보건학석사학위논문

Social defeat stress induced dysbiosis of gut microbiota and its attenuation via *Lactobacillus plantarum* treatment

2017 년 6 월

서울대학교 대학원

환경보건학과 환경보건미생물 전공

최 용 빈

락토바실러스 플란타룸이 스트레스에 의한 장내 미생물총 불균형의 완화에 미치는 효과

지도교수 고 광 표

이 논문을 보건학석사학위논문으로 제출함

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최 용 빈의 석사학위논문을 인준함

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위 원 장 백 도 명 (인)

부 위 원 장 김 성 균 (인)

위 원 고 광 표 (인)

Abstract

Social defeat stress induced dysbiosis of gut microbiota and its attenuation via *Lactobacillus plantarum* treatment

Yongbin Choi

Dept. of Environmental Health

The Graduate School of Public Health

Seoul National University

It is well known that the activation of the sympathetic nervous system and Hypothalamic-Pituitary-Adrenal (HPA) axis induced by a stressor affects many aspects of gastrointestinal tract functions such as GI motility, mucus secretion, and intestinal immune responses. We investigated the effect of social defeat stress on the gut microbiota, their metabolic functions, and host's responses. In addition, we further

investigated whether probiotics could prevent such changes. C57BL/6 mice were treated with *Lactobacillus plantarum* in the form of food pellet for 4 weeks, after which mice were exposed to a social defeat stress for a week. Host side responses were analyzed by qRT-PCR and ELISA, while the gut microbiome was analyzed via sequencing of V4-5 region of 16s rRNA. We observed that exposure to stress alters gut microbiome structure, its metabolic function and host's physiology which were effectively prevented by *L. plantarum* treatment. Here, we characterized bacterial and metabolic components which may deteriorate the symptoms of stress-induced depression through affecting host's serotonergic and immunogenic response. In particular, abundances of *Anaerosipes* spp., *Helicobateraceae*, *Mogibacteriaceae*, *Prevotella* spp., *Suterrella* spp., *Christensenella* spp., and *Parabacteroides* spp. were significantly increased in response to social defeat stress and fully prevented through *L. plantarum* treatment.

Furthermore, microbial metabolic pathways, including glycan biosynthesis were altered in response to the stress exposure. Increased level of LPS may contributes to evoke pro-inflammatory responses which eventually affects host's serotonin biosynthesis. These changes were also effectively prevented through the treatment of *L.plantarum*.

Here, this study reports a beneficial effect of *Lactobacillus plantarum* in host stress resilience and potential microbial-metabolic components ("psycho-pathogens") which affect the pathogenesis of stress induced mental illness.

Key words: Stress, Microbiota, Metabolism, Depression, Serotonin, *Lactobacillus*, Probiotics

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I. Introduction

Due to the radical changes in socio-structure and intensified competition, considerable number of today′s population suffer from mental health issue such as depression and anxiety. Suicidal rate of the South Korea is the highest among that of OECD countries and suicide is the fourth largest cause of death in the Republic of Korea. The burden of mental illness accounts for 21% of the total burden of disease in the Republic of Korea and it is 1.8 times higher than that of cancer. The number of patients with depression in the South Korea exceeds 0.6 million in 2015 and is predicted to keep increasing until 2020. Globally, more than 350 million people are suffered from depression.

There are more than one hundred trillion microbes and more than three million microbial genes exist on the human body which equals to ten times of the number of all human cells and 100 times of human genes each. Gastrointestinal tract is an incredibly dynamic ecosystem of diverse microbes (microbiota) where the microbes directly interact with hosts. It has been reported that human microbiota is associated with various metabolic syndromes, such as obesity[1] and diabetes[2],

immunogenic illness[3], intestinal illness[4], mental illness[5] and through its immuno-regulatory function and diverse metabolic pathway. More recently, the human microbiota is reported to be involved in human behavior, the etiology of mental illnesses ,such as depression, anxiety and autism, and more importantly, the regulation of host serotonin biosynthesis[6]. Probiotics are defined as microorganisms that are believed to provide health benefits when consumed [7]. It has been reported that probiotics have an influence on wide range of diseases and health status such as attenuation of high blood pressure [8], Allergy [9], decrease in respiratory-tract infections [10] and Lactose tolerance [11]. Our knowledge about the beneficial effects of probiotics is now reached to the realm of the ′Gut-Brain-Microbiota axis′, mental illness and host behavior [12-14].

