Lactobacillus plantarum prevents depressive-like behavior and immune changes evoked by chronic social defeat stress

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2019년 2월

서울대학교 보건대학원

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Abstract

Lactobacillus plantarum prevents depressive-like behavior and prevent immune changes evoked by chronic social defeat stress

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Accumulating evidence shows connection between the microbiota, immune system, and the central nervous system (CNS) in stress-related disorders. This study tested whether gut-microbes can promote serotonergic signaling, and eventually modulate depressive-like behavior, immunoregulation, and gut microbiota in chronic social defeat stress. Gut microbiota can promote the signals to modulate tryptophan hydroxylase-1 (TPH-1) expression in the gut and

subsequent serotonin (5-hydroxytryptamine, 5-HT) biosynthesis. In previous research [1], we identified the specific strain of Lactobacillus which can induce *Tph-1* gene. And we evaluated how oral administration of specific microorganism enhance the stress-resilience of the host and prevent stress-induced depression through aforementioned pathways and investigated the underlying immune regulation mechanism.

We examined the antidepressant ability of *Lactobacillus plantarum* KBL396 (LP), highest 5-HT biosynthesis ability among microorganisms, in chronic social defeat stress model using spontaneous alteration test (SAT), social interaction test (SIT), and tail suspension test (TST). Administration of LP prevented behavioral deficits by social stressor and elevated 5-HT level in the brain. In addition, diminished population in regulatory T cells and dendritic cells in mesenteric lymph node and $CD4^+/CD8^+$ ratio in splenocytes was detected in stress induced groups, while the cell population in LP treated group remained close to that of the control group. Furthermore, chronic social defeat stress altered gut-microbiome composition, but there was no difference in richness and alpha diversity between the groups. Our results suggest that chronic pretreatment of LP can have

antidepressant ability against social stressor, systemic immune regulation ability, and protective gut environment through LP induced microbial community composition.

Key words: *Lactobacillus*, 5-hydroxytryptamine (5-HT), Chronic social defeat stress, Depression, Serotonin, Immune system, Microbiome, Gut-brain axis

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I. Introduction

Stress, which is biologically defined as physical, mental, or emotional factor that causes bodily or mental tension, occurs every day affecting the behavior and well-being of the individual. It is accompanied by the adverse effect on both mental and physical health status, and at severe level, it causes various trauma-related psychopathologies in humans, such as anxiety, post-traumatic stress disorder (PTSD), and depression [2]. In the U.S., Stress and trauma-related disorders have disease burden of approximately $ 50 billion [3]. The major depressive disorder (MDD) or depression, especially, has very high disease burden and is one of the costliest psychiatric disorder affecting lives of 7% of the total population [4, 5]. Unfortunately, much of the etiology and therefore preventative and treatment measures remain unclear, due to the complexity of the disorder. However, a number of promising approaches has been made, such as serotonin and monoamine centered approach, and inflammation centered approach [6, 7].

Animal research has been used, often in conjunction with clinical studies, to test the hypotheses regarding the etiology of depression. In animal studies, the chronic social defeat stress has been widely used as a means of stress exposure, both physical and mental, and inducing

trauma-related depression in mice [8, 9]. Mice exposed to the repeated social defeat were reported to exhibit traumatic social avoidance and depressive-like behavior [10], altered monoamine homeostasis in the brain [11], pro-inflammatory immune response in the brain and GI tract [12-14], neurovascular pathology that promoting depression [15], and the dysbiosis in gut microbiota [16-18]. In accordance with these observations of the result of social defeat stress, there have been efforts to care and cure the disorder by targeting them.

  Due to the recent progress in microbiome science in last decades, it is now well known that the gut microbiota plays both causal and critical role in the regulation of host's immune system [19, 20], and host serotonin biosynthesis [21]. In addition, gut microbiota structure has been shown to correlate with the stress resilience of the host and the development of depression due to the stress exposure; recently, number of studies on the preventative effect of probiotics on the depression has been published [16, 22, 23]. However, despite the fact that probiotics treatment effectively prevents stress-induced depression in these studies, the information on the mechanism of action is still largely unknown. Thus, this study investigated the following questions. 1) Whether *Lactobacillus plantarum* KBL396 (LP) can boost host serotonin

biosynthesis in the brain, 2) Whether LP may enhance the stress-resilience of the host and prevent stress-induced depression through aforementioned pathways, 3) Whether LP may maintain immune homeostasis, and 4) Whether LP may prevent stress-induced dysbiosis in the gut microbiota.

In order to find answers to these questions, LP was selected for its ability on promoting host serotonin biosynthesis and immune regulation. This study investigated whether chronic social defeat stress (CSDS) induced mice with or without LP treatment affects depressive-like behavior. Furthermore, the influence of chronic treatment LP on stress-induced behavior, immune system, and gut-microbiota changes was investigated.

II. Materials and methods

1. Bacterial preparation

  Lactobacillus plantarum KBL396 (LP) was collected from the feces of healthy Korean adults. LP was expended from frozen stock in Man-Rogosa-Sharpe (MRS) broth medium (BD Difco, USA) at 37℃ overnight anaerobically. Frozen stocks (−80 ℃) of LP was activated 2 times of subculture (1% v/v). The LP was given in the drinking water every day for over 28 days prior to CSDS. The mice drank average of 4.4 ml of water per day, which shows daily consumption of approximately 1.9×10^9 colony forming units (CFU) of LP. The C57BL/6 mice consumed 1.9×10^9 CFU of LP and exposed to CSDS for 7days. Overall fresh LP and water were given into C57BL/6 mice daily for 35 days (Fig. 1A and Fig. S1). Viable LP stayed stabled in water as described [24]. Autoclaved water and materials were used in sterilized conditions.

2. Animals

Total 40 male C57BL/6 mice aged 3 weeks treated for 4 weeks so that 7 weeks old (20 g) were used at the start of behavior testing. Twenty six male CD-1 mice (~ 27g), aged 12 weeks, were used as an aggressor in CSDS. C57BL/6 mice and CD-1 mice were purchased from a local vendor (Orient Bio, South Korea). Animals were housed under controlled temperatures and 12-hour light/dark cycles (lights on between 9 AM– 9 PM), with autoclaved food and water. This study was approved by the Institutional Animal Care and Use Committees of Seoul National University (IACUC, SNU-170920-10-2).

