under the stomach and before the cecum. Mesenteric fat and Peyer's patches were carefully removed using fine forceps. The intestinal contents were removed in two PBS washes and then immediately frozen using dry ice. The entire brain was quickly removed and chilled in ice-cold saline. The prefrontal cortex (PFC) and hippocampus (HIP) were manually dissected on ice-cold plates and then immediately frozen using dry ice because these regions have been associated with the pathophysiology and progression of MDD (McKinnon et al., 2009; Treadway et al., 2015). Moreover, the inflammatory processes of PFC and HIP were associated with the depressive symptoms (Holmes et al., 2018; Setiawan et al., 2015). All samples were stored

205 at -80°C until needed for analysis.

206

207 **Quantitative real-time reverse transcription PCR (qRT-PCR)**

208 Total RNA was isolated using a NucleoSpin® RNA kit (Takara, Shiga, Japan) according to
209 the method outlined in a previous report (Kunisawa et al., 2018). All PCR primers were purchased
210 from Integrated DNA Technologies (Coralville, IA, USA). First-strand cDNA was synthesized using
211 the ReverTra Ace qPCR-RT kit (Toyobo, Osaka, Japan). For the quantitative PCR, SsoAdvanced^{TM}
212 Universal Probes Supermix (Bio-Rad, Berkeley, CA, USA) was used and subjected to real-time PCR
213 quantification using a StepOne^{TM} Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).
214 The PCR reaction program consisted of 50 cycles of 95°C for 30 s and 60°C for 1 min. β-actin was
215 used as a housekeeping gene to normalize all PCR data.

216 Primers used in this study were the following: IL-1β (Mm.PT.58.41616450), IL-6
217 (Mm.PT.58.13354106), TNF-α (Mm.PT.58.12575861), CD68 (Mm.PT.58.32698807), CCR2
218 (Mm.PT.58.14116710), Ym1 (Mm.PT.58.33370435), Arg1 (Mm.PT.58.8651372), CD206
219 (Mm.PT.58.42560062), IL-4 (Mm.PT.58.32703659), IL-10 (Mm.PT.58.13531087) and β-actin
220 (Mm.PT.39. a.22214843).

221

222 **Mouse tissue preparation**

223 For histological analysis, mice were deeply anesthetized with isoflurane (1 ml/ml, Wako
224 Pure Chemical Co.). Once reflex responses had disappeared, mice were transcardially perfused with
225 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were post-fixed in 4%
226 paraformaldehyde overnight at 4°C. The post-fixed tissues were cryoprotected overnight in PBS
227 containing 20% sucrose, embedded in OCT compound (Cat# 45833, Sakura Finetechnical Co.,
228 Tokyo, Japan), and cut into 20 μm sections using a cryostat (Cat# Leica CM3050; Land Hessen,
229 Germany) for immunohistochemistry.

Diacerein treatment

Diacerein (Tokyo Chemical Industry, Tokyo, Japan) as an IL-1β inhibitor, was dissolved in 1% (w/v) carboxyl methylcellulose sodium (Fujifilm Wako Pure Chemical Co., Osaka, Japan). The mice were administered per oral (p.o.) with diacerein (20mg/kg) daily 2 days before and during CSDS. The dose was used according to previous publications, in which showed that diacerein (20mg/kg; p.o.) significantly reduced IL-1β levels (Mancio et al., 2017).

Immunohistochemistry

Immunofluorescence staining was performed as described previously (Kunisawa et al. 2018). Cryosections were immunostained with a rabbit anti-Iba1 antibody (1:500; Cat# 019-19741, Wako Pure Chemical Co.). The coronal sections between 1.42 and 2.10 mm from bregma (Paxinos & Franklin 2004) were heated in a microwave in 10 mM citrate buffer (pH 6.0) up to 90°C for 5 minutes. After washing with PBS containing 0.3% Triton-X (PBST), sections were blocked with 5% fetal bovine serum (Cat# 174012, Nichirei Biscience Inc., Tokyo, Japan) in PBST for 1 hour and then incubated with primary antibody (1:500; rabbit anti-Iba1 antibody; 019-19741, Fujifilm Wako Pure Chemical Co., Osaka, Japan) in PBST at 4°C overnight. After washing with PBST, the sections were incubated with secondary antibodies (1:2000; Alexa568-conjugated goat anti-rabbit IgG; Cat# A11011, Molecular Probes, Eugene, OR, USA) and Hoechst 33342 (0.1 μg/ml; Cat# 346-07951, Dojindo, Kumamoto, Japan) for 3 hours at room temperature. Sections were then rinsed with PBST, mounted and covered with glass coverslips, and then visualized under a Zeiss LSM-710FSX100 confocal laser microscope (Olympus, Tokyo, Japan). The immunohistochemical controls were performed as described above except for the omission of the primary antibodies. No positive immunostained cells were found in any of the controls.