Serotonin (5-hydroxytryptamine, 5-HT) has been reported to be involved in a broad range of mental illness such as depression[15], anxiety[16], panic disorder[17], post-traumatic stress disorder (PTSD)[18], bulimia nervosa[19]. More than 90% of systemic serotonin is synthesized from enterochromaffin cells (EC cells), one type of enteroendocrine cell, and it is known that gut microbiota plays significant role in serotonin biosynthesis of EC cells via both direct and

indirect mechanism [7].

This study investigated ability of lactic acid bacteria in inducing host serotonin biosynthesis and whether treatment of lactic acid bacteria may attenuate the outcomes of the stress exposure via modulation of immuno-serotonergic pathway. In order to answer these questions, the host serotonin biosynthesis inducing ability was investigated in 57 strains of lactic acid bacteria. After the investigation, *in vivo* study was performed in order to analyze the beneficial effects of the gut bacteria followed by gene expression analysis as well as bioinformatic analysis.

Here, this study reports a beneficial effect of Lactobacillus plantarum in host stress resilience and potential microbial-metabolic components (“psycho-pathogens”) which affect the pathogenesis of stress induced mental illness.

II. Materials and Methods

1. Preparation of bacteria and cell-free culture supernatant(CFCS)

21 strains of *Lactobacillus* spp., 2 strains of *Lactococcus* spp. and 34 strains of *Bifidobacterium* spp. isolated from the intestine of Korean population were used in this study (Table 1). Lactic acid bacteria (LAB) were anaerobically cultured for 24 hours at 37°C in de Man-Rogosa-Sharpe (MRS) broth medium (BD DifcoTM) supplemented with 0.05% (wt/vol) L-cysteine (Sigma-Aldrich) to promote the growth of lactic acid bacteria. After performing 2 times of subculture (1% v/v) to activate LAB, culture supernatants were collected to perform down-stream *in vitro* assay. The supernatant of the culture medium was collected by centrifugation at 13,000rpm for 5 min, neutralized to pH 7.0 by the addition of 1 N NaOH. The supernatants were then passed through a 0.22-μm pore size filter unit (Millipore Co., Italy) and stored at −20°C until use.

Table 1. Lactic Acid Bacteria strains used in this study.

Lactic Acid Bacteria species	Strain	No. of isolates
Bifidobacterium animalis	KBL_500 KBL_501 KBL_612 KBL_613 KBL_637 KBL_639 KBL_640 KBL_642 KBL_646 KBL_648 KBL_649 KBL_660 KBL_662 KBL_663 KBL_664 KBL_665	16
Bifidobacterium bifidum	KBL_470 KBL_481 KBL_483 KBL_487 KBL_497	5
Bifidobacterium breve	KBL_621 KBL_624 KBL_633	3
Bifidobacterium longum	KBL_538 KBL_544 KBL_545 KBL_585 KBL_591 KBL_600 KBL_605 KBL_631 KBL_652 KBL_654	10
Lactobacillus acidophilus	KBL_402 KBL_409	2
Lactobacillus casei	KBL_382 KBL_384 KBL_385	3
Lactobacillus fermentum	KBL_374 KBL_375	2
Lactobacillus gasseri	KBL_342 KBL_381	2
Lactobacillus paracasei	KBL_383	1
Lactobacillus plantarum	KBL_396	1
Lactobacillus reuteri	KBL_346	1
Lactobacillus rhamnosus	KBL_351 KBL_354 KBL_362 KBL_363 KBL_365	5
Lactobacillus salivarius	KBL_389 KBL_391 KBL_395 KBL_397	4
Lactococcus lactis	KBL_418 KBL_419	2
Total		**57**

2. Culture of RIN14B cell line

RIN14B cells were cultured in RPMI1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Subculture of RIN14B cells was routinely performed when a confluency was above 90%. When 90% confluency was reached, growth medium was discard by suction and washed with 1X PBS. 1X Trypsin was added and cells were incubated for 10 minute at 37 °C, 5 % CO_2 incubator. Suspended cells were collected and centrifuged at 730 rpm for 3 min. Supernatnat was discarded by suction and the pellet of cells was resuspended with 10ml of RPMI1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. 1ml of cells were seeded into new T175 flask (SPL Life Sciences Co., Ltd.) with 25ml of culture medium.