3. Behavioral tests

Behavioral tests were performed in the separated place with the instructor during dark phase of the light cycle. Mouse were habituated to the testing environment for more than 30 min. Every test was recorded from above and transferred to a connected PC, which allows instructor to monitor from the separated place.
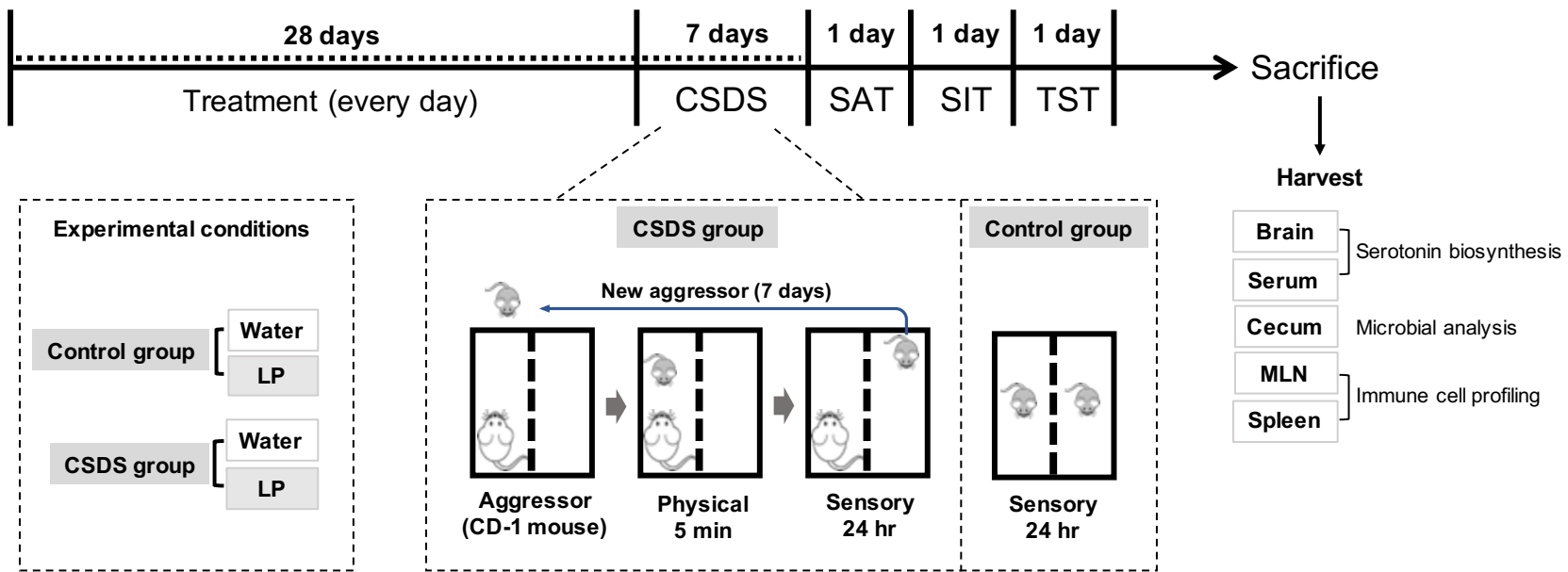
Figure 1. Experimental timeline and illustration of the chronic social defeat stress (CSDS) procedure

Experimental timeline of bacterial treatment for 35 days, followed by CSDS for 7 days and behavior tests. SAT Spontaneous alteration test, SIT Social interaction test, TST tail suspension test. 40 adult mice were used (n=10/group). Control group were housed in the same separation cage, remain undefeated. Behavioral tests were conducted 3 days in a row after CSDS.

4. Chronic social defeat stress (CSDS)

The experimental procedure of CSDS was performed as described in a previous study [8] (Fig. 1). Briefly, the CD-1 mouse was screened and selected with consistent levels of aggressive behaviors. The C57BL/6 mice described above were exposed to a different CD-1 aggressor mouse for 5 min, repeated daily for 7 days. After social defeat stress to C57BL/6 mice in the resident CD-1 aggressor's home cage compartment, the intruder mouse was transferred across the acrylic divider to the opposite half of the cage. Transparent acrylic divider allows visual, olfactory, and auditory contact for the remainder of the 24 hour period, but no physical interactions. On each consecutive 7 days, C57BL/6 mice met new CD-1 mouse to prevent habituation. Control were subject to the same procedure with housing in pairs instead of CD-1 under the same condition. LP treated group and water group did not cross-housed for any reason because of coprophagic behavior. Sterile bedding and water were used throughout the experimental procedure.

6. Spontaneous alteration test (SAT)

SAT was performed in a Y-maze (36 cm long, 3 cm (single arm width), 15 cm (single arm height), 36cm (single arm length), acrylic) with three arms at a 120° angles from each other. Each arm was named A, B and C and C57BL/6 mice were initially placed in one arm (A). The sequence (i.e. ABCBA, etc.) and the number of arm entries were recorded manually for each mouse for 8 min according to the method of Jung et al [25]. An actual alteration was defined as entries into all three arms on consecutive choices (i.e. ABC, CAB, or BCA but not CAC). Maze arm were thoroughly cleaned between the subjects to remove residual odors. Calculation for this test indicated as follows; Alteration (%) = [(number of alterations) / (number of total entries −2)] × 100.

6 Social interaction test (SIT)

Mouse was tested after social defeat using social interaction test [8]. C57BL/6 mouse explored freely alone in a white open field chamber ((W) 42 × (D) 42 × (H) 42 cm, Jeungdo B&P, South Korea) with inverted empty wire cup (Diameter: 10 cm, (H) 10 cm, 1 cm interval) for 180 s (Aggressor absent condition) (Fig. S2). Then C57BL/6 mouse removed from the testing area into a holding cage. After that, a novel CD-1 male mouse was enclosed in the wire cup and the same C57BL/6 mice were put into the acrylic chamber for 3 min (Aggressor present condition). The wire mesh physically separated CD-1 mouse and the defeated C57BL/6 mouse but sensory contact was available. Between every session, open field chamber and wire cup were cleaned with 70 % ethanol and distilled water. Time spent in the interaction zone, which is 8 cm wide area surrounding the wire cup, was counted. Also, the time spent in the corner zone (9 × 9 cm) of the testing area, both corners on the opposite side of interaction zone, was counted with EthoVision (Noldus, USA). Time was counted only the last 2.5 min from each session. The social interaction ratio (SI ratio) is indicated as follows; SI ratio=[(the time spent in the interaction zone with CD-1 present)/(the time spent in the interaction zone absence of CD-1)] [8]

7. Tail suspension test (TST)

The tail suspension test is a behavioral procedure when mice were individually suspended by the tail on a horizontal bar (30 cm above from the ground). Adhesive tape was attached on the tip of the mice tail (2 cm). The movement of C57BL/6 mouse is comprised of agitation and immobility during 6 min of test and performed as a method described by Steru et al [26]. Antidepressant treatment can decrease the time of immobility posture in this test, which is a useful test for assessing the behavioral effects of the treatment [27]. Total immobile time counts only the last 4 min of a 6 min test session and the periods with complete motionless on four limbs. Apparatus was cleaned thoroughly with 70 % ethanol and distilled water after each subject. Observers were blind to the experimental conditions scored the behavioral recordings.

8. Quantitative Real time Polymerase Chain Reaction (qRT-PCR)

For RNA quantification, tissues were homogenized using a TissueLyser II (Qiagen, USA) in Lysis buffer (Easy spin™ Total RNA Extraction Kit, iNtRON biotechnology, South Korea). RNA was extracted according to manufacturer's protocol (iNtRON Biotechnology). cDNA was synthesized with the High Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher Science, USA). To estimate expression of various genes, cDNA was amplified with Power SYBR Green PCR Master Mix (Thermo Fisher Science) according to manufacturer's instructions. GAPDH was measured as a normalizer for each sample. Thermo cycling conditions were hold at 95 ℃ for 10 min, 40 cycles of PCR stage (denaturation at 95 ℃ for 15 min, annealing and extension at 60 ℃ for 1 min), and continuous melt curve stage at 95 ℃ for 15 min and 60 ℃ for 1 min. The relative quantity (ΔΔCt) method was used for calculating the relative quantification of gene expression. All primers were purchased from MacrogenDNA. Primers used in this study are listed in Table 1.