The number, area, and length of Iba1-positive cells for immunoreactivities were analyzed

using ImageJ software. The average of at least three slices in each mouse was calculated in a 360 μm × 260 μm of the PFC (prelimbic area) and HIP (CA1 area) and used for statistical analysis.

Data analyses

Statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, USA). Significant differences in comparisons of the two groups were analyzed using Student's t-test. Multiple group comparisons were performed by an analysis of variance (ANOVA) followed by the post hoc tests which are indicated in the figure legends. Microbiome data were analyzed using a permutational multivariate analysis of variance (PERMANOVA) test. Outliers were statistically determined by the Smirnov–Grubbs test and excluded the experimental analysis (p <0.05). The criterion for a significant difference was $p < 0.05$ for all statistical evaluation. All data were expressed as the mean ± SEM.

Results

CSDS induces deficits in social interaction

Adult male C57BL/6J mice were exposed to CSDS for five consecutive days as described in our previous studies (Hasegawa et al., 2019; Mouri et al., 2018). The mice were subjected to the social interaction test one day after their last exposure to CSDS to confirm the development of a depression-like behavior (Figure 1A; D6). The time spent by the mice in the interaction and corner zones was measured (Figure 1B). In the presence of the target ICR mouse, CSDS mice spent significantly less time in the interaction zone compared with the control mice (Figure 1C: Two-way ANOVA, CSDS, $F_{(1, 60)} = 4.283$, $p < 0.05$; session, $F_{(1, 60)} = 5.836$, $p < 0.05$; CSDS × session, $F_{(1, 60)} = 8.644$, $p < 0.05$). Inversely, the time spent in the corner areas was increased in CSDS mice (Figure 1D: Two-way ANOVA, CSDS, $F_{(1, 60)} = 8.410$, $p < 0.05$; session, $F_{(1, 60)} = 8.752$, $p < 0.05$; CSDS × session, $F_{(1, 60)} = 5.770$, $p < 0.05$). However, no changes in the time spent in the interaction and corner zones were observed between the control and CSDS groups in the absence of the target ICR mouse (Figure 1C and D). These results indicated that CSDS impaired mouse social interactions and that the experimental paradigm constituted a mouse model of mild depression.

CSDS alters the composition of the mouse gut microbiota as measured in feces

Recent studies have demonstrated that abnormal microbiota composition might contribute to MDD (Jiang et al., 2015; Wong et al., 2016). To distinguish phylotypes in the gut microbiota, LEfSe analysis was performed on genomic DNA isolated from fecal samples of control and CSDS mice one day after the last exposure to CSDS (Figure 2A). CSDS increased the abundance of Bacilli, Bacteroidia, Mollicutes, and Verrucomicrobiae classes (log10 [LDA score] > 2.0), whereas it decreased the levels of the class Erysipelotrichi (log10 [LDA score] > 2.0; Figure 2B and C). Mice exposed to CSDS for 10 consecutive days showed severe depression-like behavior, social impairment, and alterations of the gut microbiota composition (Supplemental Figure 1A–E). These

results indicated that CSDS altered the gut microbiota composition.

Heat-sterilized *B. breve* M-16V prevents the impairment of social interaction induced by CSDS

To evaluate the effect of heat-sterilized *B. breve* M-16V on preventing the depression-like behaviors induced by CSDS, mouse food was supplemented with heat-sterilized *B. breve* M-16V for 33 days (Figure 3A). First, we investigated whether the supplementation affected the gut microbiota composition changes induced by CSDS. LEfSe analysis one day before the first exposure to CSDS showed that the supplementation with heat-sterilized *B. breve* M-16V increased the abundance of Bifidobacterium (log10 [LDA score] > 2.0; Supplemental Figure 2A–C). Next, the effects of the supplementation on the depression-like behavior induced by CSDS were investigated. Mice exposed to CSDS spent significantly less time in the interaction zone and significantly more time in the corner zones one day and two weeks after the last exposure to CSDS than control mice (Figure 3B–E). Interestingly, a 33-day supplementation with heat-sterilized *B. breve* M-16V significantly reversed the effect of CSDS on the time spent in the interaction (Figure 3D: Two-way ANOVA, CSDS, $F_{(1, 47)} = 0.2377$, $p = 0.6282$; treatment, $F_{(1, 47)} = 17.53$, $p < 0.01$; CSDS × treatment, $F_{(1, 47)} = 5.400$, $p < 0.01$) and corner zones (Figure 3E: Two-way ANOVA, CSDS, $F_{(1, 47)} = 4.057$, $p < 0.01$; treatment, $F_{(1, 47)} = 7.282$, $p < 0.01$; CSDS × treatment, $F_{(1, 47)} = 3.416$, $p = 0.0709$), whereas a 19-day supplementation had no effect (Figure 3B: Two-way ANOVA, CSDS, $F_{(1, 47)} = 4.138$, $p < 0.05$; treatment, $F_{(1, 47)} = 2.594$, $p = 0.1139$; CSDS × treatment, $F_{(1, 47)} = 0.7471$, $p = 0.3918$; Figure 3C: CSDS, $F_{(1, 47)} = 8.056$, $p < 0.01$; treatment, $F_{(1, 47)} = 1.567$, $p = 0.2168$; CSDS × treatment, $F_{(1, 47)} = 0.4473$, $p = 0.5069$). There were no changes in body weight for the four groups (Figure 3F: Two-way ANOVA, group, $F_{(3, 516)} = 5.550$, $p < 0.01$; date, $F_{(11, 516)} = 77.96$, $p < 0.01$; group × date, $F_{(33, 516)} = 0.4846$, $p = 0.9937$). Taken together, these results suggested that long-term supplementation with heat-sterilized *B. breve* M-16V prevented social impairment induced by CSDS.