3. *In vitro* TPH-1 expression assay

RIN14B cells were seeded in 24-well plates at the rate of 4.5 × 10^5cells/0.5 mL RPMI1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin /well and cultured for 24 h. The medium was removed before washing the cells with HBSS containing 0.1% BSA and 2 μM fluoxetine (Tocris). The HBSS was again removed and replaced with 0.4 ml CFCS, MRS as a negative control and MRS containing 25uM deoxycholate as a positive control, after which the solution was incubated further for 1 hour at 37 °C. Supernatants were collected and centrifuged for 5 min to remove any detached cells. The supernatants were collected and stored at −80 °C until 5-HT measurement using a serotonin ELISA kit (5-HT enzyme-linked immunosorbent assay kit, Eagle Bioscience). Remaining adherent RIN14B cells were lysed with 0.5ml of lysis buffer/well (easy-spin™ Total RNA Extraction Kit, Intron biotechnology). Then, total RNA was extracted according to protocol provided by manufacturer (easy-spin™ Total RNA Extraction Kit, Intron biotechnology) and converted to cDNA using High Capacity RNA-to-cDNA kit (applied biosystems, Thermo Fisher Science) for further gene expression analysis using quantitative real-time PCR (qRT-PCR).

4. Quantative Real Time Polymerase Chain Reaction (qRT-PCR)

To estimate expression of various genes, Rotor-Gene SYBR Green PCR Kit (Qiagen) were used. GAPDH was used as an internal control. Thermo-cycling conditions were hold at 95°C for 5 min and 40 cycles of denaturation at 95°C for 5 sec, with annealing and extension at 60°C for 10 seconds. Primers used in this study are listed in Table 2. The $2^{(-\Delta\Delta C_T)}$ method ($\Delta\Delta CT=(C_{T,Target}-C_{T,Ref})_{treated}-(C_{T,Target}-C_{T,Ref})_{control}$) was used (where C_T is the threshold cycle) for calculating the relative gene expression

5. Animals

Conventional C57BL/6 (3 weeks old) male mouse were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were maintained on a 12 hours light/dark cycle with lights on at 9 a.m. Mice were housed 3 per cage and cages were randomly assigned to control or experimental group. Animal experiment protocols were approved by the Seoul National University Institutional Animal Care and Use Committees (IACUC). 6 mice were assigned to each experimental group.

Table 2. List of primers for gene expression analysis

Name	Primer sequence (5' -> 3')	Target
V4_F	GCC AGC AGC CGC GGT AA	16S_V4
V4_R	GAC TAC CAG GGT ATC TAA T	
Lc_F	TGAAGAATTGATGGAACTCG	elongation factor Tu (tuf) gene
Lc_R	CATTGTGGTTCACCGTTC	
LPrecAF	GTGGTGCGGTCGATATTTTAGTT	recA gene
LPrecAR	TCAGCCGCGCTTGTAACC	
Tph-1_F	AACAAAGACCATTCCTCCGAAAG	Mouse TPH-1
Tph-1_R	TGTAACAGGCTCACATGATTCTC	
GAPDH_F	AACTTTGGCATTGTGGAAGG	Mouse GAPDH
GAPDH_R	GGATGCAGGGATGATGTTCT	
TNFa_F	TCTCATGCACCACCATCAAGGACT	Mouse TNFa
TNFa_R	TGACCACTCTCCCTTTGCAGAACT	
IL-1b_F	AAGGGCTGCTTCCAAACCTTTGAC	Mouse IL-1b
IL-1b_R	ATACTGCCTGCCTGAAGCTCTTGT	
IL-18_F	GCCTCAAACCTTCCAAATCA	Mouse IL-18
IL-18_R	TGGATCCATTTCCTCAAAGG	
NF-kB_F	TTCTGAACTAAGTTGCGTTGTGCTG	Mouse NF-kB
NF-kB_R	CACGGTCTGGGAACTCTGGAA	
IL-6_F	TTCCATCCAGTTGCCTTCTT	Mouse IL-6
IL-6_R	CAGAATTGCCATTGCACAAC	
IL-10_F	GGTTGCCAAGCCTTATCGGA	Mouse IL-10
IL-10_R	ACCTGCTCCACTGCCTTGCT	
IDO_F	ACTGTGTCCTGGCAAACTGGAAG	Mouse IDO
IDO_R	AAGCTGCGATTTCCACCAATAGAG	
SLC6A4_F	TATCCAATGGGTACTCCGCAG	Mouse SLC6A4 (serotonin transporter)
SLC6A4_R	CCGTTCCCCTTGGTGAATCT	

'F' and 'R' in the name of primers represents 'Forward' and 'Reverse' respectively.