Table 1. List of primers.

Target		Primer sequence
Tph-1	Fw[a]	GGCTTTGAGGTCCTCTTTCCA
Tph-1	Rw[b]	CCCCCTTTCTGAGGAATGGTC
Tph-2	Fw	CAGTCCACGAAGATTTCGACTT
Tph-2	Rw	GCAAGACAGCGGTAGTGTTCT
slc6a4	Fw	TATCCAATGGGTACTCCGCAG
slc6a4	Rw	CCGTTCCCCTTGGTGAATCT
5-HT$_{2a}$	Fw	GAGGCTTCGGAAGTGTTAGCA
5-HT$_{2a}$	Rw	TAATGCAATTAGGTGACGACTCG
GAPDH	Fw	AACTTTGGCATTGTGGAAGG
GAPDH	Rw	GGATGCAGGGATGATGTTCT

[a]Fw represents sequences of a forward primer

[b]Rw represents sequences of a reverse primer

9. FACs and ELISAs

Splenocytes and mesenteric lymph nodes (MLN) were harvested and minced into single cell suspension using a cell strainer (70 μm, Corning, USA) in cold fluorescence-activated cell sorting (FACS) buffer (2 % inactivated fetal bovine serum, 2 mM EDTA in PBS). Cell suspensions were centrifuged 1500 rpm for 5 min at 4 ℃. The pellet was resuspended in FACS buffer. Splenocytes were re-suspended in red blood cell (RBC) lysis buffer for 1 – 2 min. MLN and splenocytes were washed with 9 ml of FACs buffer and centrifuged. Viable cell numbers were counted by adding Trypan blue and diluted with FACs buffer to 5.0×10^5 cells/well in 96 well plate. Intracellular staining was performed according to manufacturer's instructions (BD Biosciences, USA). The cells were incubated with labeled monoclonal antibodies (eBioscience, USA). We validated the flowcytometric identification of $CD3^+$ (T cells), $B220^+$ (B cells), $CD11C^+$ (Dendritic cells), and $CD3^+CD4^+CD25^{hi}Foxp3^+$ Cells (Treg cells) among MLN and spleenocytes. Flow cytometry data were acquired by FACsVerse™ (BD Bioscience) and analyzed by FlowJo (TreeStar, USA).

Whole brain sample was homogenized using a TissueLyser II (Qiagen, USA) in T-PER Tissue Protein Extraction reagent (Thermo Scientific).

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Sample was duplicated for the measure of Pierce^{TM} BCA Protein assay kit (Thermo Fisher, USA) and mouse IL-1b ELISA kit (Sigma-Aldrich, USA).

Blood was collected and waited 30 min in RT for clotting. Clotted sample was centrifuged at 1,800 rpm for 5 min at 4℃. After that, serum supernatant is obtained and stored at -80℃. Sample was duplicated for the measure of BCA Protein assay kit (Thermo Fisher) and Serotonin Ultrasensitive ELISA kit (Eagle BioScience, USA).

10. Fecal DNA extraction and Illumina sequencing

Mouse cecum was stored at −80℃ until used. Bacterial DNA was extracted from cecum using a QIAamp Stool Mini Kit (Qiagen, USA), according to the manufacturer's instruction. For 16S rRNA sequencing, the V3-V4 region of the 16S rRNA gene was amplified using Illumina-adapted universal primers from manufacturer (Illumina, USA). KAPA Library Quantification kit (KAPA Biosystems, USA) was used to quantify the amplicons. The amplicons were purified, pooled in equal quantities, and then sequenced on the Illumina Miseq platform. Analyzed by Quantitative Insights into Microbial Ecology (QIIME) 1.8 software package [28]. The sequences were clustered into operational taxonomic units (OTUs) at 97 % identity. Alpha-diversity indexes (Chao1, Shannon, Simpsons index) were analyzed from OTU table. Taxa profiles were visualized as a heatmap using R package.

11. Statistical analysis

All statistical analyses between two groups were performed in GraphPad Prism 6 (USA) using one-tailed Mann-Whitney U-test, Two-way ANOVAs, with Tukey's multiple comparison were used. In vitro LAB screening results were analyzed using ANOVA (One-way by Tukey's multiple comparison). Behavioral results were analyzed using ANOVA (two-way, Tukey's multiple comparison) in SIT, SI ratio, SAT. Mann-Whitney U-tests were used for analysis in TST, serotonin results, FACs data, qPCR, and microbiome analysis data. The results of the statistical tests are presented within the results section. Results in figures are expressed as mean $\pm$ standard error of the mean (SEM) with 95% confidence limits. P values less than 0.05 ($P < 0.05$) were used and denoted as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

III.   Result

1. Pretreatment of *Lactobacillus plantarum* KBL396 (LP) ameliorates depression-like behavior in Chronic Social Defeat Stress (CSDS)

Since LP increased the most serotonergic activities in the host among other LAB strains in previous research, we examined the effect of chronic LP pretreatment on depression-like behavior after subjected to CSDS (Fig. 1). In the social interaction test without CD-1 mouse, there was no significant difference in total interaction time among the four groups (Fig. 2B). A two-way ANOVA, with stress (control vs. CSDS) and treatment (water vs. LP) as independent variables, demonstrates the time in interaction zone was influenced by the CSDS and the treatment (stress: $F(1,36)=4.843$, $p=0.0342$; treatment: $F(1,36)=11.21$, $p=0.0019$; stress $\times$ treatment interaction: $F(1,36)=6.301$, $p=0.0167$; Fig. 2A). As showed in Fig. 2A, the mice subjected to CSDS spent less time in interaction zone (CD-1 present) in non-treated group but restored in LP treated CSDS group ($p<0.01$). Total interaction time in CD-1 present was significantly decreased in water group after CSDS (Fig. 2A). Control mice and CSDS LP treated mice spent more time with unfamiliar CD-1 than in absent period (Fig. 2A and 2B). LP treated