Heat-sterilized *B. breve* M-16V affects the gut microbiota changes induced by CSDS

We examined whether heat-sterilized *B. breve* M-16V modulated the alteration of the gut microbiota composition induced by CSDS using Bray-Curtis and Jaccard dissimilarity measures one day after the last exposure to CSDS (Figure 4A). PCoA plots showed distinct clustering between control and CSDS mice, suggesting significant differences between the groups (β-diversity: Bray-Curtis dissimilarity index [Figure 4B], Jaccard dissimilarity index [Figure 4C], and permutational multivariate analysis of variance [PERMANOVA, $p < 0.01$; Figure 4B and C]). However, the microbiota diversity in mice receiving heat-sterilized B. breve M-16V was still different than the one from control mice. LEfSe analysis was performed to further examine the phylotypes in the gut microbiota of CSDS mice eating food with or without heat-sterilized *B. breve* M-16V supplementation. Heat-sterilized *B. breve* M-16V supplementation increased the abundance of the class Bifidobacterium (-log10 [LDA score] > 2.0) and decreased the levels of the Bacteroidia class ((log10 [LDA score] > 2.0; Figure 4D and E). These results suggested that heat-sterilized *B. breve* M-16V improved social impairments by affecting the gut microbiota alteration in CSDS mice.

Heat-sterilized *B. breve* M-16V suppresses neuroinflammation induced by CSDS

It is well known that neuroinflammation is an important factor in MDD pathology (Raison et al., 2006; Schiepers et al., 2005). The increase of inflammatory cytokines induced by activated microglia in the PFC and HIP plays a critical role in the development of the depression-like behavior induced by CSDS (Nie et al., 2018; Song et al., 2020). Furthermore, excessive activation of M1 and M2 microglia contributes to MDD pathology (Kobayashi et al., 2013). To gain insight into the mechanisms involved in the effect of heat-sterilized B. breve M-16V on social interactions, the levels of M1 microglia-related genes, such as IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and CD68 were measured in the PFC and HIP. Increased IL-1β amounts were detected in PFC and HIP after CSDS exposure and this increase was significantly prevented by heat-sterilized *B. breve* M-16V