mice showed increased SI ratio in stressed mice after CSDS compared with water group mice after CSDS (stress: $F(1,28)=3.912$, $p=0.0579$; treatment: $F(1,28)=7.393$, $p=0.0111$; stress × treatment: $F(1,28)=6.089$, $p=0.02$; Fig. 2C). In the TST, CSDS augmented immobility time in untreated group ($p=0.0325$) (Fig. 2D). Fig. 2D shows defeated LP treatment group significantly reduced immobility time when compared to defeated water group ($p=0.0206$). A high rate of alteration indicates that the rodent can remember which arm was entered last. Chronic treatment of LP elevated short-term spatial memory (stress: $F(1,28)=0.0301$, $p=0.8635$; treatment: $F(1,28)=10.54$, $p=0.003$; stress × treatment: $F(1,28)=1.357$, $p=0.2539$; Fig. 2E).
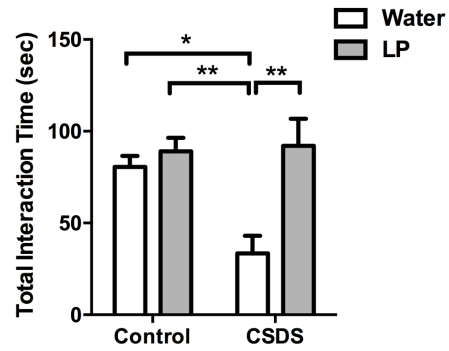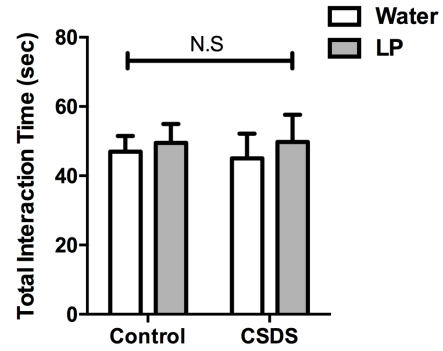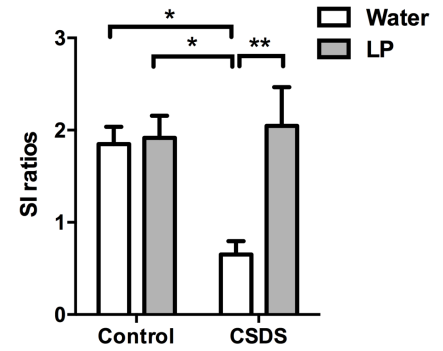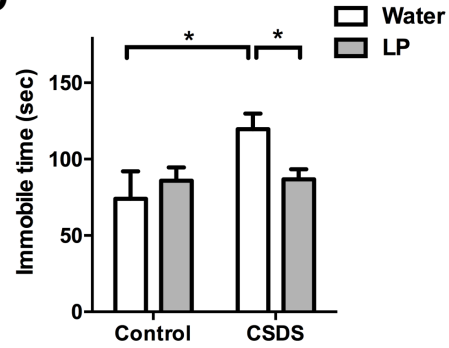
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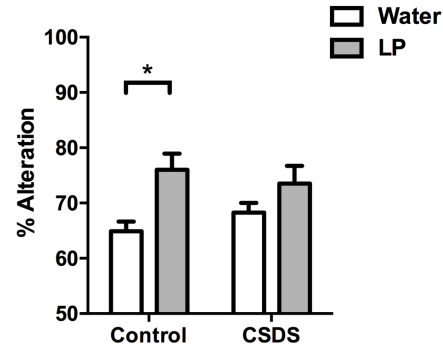
Figure 2. *L. plantarum* **KBL396 (LP) treatment attenuate stress-induced alterations in behavior and enhanced cognitive function**

(A) SIT with aggressor (CD-1). In CSDS LP treated group, there is a significant interaction time increase than CSDS water treated group. (B) SIT without aggressor (CD-1). (C) Social Interaction ratio (SI ratio). (D) TST. CSDS water group spent more time in immobile phase ($p<0.05$). (E) Spontaneous Alteration Test (SAT). Data are represented as mean±SEM. (n=6-8/group) *P<0.05, **P<0.01, ***P<0.001.

2. Chronic *L.plantarum* KBL396 (LP) administration alters serotonin production in Male C57BL/6 mouse

To investigate the serotonin synthesis mechanisms, we examined the changes in expression of genes that are related to 5-HT in the brain. *Tph2* is a rate-limiting enzyme that is entirely responsible for the 5-HT synthesis in the brain and tryptophan (Trp) is converted into 5-hydroxytryptophan (5-HTP), which changed to 5-HT by the amino acid decarboxylase (ADCC) [29]. *Tph2* mRNA level was increased in non-stressed LP treatment group (p=0.0476) (Fig. 3A), which leads to considerable increase in the 5-HT level in brain (p=0.0079) (Fig. 3B). 5-HT tended to increase in CSDS group in fig. 3B (p=0.0754). There was no change in the level of serotonin in serum (Fig. 3C). LP treatment up regulated in serotonin transporter (*slc6a4*) (p=0.0278) and serotonin 2A receptor (*5-HT$_{2A}$*) (p=0.0278) mRNA expression in control group (Fig. 3D and 3E).

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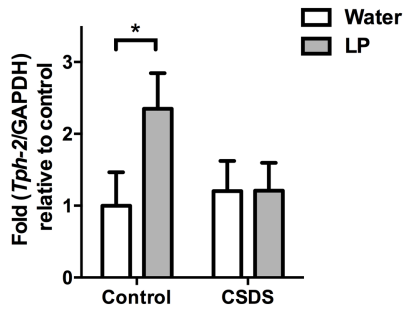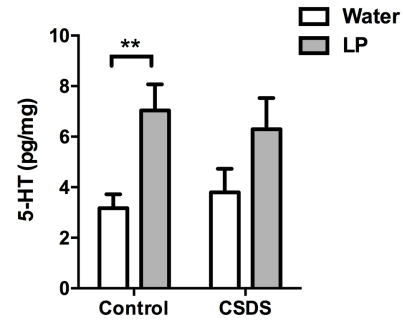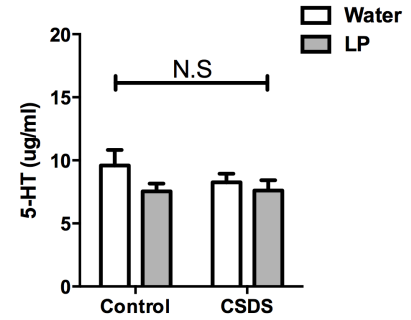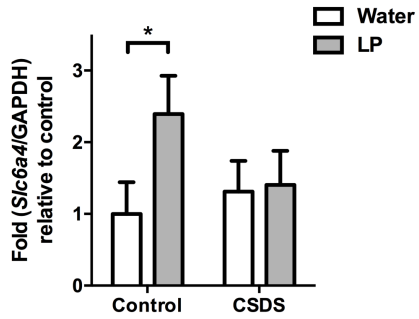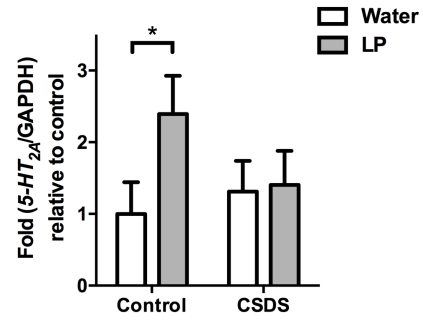
Figure 3. Host 5-HT biosynthesis inducing ability of LP

(A) *Tph-2* expression in the brain. (B) 5-HT (pg/mg) level in the brain. (C) 5-HT (µg/ml) level in serum. (D) Serotonin reuptake receptor (*Slc6a4*) expression in the brain. (E) Serotonin receptor (*5-HT$_{2A}$*) expression in the brain. Data are represented as mean±SEM. (n=5/group) *P<0.05, **P<0.01.

3 LP treatment ameliorates CSDS induced alteration in the immune functions

Immune systems interact as a proposed pathway of the microbiota-gut-brain axis [30, 31]. $CD4^+/CD8^+$ ratio reduction was more increased in stressed water group ($p=0.0079$) than stressed LP treatment group ($p=0.0278$) (Fig. 4A). In CSDS group, LP treated mouse tended to increase in $CD4^+/CD8^+$ ratio ($p=0.0754$) (Fig. 4A). $CD4^+$ T cells were decreased ($p=0.004$) and $CD8^+$ T cells were increased ($p=0.0119$) in stressed control group (Fig. 4B). The alteration in immune function of LP was not restricted to adaptive immune response. MLN DCs were associated with the induction of Foxp3+ Treg cells [32]. $CD4^+CD25^+Foxp3^+$ Tregs in MLN were decreased in stressed non-treated group ($p=0.0278$) (Fig, 5A). $CD11c^+$ DC in MLN were decreased in CSDS water group ($p=0.0119$), while LP treated CSDS group did not decreased to that of control group ($p=0.0203$) (Fig. 5B)
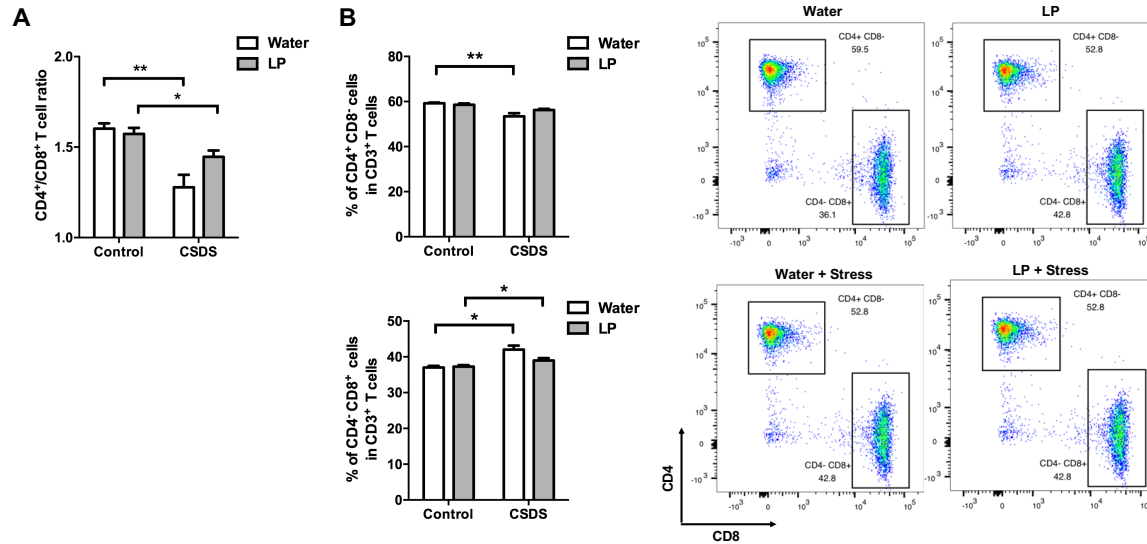
Figure 4. Effect of chronic LP treatment and CSDS on lymphoid cell subtypes

(A) CD4$^+$/CD8$^+$ ratio in splenocytes in mice following exposure to CSDS and LP treatment. (B) Population of T cell (CD4$^+$ CD8$^-$ in CD3$^+$ T cells in splenocytes) and B cell (CD4$^-$ CD8$^+$ in CD3$^+$ T cells in splenocytes). FACS gating strategy for CD4$^+$/CD8$^+$ ratio in splenocytes. Statistical analysis was performed using a one-tailed Mann-Whitney U-test. Data are represented as mean±SEM. *P<0.05, **P<0.01.
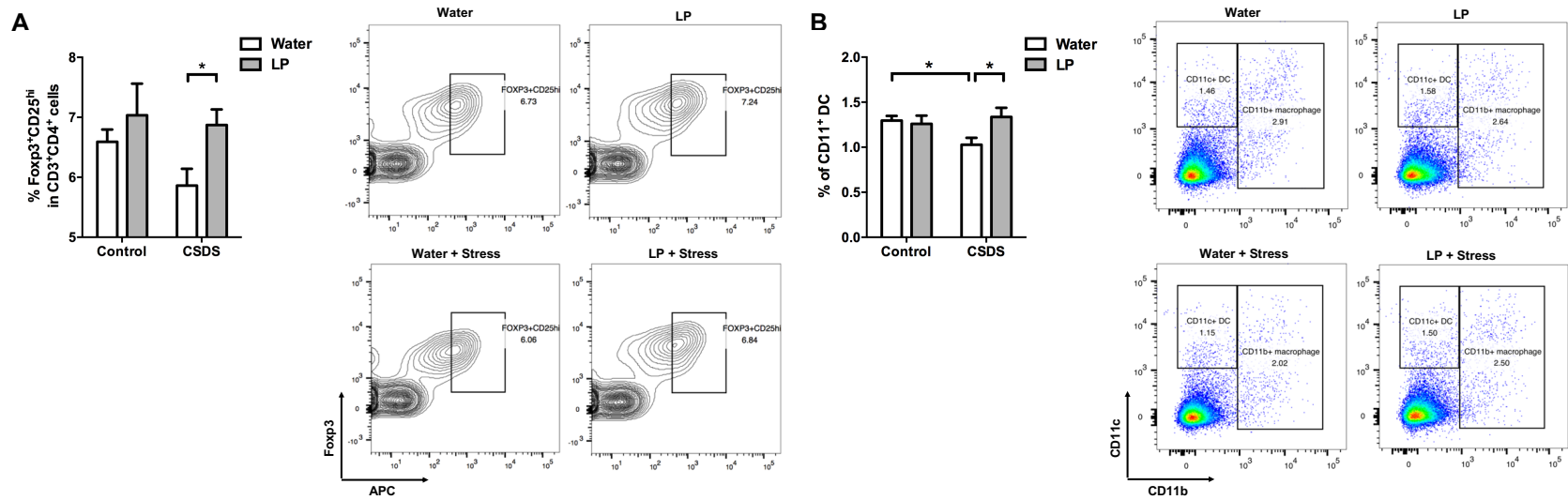
Figure 5. Effect of chronic LP treatment and CSDS on regulatory T cells and dendritic cells

(A) LP treatment prevents stress induced decrease in natural regulatory T cells (CD4$^+$ C25$^+$ Foxp3$^+$ Treg cells) in MLN of CSDS group. (B) CD11c$^+$ DC. The number of CD4$^+$CD25$^+$Foxp3$^+$ Tregs and DCs were calculated based on percentage of total MLN cell counts. Statistical analysis was performed using a one-tailed Mann-Whitney U-test (n=5/group). Data are represented as mean±SEM. *P<0.05.