supplementation (Figure 5A: Two-way ANOVA, CSDS, $F_{(1, 31)} = 5.008$, $p < 0.05$; treatment, $F_{(1, 31)} = 3.819$, $p = 0.0597$; CSDS × treatment, $F_{(1, 31)} = 4.207$, $p < 0.05$; Figure 5E: CSDS, $F_{(1, 31)} = 8.919$, $p < 0.01$; treatment, $F_{(1, 31)} = 4.352$, $p < 0.05$; CSDS × treatment, $F_{(1, 31)} = 7.036$, $p < 0.05$). The IL-1β antagonist diacerein significantly attenuated the depression-like behavior induced by CSDS (Supplemental Figure 3: Two-way ANOVA, CSDS, $F_{(1, 40)} = 5.659$, $p < 0.05$; diacerein, $F_{(1, 40)} = 6.912$, $p < 0.05$; CSDS × diacerein, $F_{(1, 40)} = 6.466$, $p < 0.05$). No differences in IL-6, TNF-α, and CD68 levels were detected between control and CSDS groups with or without heat-sterilized *B. breve* M-16V supplementation (Figure 5B: Two-way ANOVA, CSDS, $F_{(1, 31)} = 0.3877$, $p = 0.5381$; treatment, $F_{(1, 31)} = 1.300$, $p = 0.2630$; CSDS × treatment, $F_{(1, 31)} = 0.9921$, $p = 0.3269$; Figure 5C: CSDS, $F_{(1, 31)} = 0.1848$, $p = 0.6702$; treatment, $F_{(1, 31)} = 0.7529$, $p = 0.3922$; CSDS × treatment, $F_{(1, 31)} = 0.002605$, $p = 0.9596$; Figure 5D: CSDS, $F_{(1, 31)} = 9.006$, $p < 0.05$; treatment, $F_{(1, 31)} = 0.1250$, $p = 0.7260$; CSDS × treatment, $F_{(1, 31)} = 1.162$, $p = 0.2893$; Figure 5F: CSDS, $F_{(1, 31)} = 1.474$, $p = 0.2339$; treatment, $F_{(1, 31)} = 2.908$, $p = 0.0981$; CSDS × treatment, $F_{(1, 31)} = 0.7147$, $p = 0.4044$; Figure 5G: CSDS, $F_{(1, 31)} = 0.7886$, $p = 0.3814$; treatment, $F_{(1, 31)} = 0.2350$, $p = 0.6312$; CSDS × treatment, $F_{(1, 31)} = 1.781$, $p = 0.1918$; Figure 5H: CSDS, $F_{(1, 31)} = 2.669$, $p = 0.1124$; treatment, $F_{(1, 31)} = 0.0039$, $p = 0.9503$; CSDS × treatment, $F_{(1, 31)} = 0.0372$, $p = 0.8483$). Moreover, heat-sterilized *B. breve* M-16V prevented the increase of M2 microglia-associated chemokine receptor 2 (CCR2) and Ym1 in the PFC and HIP after CSDS exposure (Figure 5I: Two-way ANOVA, CSDS, $F_{(1, 31)} = 7.602$, $p < 0.01$; treatment, $F_{(1, 31)} = 2.578$, $p < 0.01$; CSDS × treatment, $F_{(1, 31)} = 16.05$, $p < 0.01$; Figure 5J: CSDS, $F_{(1, 30)} = 4.101$, $p = 0.0518$; treatment, $F_{(1, 30)} = 3.142$, $p = 0.0864$; CSDS × treatment, $F_{(1, 30)} = 6.764$, $p < 0.05$; Figure 5M: CSDS, $F_{(1, 31)} = 7.491$, $p < 0.05$; treatment, $F_{(1, 31)} = 6.446$, $p < 0.05$; CSDS × treatment, $F_{(1, 31)} = 3.616$, $p = 0.0666$; Figure 5N: CSDS, $F_{(1, 29)} = 9.151$, $p < 0.01$; treatment, $F_{(1, 29)} = 6.004$, $p < 0.05$; CSDS × treatment, $F_{(1, 29)} = 6.448$, $p < 0.05$), whereas there were no differences in the arginase (Arg) and CD206 between the control and CSDS groups with or without heat-sterilized *B. breve* M-16V supplementation (Figure 5K: Two-way ANOVA, CSDS, $F_{(1,}$

$_{31)}$ = 1.172, p = 0.2874; treatment, $F_{(1, 31)}$ = 0.1985, p = 0.6590; CSDS × treatment $F_{(1, 31)}$ = 0.01346, p = 0.9084; Figure 5L: CSDS, $F_{(1, 31)}$ = 9.005, p = 0.0053; treatment, $F_{(1, 31)}$ = 0.1250, p = 0.7260; CSDS × treatment, $F_{(1, 31)}$ = 0.2893, p = 2893; Figure 5O: CSDS, $F_{(1, 31)}$ = 1.649, p = 0.2086; treatment, $F_{(1, 31)}$ = 2.858, p = 0.1010; CSDS × treatment, $F_{(1, 31)}$ = 0.2971, p = 0.5896; Figure 5P: CSDS, $F_{(1, 31)}$ = 0.3506, p = 0.5580; treatment, $F_{(1, 31)}$ = 0.8274, p = 0.3700; CSDS × treatment, $F_{(1, 31)}$ = 0.5268, p = 0.4734).

Next, we examined the microglial morphology in CSDS mice by performing an Iba-1 (microglia marker) immunostaining in the PFC and HIP (Supplemental Figure 4A and 3E). Quantification analyses showed that there were no differences in the number, area, and length of Iba-1-positive cells in PFC and HIP between control and CSDS mice after 33 days of *B. breve* M-16V supplementation (Supplemental Figure 4B: Two-way ANOVA, CSDS, $F_{(1, 8)}$ = 3.992, p = 0.0808; treatment, $F_{(1, 8)}$ = 0.8458, p = 0.3846; CSDS × treatment, $F_{(1, 8)}$ = 2.792, p = 0.1333; Supplemental Figure 4C: CSDS, $F_{(1, 8)}$ = 0.0162, p = 0.9020; treatment, $F_{(1, 8)}$ = 4.456, p = 0.0678; CSDS × treatment, $F_{(1, 8)}$ = 1.320, p = 0.2838; Supplemental Figure 4D: CSDS, $F_{(1, 8)}$ = 0.0464, p = 0.8348; treatment, $F_{(1, 8)}$ = 2.915, p = 0.1252; CSDS × treatment, $F_{(1, 8)}$ = 0.1099, p = 0.7488; Supplemental Figure 4F: CSDS, $F_{(1, 8)}$ = 0.1118, p = 0.7467; treatment, $F_{(1, 8)}$ = 1.580, p = 0.2442; CSDS × treatment, $F_{(1, 8)}$ = 2.275, p = 0.1699; Supplemental Figure 4G: CSDS, $F_{(1, 8)}$ = 4.699, p = 0.0620; treatment, $F_{(1, 8)}$ = 2.012, p = 0.1938; CSDS × treatment, $F_{(1, 8)}$ = 3.195, p = 0.1117; Supplemental Figure 4H: CSDS, $F_{(1, 8)}$ = 8.894, p = 0.0175; treatment, $F_{(1, 8)}$ = 7.219, p = 0.0276; CSDS × treatment, $F_{(1, 8)}$ = 0.4880, p = 0.5046).

Chronic stress is known to disrupt the integrity of the gut barrier, resulting in an increased inflammation in the intestine and abnormal behaviors (Lv et al., 2019). Therefore, we investigated inflammation-related genes, such as IL-1β, IL-6, and TNF-α, in the intestine of CSDS mice. There were no differences in the levels of these cytokines between control and CSDS mice with or without heat-sterilized *B. breve* M-16V supplementation (Supplemental Figure 5A: Two-way ANOVA,

CSDS, $F_{(1, 31)} = 5.008$, $p < 0.05$; treatment, $F_{(1, 31)} = 3.819$, $p = 0.0597$; CSDS × treatment, $F_{(1, 31)} = 4.207$, $p < 0.05$; Supplemental Figure 5B: CSDS, $F_{(1, 31)} = 0.3877$, $p = 0.5381$; treatment, $F_{(1, 31)} = 1.300$, $p = 0.2630$; CSDS × treatment, $F_{(1, 31)} = 0.9921$, $p = 0.3269$; Supplemental Figure 5C: CSDS, $F_{(1, 31)} = 0.1848$, $p = 0.6702$; treatment, $F_{(1, 31)} = 0.7529$, $p = 0.3922$; CSDS × treatment, $F_{(1, 31)} = 0.002605$, $p = 0.9596$). Taken together, our results suggest that heat-sterilized *B. breve* M-16V improves social impairment at least partly by suppressing neuroinflammation induced by CSDS in the PFC and HIP of mice.

Discussion

Recent evidence suggested a crucial role of the gut microbiota in the pathophysiology of MDD. Abnormalities in gut microbiota composition have been shown in patients with MDD and in an MDD mouse model (Bailey et al., 2011; Cryan and Kaupmann, 2005; Jiang et al., 2015). Moreover, fecal transfer of MDD microbiota to the gut flora of control mice resulted in depression-like behaviors (Zheng et al., 2016). In the present study, we show that the homeostasis of the gut microbiota is altered in mice exposed to CSDS (Figure 2), suggesting that alterations of the gut microbiota composition play a role in the pathophysiology of depression-like behavior induced by CSDS.

B. breve M-16V is a probiotic strain commonly used as supplement in baby formula. Some studies have reported that *B. breve* M-16V alleviated allergic disorders and protected premature infants against necrotizing enterocolitis (Kostadinova et al., 2016; Patole et al., 2016). Importantly, not only living but also heat-sterilized *B. breve* M-16V modulated immunity and suppressed the production of inflammatory cytokines (Sugahara et al., 2017). Heat-sterilized, nonviable forms of probiotics are safer, as the risk of secondary bacterial infection is reduced (Taverniti and Guglielmetti, 2011). Therefore, they might be used in a wide range of products and are easy to implement. Here we induced CSDS in mice, resulting in social impairment, as a model of the social withdrawal displayed by patients with MDD (Bagot et al., 2017; Chaouloff, 2013; Wood et al., 2012). A previous study reported that the social behavior deficit induced by 10 consecutive days of CSDS persisted four weeks after the stress (Venzala et al., 2012). Thus, the decreased time spent in the interaction zone in the second session likely reflected a strict impairment in CSDS mouse sociability rather than a learning effect from the recent stress exposure (Venzala et al., 2012). Therefore, CSDS constitutes a reliable model for investigating the pathophysiology of MDD. In this study, the prophylactic effects of heat-sterilized *B. breve* M-16V were assessed on a mild depression-like behavior induced by CSDS for five consecutive days. The supplementation with heat-sterilized *B. breve* M-16V significantly prevented the deficit in social interactions observed two weeks after the last exposure to

CSDS (Figure 3D, E), but had no effect one day after CSDS (Figure 3B, C). Fecal samples are often collected one day after CSDS (Bastiaanssen et al., 2020; Bharwani et al., 2017; McGaughey et al., 2019; Werbner et al., 2019). Thus, we performed the behavioral tests and collected fecal samples for microbiota profiling one day after CSDS. However, fecal sample content at this time point might have reflected changes not only induced by CSDS but also by acute stress. Further work is needed to investigate 1) the microbiota profiling in the fecal samples collected two weeks after CSDS, 2) the prophylactic effect of heat-sterilized *B. breve* M-16V on sustained and severe depression-like behaviors induced by CSDS for 10 consecutive days, and 3) the prophylactic effect of heat-sterilized *B. breve* M-16V on other behavioral tasks, such as the forced swimming and sucrose preference tests. However, our results provide evidence that heat-sterilized *B. breve* M-16V might contribute to CSDS resilience and might constitute an ingredient of functional food preventing MDD.