4. CSDS elicited shifts in microbial communities

According to previous research, chronic stress induces dysbiosis in gut microbiome[17, 18] and certain bacteria treatment can restore microbiota changes[33]. We analyzed the diversity and composition of cecum microbial profile following CSDS or treatment of LP. There was no significant difference in Chao1 richness, Shannon index, and Simpson index (Fig. 6A, B, C). However, Principal component analysis shows a discernable shift in gut microbiota by each factor (Stress vs. treatment; Fig. 6D). Stress group are distinctly clustered from control group (Fig 6D). Regardless of stress, *Lactobacillales* (Water vs. LP: $p<0.0001$; CSDS Water vs. CSDS LP: $p=0.0005$) was significantly increased in LP treated groups (Fig. 7A). *Verrucomicrobiales* was increased in CSDS induced LP group ($p=0.0188$; Fig. 7A). Unclassified genus in S24.7 was considerably increased in stress-induced groups ($p=0.0001$; Fig. 7B). *Akkermansia* was decreased in the stressed water group ($p=0.014$), whereas the stressed LP treated group restored abundance just as control group ($p=0.011$) (Fig. 7B). *Parabacteroides* was decrease in stress-induced group ($p=0.0028$) (Fig. 7B).
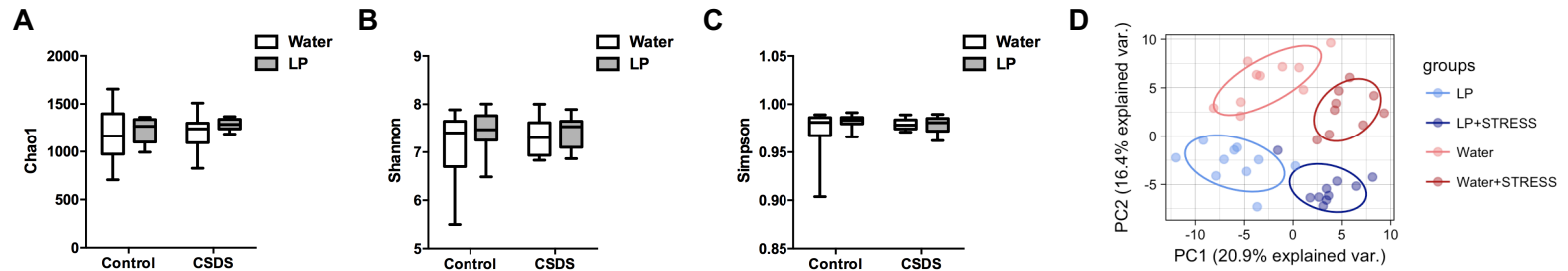
Figure 6. CSDS alters microbial compositions

(A, B, C) The Chao1 richness, Shannon, Simpson diversity index estimated from the rarefied 16s rRNA data. Alpha diversity was not affected by the stress exposure or LP treatment. Data are shown as mean±SEM. (n= 9-10/group) (D) Principal component analysis (PCoA) plot of cecal microbiota structure shows significant shifts in response to CSDS and LP treatment in family level of four groups (LP; LP + Stress; Water; Water + Stress; n=9 -10/group, weighted UniFrac distance). The PCoA plot was based on genus level relative operational taxonomic unit (OTU) data. Data are represented as mean±SEM.
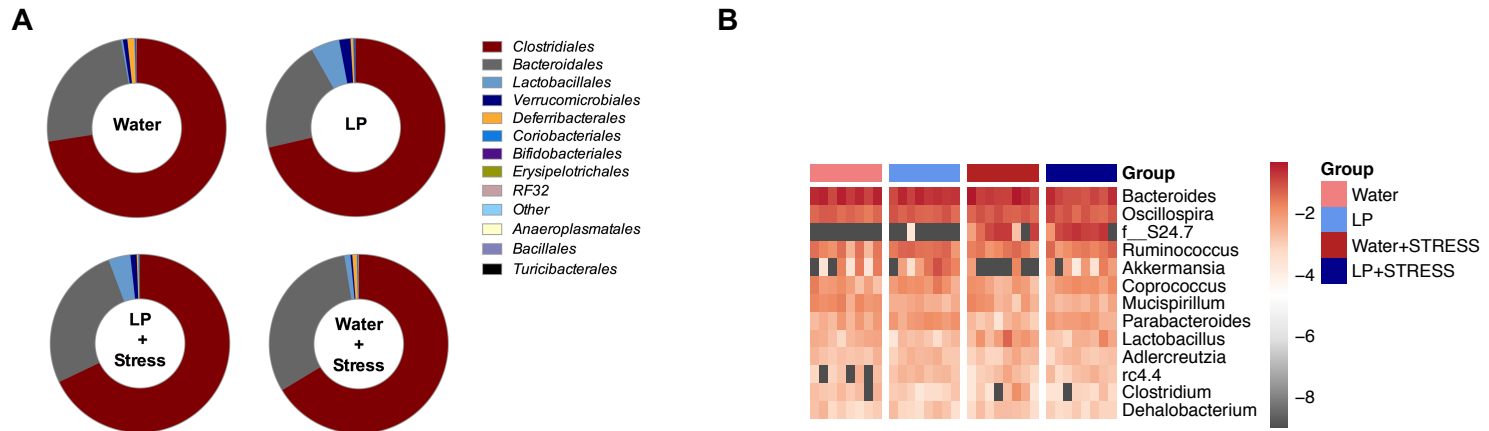
Figure 7. Stress induced structural changes in the microbiota community

(A) Average relative abundance of taxa at the order level. (B) The heatmap of relative abundance of taxa at the genus level (x axis: 9-10 mouse/group; y axis: bacterial genus; OTUs with abundance less than 0.05 % of the total read count was removed).

IV. Discussion

This study exhibits that chronic treatment with LP by oral administration elevates 5-HT level and serotonergic signaling in the mice brain. LP administration alleviates depression-like behavior in CSDS mice and improves spatial memory. Furthermore, LP restores stress-induced imbalance of immune homeostasis and immunoregulatory system. Gut-microbiota was shifted with the stress exposure, and LP treatment. These data indicate the close link between microbiota and bidirectional gut-brain axis.

Previous research validated the ability of LAB to produce host 5-HT biosynthesis [1]. In this study, LP produced the highest level of 5-HT among LAB. Chronic administration of LP promoted 5-HT biosynthesis in the brain, but not in serum. 5-HT is not known to cross the blood-brain-barrier, which explains that 5-HT was synthesized in the brain of LP treated mouse. The serotonergic neurotransmission system [34] and SERT (serotonin transporter) [35] are closely related to anxiety and depression phenotypes. In our research, the level of 5-HT increased with the effect of LP and that of 5-HT receptor and SERT changed. In stressed group, 5-HT level in the brain was tended to increase with the chronic pretreatment of LP. The effect of microbial

pretreatment on the expression of 5-HT receptors was significantly increased in the control group, but there was no effect of microbe treatment in the stressed group. However, the effect of microbial treatment on receptor expression in the prefrontal cortex, nucleus accumbens (NAc) and hippocampus was investigated with the recovery of behavior phenotypes [36]. Serotonergic fibers and mass of serotonergic receptors are expressed are located in hippocampus [37]. These results demonstrate that further gene expression in various brain regions should be examined.

Intriguingly, social avoidance and depressive-like behavior were reversed with the chronic treatment of probiotics. CSDS decreases social behavior and increases social despair behavior [38]. However, chronic pretreatment of LP recovers social interaction time with social aggressor in CSDS induced mice and reduces immobility time in TST. This indicates that chronic intake of LP prevents depression-like behavior in mice and improves spatial memory. In previous research, *Lactobacillus* strain has beneficial effect on anxiety and fear-related behavior [39] and on cognition function [40]. Chronic probiotic intake may be an option for MDD patients − 67 % of cumulative remission rate in multistep treatment [41] and treatment-resistant depression

(TRD), which has insufficient response to multiple antidepressant [42]. The role of neurotrophins contributes to the survival and maintenance of neurons. According to Koponen, E. *et al* [43], rodents with increased *Bdnf* and *Trkb* expression in the brain showed attenuated depressive-like behavior and anxiety-like behaviors. Chronic LP pretreatment increased *Bdnf* and *Trkb* expression in the brain, suggesting that bacteria would be expected to have anti-depressant effect (Fig. S3). The mechanism of microbes modulating the depressive-like behavior is not well known, but bacteria strain itself can control gut-microbiota composition and immune status. These results emphasize the effect of LP exposure on gut-brain signaling, and further assessment in multiple brain regions and neuronal pathways is warranted.

It is well known that, gut-microbiome regulates systemic and intestinal immune system [44]. Similar to our results, peripheral blood CD4$^+$CD25$^+$Treg cells diminished in patients with major depression [45] and stressed mice had a significantly decreased CD4$^+$CD25$^+$Treg cell population compared to non-stressed mice [46]. DCs activates T cells, which can differentiate into regulatory T cells. Alteration of T cells into Foxp3$^+$ Tregs is mediated by DCs and Treg cells in MLN can migrate to the distant regions [47]. Social defeat downregulated DCs and Treg

cells in MLN, which was prevented by chronic LP treatment. Similar to our research, severe stress exposed patients displayed significant reduction in Treg cells [48]. Chronic stress may be relevant to immune dysfunction. Probiotics have a capability to induce regulatory T cells and IL-10 secretion [49]. Consistent with the immunomodulation of gut bacteria, previous studies validated the ability of LP to induce IL-10, anti-inflammatory cytokine, in the LPS induced macrophage cells [50]. Immunomodulation capability of LP may be relevant to stress-resistant behavior. Unlike what we expected, pro-inflammatory cytokines like IL-1b did not reach the statistical significance (p=0.0542) in the brain of CSDS group (Fig. S4). Even though some research shows that SSRI treatment increased the pro-inflammatory cytokines in the brain [51]; chronic LP treatment itself did not altered pro-inflammatory cytokine levels in the brain, suggesting a safer treatment. Pretreatment of LP modulate the host immunoregulatory responses, which may be causative to the anti-depressant effect [52]. Our study is limited to T cells and DCs. Further immune cell types should be assessed in future studies.

Specific bacteria in gut microbiota have a capacity to restore microbial dysbiosis [53]. Severe psychological stress have been

associated with gastrointestinal diseases resulting neurological diseases [54] and imbalance in gut-brain axis can cause mood disorders and depression [40]. In our study, stress did not alter richness of the gut-microbiota but PCA plot reveals distinct clustering of each group. Administration of LP increased in *Lactobacillales*, which led to another gut microbiome composition in CSDS induced group. The relative abundance of *Akkermansia* and *parabacteroides* decreased in the social disruption mouse [55], which was similar to our results. Chronic stress altered the composition of gut microbiota. However, LP administrated group did not shifted to the stressed group composition. Treatment with LP changed the gut microbiota in stressed environment, and changes among groups should be used for further study.

Our study shows the ability of lactic acid bacteria to promote serotonin synthesis; Supplement with LP changes the microbiota to maintain immune homeostasis under stressed condition and shifted microbial composition shows stress resilience and neuroprotective function. Taken together, pretreatment of LP has stress resilience and immuno-modulating effect by modifying gut microbiota composition. Through these findings, further investigation on the effects of LP on depression in humans is needed.

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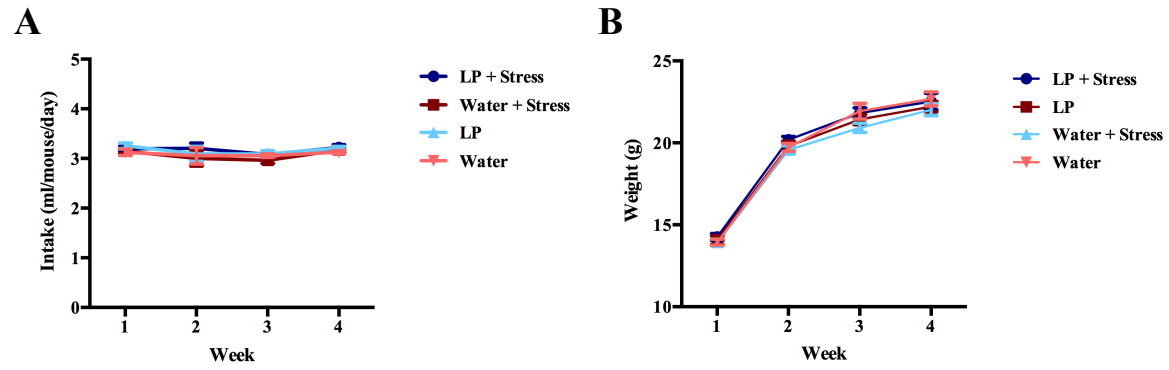
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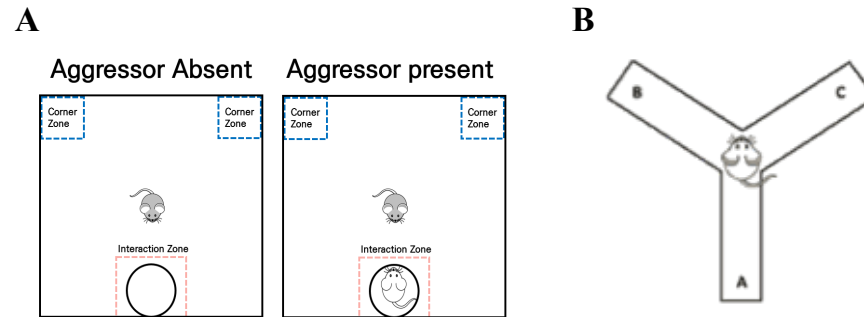
Supplementary Figure 1. Water intake and body weight of mice administrated with *L.plantarum* or drinking water.

(A) Water intake (ml/mouse/day). (B) Body weight. No difference among experimental groups. Data are shown as mean±SEM. (n=10/group).
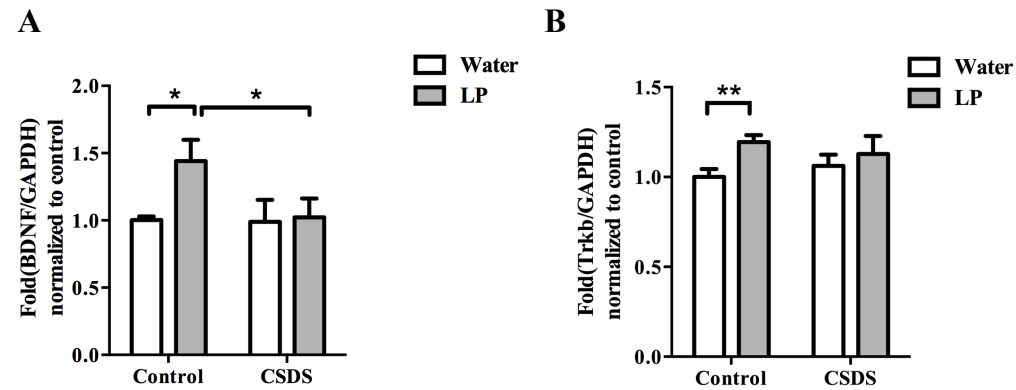
[Appendix 2] Supplementary figure 2

A

Aggressor Absent   **Aggressor present**

B

Supplementary Figure 2. Schematic depiction ot the experimental set up for the social interaction test (SIT) and spontaneous alteration test (SAT)

(A) SIT for evaluation of defeated mice. The behavior was monitored total 6 min (3 min without CD-1 mice and 3 min with CD-1 mice). (B) Y-maze is used to assess SAT for 8 min. Total number of entries and sequence of entries were recorded.