A strong link between neuroinflammation and MDD has been established (Hashimoto, 2015; Hodes et al., 2015; Wohleb et al., 2016; Yirmiya et al., 2015; Zhang et al., 2016). Excessive activation of M1 and M2 microglia was involved in the pathophysiology of MDD (Kobayashi et al., 2013). Here, CSDS exposure induced an increased expression of four genes, namely the M1-related gene IL-1β and the M2-related genes CCR2 and Ym1 in the PFC and HIP as well as CD206 in the PFC. Heat-sterilized *B. breve* M-16V supplementation significantly prevented the expression of these genes, except CD206 (Figure 5A, E, I, J, L, M, and N). In contrast, there was no difference in the expression of other microglia-related genes in the control and CSDS mice with or without Heat-sterilized *B. breve* M-16V supplementation (Figure 5). Elevated levels of IL-1β are found in postmortem brains of patients with MDD (Raison et al., 2006; Schiepers et al., 2005). An increase of IL-1β in the brain promotes a depression-like behavior in stress models, whereas IL-1 receptor knockout mice do not present a depression-like behavior after CSDS exposure (McKim MD et al., 2018; Wohleb ES et al., 2014). Here, the IL-1β antagonist diacerein significantly attenuated the depression-like behavior induced by CSDS (Supplemental Figure 3). As the inflammatory response

was widely spread across the brain (Figure 5), it is difficult to evaluate whether microinjections of IL-1β into the brain would prevent the protective effect of heat-sterilized *B. breve*. However, our results suggest that heat-sterilized *B. breve* M-16V ameliorates depression-like behavior by suppressing IL-1β expression induced by CSDS. The mechanisms activated by heat-sterilized *B. breve* M-16V to suppress IL-1β expression induced by CSDS remain to be investigated. Interestingly, CSDS mobilizes monocytes in the brain and consequently exacerbates inflammation (Ishikawa et al., 2021; Zhang et al., 2021). CSDS-induced depression-like behavior is caused not only by resident microglial activation but also by the recruitment of monocytes to the brain (Wohleb et al., 2014a). CCR2 and Ym1 play an important role in monocyte recruitment to the brain during inflammatory processes (Ikeda et al., 2018). Although the role of CCR2 and Ym1 in CSDS-induced depression-like behavior is not fully understood, these reports suggest that heat-sterilized *B. breve* M-16V suppresses CSDS-induced inflammation, including IL-1β production, the recruitment of monocytes to the brain by inhibiting CCR2 and Ym1. There was no difference in microglia morphology between control and CSDS mice with or without Heat-sterilized *B. breve* M-16V supplementation (Supplemental Figure 4). A recent study showed that chronic stress-induced microglial proliferation was followed by microglial deactivation and apoptosis (Kreisel T et al., 2014). Therefore, heat-sterilized *B. breve* M-16V might suppress microglia morphological changes at an earlier time point after CSDS.

To understand the mechanism of heat-sterilized *B. breve* M-16V, we investigated whether heat-sterilized *B. breve* M-16V modulates the alteration of the gut microbiota in CSDS mice (Figure 4D, E). Indeed, metabolites modulated by gut microbiota might be involved in brain inflammation. It has been shown that treatment with probiotics affected bacterial metabolic pathway and modulated the host metabolome (Holmes et al., 2012), inhibiting neuroinflammation (Fung et al., 2017; Rothhammer et al., 2016). Circulating bacterial metabolites cross the blood–brain barrier and directly impact neuronal function (Rooks and Garrett, 2016). Alterations of various metabolites were reported in mice treated with heat-sterilized *B. breve* M-16V (Sugahara et al., 2017). Thus, heat-sterilized *B.*

breve M-16V might modulate the immune response in the brain by regulating the metabolism in the gut. The causal relationship between microbiota alteration and depression-like behavior in CSDS mice remains to be determined.

In conclusion, mice exposed to CSDS presented alteration of the gut microbiota composition, increased IL-1β expression levels, and a depression-like behavior. Heat-sterilized *B. breve* M-16V supplementation affected these phenotypes induced by CSDS. Taken together, these results suggest that heat-sterilized *B. breve* M-16V might increase resilience to stress and prevent or alleviate persistent MDD by inhibiting IL-1β expression induced by CSDS. Therefore, heat-sterilized *B. breve* M-16V supplementation might constitute an attractive strategy for MDD prevention.

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Author contributions

AK devised the project and the main conceptual ideas, conducted all the experiments, and wrote the manuscript. KK supervised the work and wrote the manuscript. SA, YS, KS, TI, and TK assisted the experiments. BW, YY, and KS contributed to the manuscript discussion. TN and AM supervised the work and finalized the manuscript.

Figure Legends

Figure. 1 CSDS induces impairment of social interaction.

(A, B): Experimental protocol (A) and apparatus (B) of CSDS. C57BL/6J (adult; 7-week-old) mice were exposed to aggressor ICR mice for 5 consecutive days. Behavioral analyses were performed 1 day after the last stress exposure of CSDS (D6). (C, D): The time spent in interaction (C) and corner (D) zones was measured by the social interaction test. Data are mean±SEM. (n = 11-22 mice each). $^{**}p < 0.01$ compared with control group (Target). $\#p < 0.05$, $\#\#p < 0.01$ compared with CSDS group (No target).

Figure. 2 CSDS alters the gut microbiota composition in mouse feces.

(A): Experimental protocol of gut microbiota analyses. C57BL/6J (adult; 7-week-old) mice were exposed to aggressor ICR mice for 5 consecutive days. Gut microbiota analyses were performed 1 day after the last stress exposure of CSDS (D6). (B): Cladogram based on LEfSe analysis indicating differences at phylum, class, order, family, and genus levels in the feces between control and CSDS mice. (C): Control (green)- and CSDS (red)-enriched taxa represent as a positive and negative-LDA score, respectively. The taxa with an LDA score > 2. Although negativity or positivity is determined by alphabetical order of the groups, the absolute values of the effect size indicate the scale of the difference between 2 groups regardless of the positivity or negativity.

Figure. 3 Heat-sterilized *B. breve* M-16V prevents the impairment of social interaction induced by CSDS.

(A): Experimental protocol of social interaction test. C57BL/6J (adult; 7-week-old) mice were exposed to aggressor ICR mice for 5 consecutive days. C57BL/6J (5-week-old) mice were fed a diet with or without heat-sterilized *B. breve* M-16V starting from 2 weeks (Day 0-14) before CSDS (Day 15-19) to the end of experiments (D33). Behavioral analyses were performed 1 day (D20) or 2 weeks

(D33) after the CSDS. (B, C): The time spent in interaction (B) and corner (C) zones were measured 1 day after the last exposure to CSDS by the social interaction test (two-way ANOVA followed by Tukey's multiple comparison test, interaction zone. (D, E): The time spent in interaction (D) and corner (E) zones were measured 2 weeks after the last exposure to CSDS by the social interaction test. (F): The body weight of each mouse was measured every 3 days to the end of the experiment. Data are mean±SEM. (n = 12-28 mice each) *$p < 0.05$ compared with control group (Target), #$p < 0.05$ compared with CSDS group (Target)

Figure. 4 Heat-sterilized *B. breve* M-16V affected on the alteration of gut microbiota composition induced by CSDS.

(A): Experimental protocol of gut microbiota analyses. C57BL/6J (adult; 7-week-old) mice were exposed to aggressor ICR mice for 5 consecutive days. C57BL/6J (5-week-old) mice were fed a diet with or without heat-sterilized *B. breve* M-16V starting from 2 weeks (Day 0-14) before CSDS (Day 15-19) to the end of experiments (D33). Gut microbiota analyses were performed 1 day after the CSDS (D20). Microbiota profiles in fecal samples from Control, Control + heat-sterilized *B. breve* M-16V, CSDS, and CSDS + heat-sterilized *B. breve* M-16V mice at the family level (n = 5-6 mice each). (B, C): PCoA plots of Bray-Curtis (B) and Jaccard (C) dissimilarity among samples. Ellipses represent 95% confidence (PERMANOVA, Bray-Curtis dissimilarity ($p < 0.01$), Jaccard dissimilarity ($p < 0.01$)). (D): Cladogram based on LEfSe analysis indicating differences at phylum, class, order, family, and genus levels in the feces between CSDS and CSDS + heat-sterilized *B. breve* M-16V mice. (E): CSDS (green)- and CSDS + heat-sterilized *B. breve* M-16V (red)-enriched taxa represent as a positive and negative LDA score, respectively. The taxa with an LDA score > 2. Although negativity or positivity is determined by alphabetical order of the groups, the absolute values of the effect size indicate the scale of the difference between 2 groups regardless of the positivity or negativity.

Figure. 5 Heat-sterilized *B. breve* M-16V suppresses the neuroinflammation induced by CSDS.

(A-D): Effect of heat-sterilized *B. breve* M-16V on the M1 microglia-related gene expressions of IL-1β (A), IL-6 (B), TNF-α (C), and CD68 (D) in the PFC of control and CSDS mice, were analyzed by qRT-PCR 2 weeks after the last exposure to CSDS. (E-H): Effect of heat-sterilized *B. breve* M-16V on the M1 microglia-related gene expressions of IL-1β (E), IL-6 (F), TNF-α (G), and CD68 (H) in the HIP of control and CSDS mice, were analyzed by qRT-PCR 2 weeks after the last exposure to CSDS. (I-L): Effect of heat-sterilized *B. breve* M-16V on the M2 microglia-related gene expressions of CCR2 (I), Ym1 (J), Arg (K), and CD206 (L) in the PFC of control and CSDS mice, were analyzed by qRT-PCR 2 weeks after the last exposure to CSDS. (M-P): Effect of heat-sterilized *B. breve* M-16V on the M2 microglia-related gene expressions of CCR2 (M), Ym1 (N), Arg (O), and CD206 (P) in the HIP of control and CSDS mice, were analyzed by qRT-PCR 2 weeks after the last exposure to CSDS. Data are mean±SEM. (n = 8-9 mice each).