[Appendix 3] Supplementary figure 3
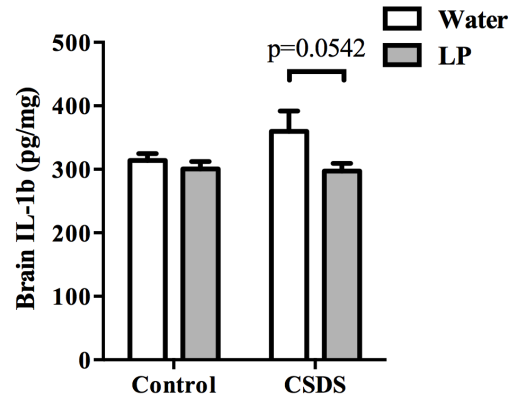

A

B

Supplementary Figure 3. Brain *Bdnf* expression level

(A) *Bdnf* expression is upregulated in the control stressed group. Chronic LP treatment did not show dysregulation in *Bdnf* expression. (B)

Trkb expression in the brain. Data are shown as mean±SEM. (n=5/group). Statistical analysis was performed using Mann-Whitney U-test.
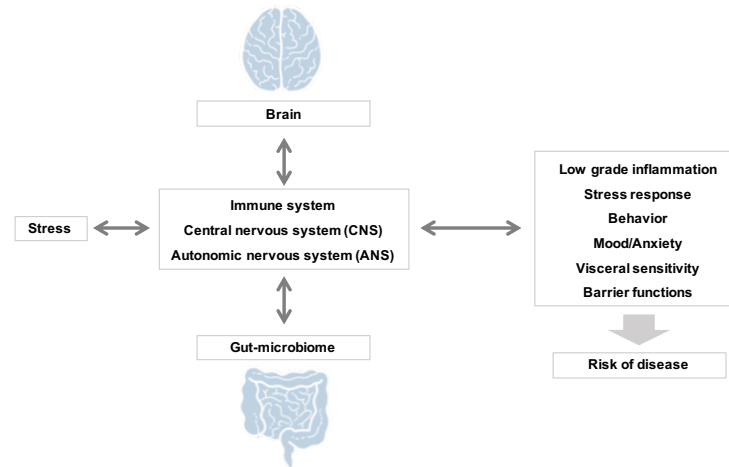
*P<0.05, **P<0.01.

[Appendix 4] Supplementary figure 4

Supplementary Figure 4. Brain IL-1b level

Protein level of IL-1b in the brain didn't reach statistical difference in CSDS group. Data are shown as mean±SEM. Statistical analysis was performed using one-tailed student t-test. (n=5/group).

[Appendix 5] Supplementary figure 5

Supplementary Figure 5. Gut-brain axis

Bidirectional communication between gut and brain. Gut-brain axis plays important role in balance between homeostasis and disease. Alteration in immune system, central nervous system (CNS), and autonomic system (ANS) by gut-microbiome may lead to systemic inflammation, increase in stress response, and increase anxiety and depressive-like behaviors [40].

[Appendix 6] Abbreviation

Central nervous system (CNS)

5-hydroxytryptamine (5-HT)

Social interaction test (SIT)

Tail suspension test (TST)

Spontaneous alteration test (SAT)

Post-traumatic stress disorder (PTSD)

Major depressive disorder (MDD)

Chronic social defeat stress (CSDS)

Mesenteric lymph nodes (MLN)

Phosphate buffer solution (PBS)

Man, Rogosa and Sharpe (MRS)

Fetal bovine serum (FBS)

Fluorescence-activated cell sorting (FACS)

Quantitative Insights into Microbial Ecology (QIIME)

Operational taxonomic units (OTUs)

Principle coordinate analysis (PCoA)

Standard error of the mean (SEM)

Nucleus accumbens (NAc)

Treatment-resistant depression (TRD)

Brain-derived neurotrophic factor (BDNF)

Tropomyosin receptor kinase B (Trkb)

Autonomic nervous system (ANS)

국문초록

락토바실러스 플란타룸의 만성 스트레스 유도로 인한 우울 유사 증상과 면역체계 변화의 예방 효과

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장-뇌 축이란, 장내 미생물과 중추신경계와의 양방향 소통을 의미한다. 장내미생물이 신경망을 통해 뇌에 영향을 주고, 면역세포를 자극하여 뇌와 소통하는 등 장과 뇌 사이에 서로 신호를 주고 받는 다는 연구들이 밝혀지고 있다. 이 연구는 스트

레스로 인한 행동 증상을 장내 미생물이 면역 조절과 세로토닌 생합성 능력을 통해서 조절할 수 있는지를 확인하였다. 한국인 대변에서 분리한 새로운 균주들 가운데, 락토바실러스 플란타룸 KBL396은 선행 연구를 통해 세로토닌 생합성 능력이 가장 높아서 선정되었다. 락토바실러스 플란타룸 KBL396을 35일간 만성적으로 제공하고하였다. C57BL/6 마우스에 7 일간 사회적 패배 스트레스를 유도하였고, 우울 유사 증상 및 공간 기억력을 사회적 친밀감 실험, 꼬리 매달기 실험, 그리고 미로 실험을 이용하여 확인하였다. 사회적 패배 모델을 이용한 우울증 유도 마우스 실험에서 락토바실러스 플란타룸 KBL396을 투여한 그룹은 우울 사회적 친밀도 감소의 예방, 우울 유사 증상의 예방, 그리고 공간 기억에 대한 변화를 확인할 수 있었다. 마우스 뇌에서의 세로토닌의 양, 세로토닌 수용체의 발현량, 세로토닌 재흡수 수용체의 발현량의 변화를 보았다. 사회적 패배 스트레스는 비장의 $CD4^+$/$CD8^+$ T 세포의 비율을 감소시켜 면역력을 낮추지만, 락토바실러스 플란타룸 KBL396 투여군은 비율을 유지하는 결과를 보였다. 생체 내

부적절한 면역반응을 조절 및 감시하는 역할을 하는 조절 T 세포와 항원 전달 세포인 수지상세포의 장간막 림프절에서 비율이 스트레스를 받으면 감소하지만, 락토바실러스 플란타룸 KBL396 투여군에서는 감소를 예방하였다. 마이크로비옴 분석 결과 장내 균 다양성은 그룹 간 차이가 없었지만, 장내 균의 군집 구조가 다르다는 것이 확인하였다. 락토바실러스 플란타룸 KBL396의 투여군은 아커만시아와 파라박테로이데스의 증가를 확인하였다. 스트레스로 인한 면역 체계의 변화와 장내 미생물 군집의 변화는 락토바실러스 플란타룸 KBL396의 35 일간의 투여로 인한 예방 효과를 보여주었다.

주요 단어: 락토바실러스, 5-hydroxytryptamine (5-HT), 만성 스트레스 유도, 우울증, 세로토닌, 면역 시스템, 마이크로비옴, 장-뇌 축

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