$^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with CSDS group

Supplemental Figure. 1 Severe CSDS also induces impairment of social interaction and alteration of the gut microbiota composition in mouse feces.

(A): Experimental protocol of CSDS for 10 consecutive days. C57BL/6J (adult; 7-week-old) mice were exposed to aggressor ICR mice for 10 consecutive days. Behavioral and gut microbiota analyses were performed 1 day after the last stress exposure of CSDS (D11). (B, C): The time spent in interaction (B) and corner (C) zones was measured by the social interaction test. Data are mean± SEM. (n = 16-18 mice each). (D): Cladogram based on LEfSe analysis indicating differences at phylum, class, order, family, and genus levels in the feces between control and CSDS mice. (E): Control (green)- and CSDS (red)-enriched taxa represent as a positive–and negative LDA score,

581 respectively. The taxa with an LDA score > 2. Although negativity or positivity is determined by
582 alphabetical order of the groups, the absolute values of the effect size indicate the scale of the
583 difference between 2 groups regardless of the positivity or negativity.
584 *$p < 0.05$, **$p < 0.01$ compared with control group.
585
586 **Supplemental Figure. 2 Heat-sterilized *B. breve* M-16V increased the abundance levels of**
587 **Bifidobacterium in mouse feces.**
588 (A): Experimental protocol of gut microbiota analyses. C57BL/6J (5-week-old) mice were fed a diet
589 with or without heat-sterilized *B. breve* M-16V starting from 2 weeks (Day 0-14) before CSDS (Day
590 15-19) to the end of experiments (D33). Gut microbiota analyses were performed 1 day before the
591 first exposure of CSDS (D15). (B): Cladogram based on LEfSe analysis indicating differences at
592 phylum, class, order, family, and genus levels in the feces between control and control +
593 Heat-sterilized *B. breve* M-16V mice. (C): Control (red)- and Control + Heat-sterilized *B. breve*
594 M-16V (green)-enriched taxa represent as a negative and positive LDA score, respectively. The taxa
595 with an LDA score > 2. Although negativity or positivity is determined by alphabetical order of the
596 groups, the absolute values of the effect size indicate the scale of the difference between 2 groups
597 regardless of the positivity or negativity.
598
599
600 **Supplemental Figure. 3 IL-1β inhibitor prevented the impairment of social interaction induced**
601 **by CSDS.**
602 (A): Experimental protocol of social interaction test. C57BL/6J mice were exposed to aggressor ICR
603 mice for 5 consecutive days. C57BL/6J mice were treated with diacerein (20mg/kg, p.o.) daily from
604 2 days (Day -2 - 0) before CSDS (Day 1-5) to the end of experiments (D5). Behavioral analyses were
605 performed 1 day (D6) after the CSDS. (B): The time spent in interaction zone was measured 1 day

after the last exposure to CSDS by the social interaction test (two-way ANOVA followed by Tukey's multiple comparison test, interaction zone. Data are mean±SEM. (n = 7-15 mice each).

Supplemental Figure. 4 No difference in the microglial morphology by CSDS and heat-sterilized *B. breve* M-16V between control and CSDS mice.

(A): Representative images of immunostaining for Iba-1 (red) in the PFC. Scale bar: 100 μm. (B-D): Effect of heat-sterilized *B. breve* M-16V on the number (B), area (C), and process length (D) of Iba-1^+ cells in the PFC of control and CSDS mice, were quantified 2 weeks after the last exposure to CSDS (two-way ANOVA followed by Tukey's multiple comparison test, number. (E): Representative images of immunostaining for Iba-1 (red) in the HIP. Scale bar: 100 μm. (F-H): Effect of heat-sterilized *B. breve* M-16V on the number (F), area (G), and process length (H) of Iba-1^+ cells in the HIP, were quantified 2 weeks after the last exposure to CSDS. Data are mean±SEM. (n = 3 mice each).

Supplemental Figure. 5 No difference in the inflammatory cytokines in the intestine by CSDS exposure and heat-sterilized *B. breve* M-16V between control and CSDS mice.

(A-C): Effect of heat-sterilized *B. breve* M-16V on the inflammation-related gene of IL-1β (A), IL-6 (B), and TNF-α (C) in the intestine of control and CSDS mice, were analyzed by qRT-PCR 2 weeks after the last exposure to CSDS. Data are mean±SEM. (n = 8-9 mice each).

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