6. Preparation of Lactic Acid Bacteria (LAB) containing food pellet

LAB were cultured as described above. After the activation of bacterial cells via two times of subculture (1% v/v), the culture of bacteria was inoculated into new MRS broth and further cultured for 8 to 10 hours according to growth-rate of each bacteria. Cultured bacteria were then centrifuged at 8000rpm, 4℃ for 20 min. Supernatant was discard and the pellet was resuspended with ice-cold 1X PBS after which the resuspended solution was centrifuged and PBS washed again as described. Pellets were resuspended with ice-cold 1X PBS as desired volume for further kneading step. Radiated food pellet were grinded with a blender and mixed with the bacteria containing PBS solution. The mixture was then kneaded and made into ball-shape food pellets (‘Bacto-balls’; ‘Lacto-balls’). Bacto-balls were then deep freezed at -80℃ for 24 hours, followed by 48 hours of lyophilization. Lyophilized Bacto-balls were stored at -80℃ until provided to mice.

7. Social defeat stress

After 4 weeks of *Lactobacillus plantarum* treatment, mice were exposed to social defeat stress as previously described[20]. ICR mice, selected on the basis of their attack latencies were used as aggressive residents. Briefly, C57BL/6J mice, aged ～8 wk, were exposed to a different ICR aggressor mouse each day, 5 min/day for 7 days. Each C57BL/6J mice were moved to different cage and exposed to different ICR mice every day(Figure 2).

8. Serum serotonin measurement

Blood sample were collected by cardiac puncture by using 3ml syringe with needle (309570, BD) according to general protocol [21]. Serum was separated from whole blood by allowing the blood to clot at room temperature for 30 minutes, then centrifuging at 8000 rpm for5 minutes at 4℃. The resulting serum supernatant was then stored in -20℃ until use. Serum levels of serotonin were measured using commercial ELISA assay (Eagle Biosciences, USA) according to the manufacturer's instructions.

9. **Animal tissue sampling and analysis**

Since serotonin biosynthesis in the intestine has been reported to be restricted to colon, which is also verified by our preliminary test(supplementary figure 4.), colon tissues were sampled to evaluate the serotonin biosynthesis of each mice. In addition, cecum samples were used to evaluate the composition of the gut microbiome because it is home to the commensal microbes[22].

To evaluate the host's immune response, Messenteric Lymph Node (MLN) and Peyer's patch (PP) were sampled. MLN and PP has been known to represent a systemic immune responses and enteric immune responses respectively[23, 24].

Brain tissues, including Hippocampus, Nucleus Accumbens and Dorsal Raphe Nucleus, were also sampled to evaluate serotonin biosynthesis and immune response in the brain.

10. **Bioinformatics and statistical analysis**

Linear discriminant analysis (LDA) effect size (LEfSe) was performed to discover specific microbial biomarker and characterize statistically significant differences between groups. Web-based galaxy module was used for LEfSe analysis. In this process, the Kruskal-Wallis and Wilcoxon test were used and threshold of logarithmic LDA score was set to 3.0[25].

Comparing microbial community between samples, principle coordinate analysis (PCoA) was performed using the *ggbiplot* package in R. To evaluate metabolic function of each microbiota, PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) was performed in the galaxy web-based platform [26]. For calculating relative gene expression, the $2^{(-\Delta\Delta C_T)}$ method ($\Delta\Delta C_T=(C_{T,Target}-C_{T,Ref})_{treated}-(C_{T,Target}-C_{T,Ref})_{control}$) was used (where C_T is the threshold cycle).

All data were analyzed with Prism 5 (GraphPad Software, San Diego, CA). Statistical significance was measured using Mann-Whitney test when comparing two groups, two-way ANOVA with Bonferroni post-tests comparing various groups. In all graphs, data were presented as mean ± sem.

III. Results

1. TPH-1 expression can be induced by lactic acid bacteria

Recently, it is reported that the gut microbiota regulates the host's serotonin biosynthesis[6]. On the basis of this discovery, we wanted to know whether lactic acid bacteria can induce the host serotonin biosynthesis. To do this, we evaluated the ability of lactic acid bacteria in inducing TPH-1 (Tryptophan hydroxylase – 1, rate limiting enzyme of serotonin (5-hydroxytryptamine; 5-HT) biosynthesis) expression in RIN 14B cell line. It is well known that colonic enterochromaffin cells are responsible for synthesizing more than 95 % of serotonin in humans [27]. RIN 14B cell line is well-established model cell line of the enterochromaffin cells [28]. Thus, we used RIN 14B cell line and see if lactic acid bacteria can induce TPH-1 expression or not. Total of 57 strains of lactic acid bacteria (Table 1.) were tested and 8 strains, one strain of *Lactobacillus plantarum* (KBL396), two strains of *Lactococcus lactis* (KBL418 and KBK419) and three strains of *Bifidobacterium bifidum* (KBL470, KBL483 and KBL487), showed significant TPH-1 inducing ability (Figure 1.). The assay was performed three times independently and duplicate each time .

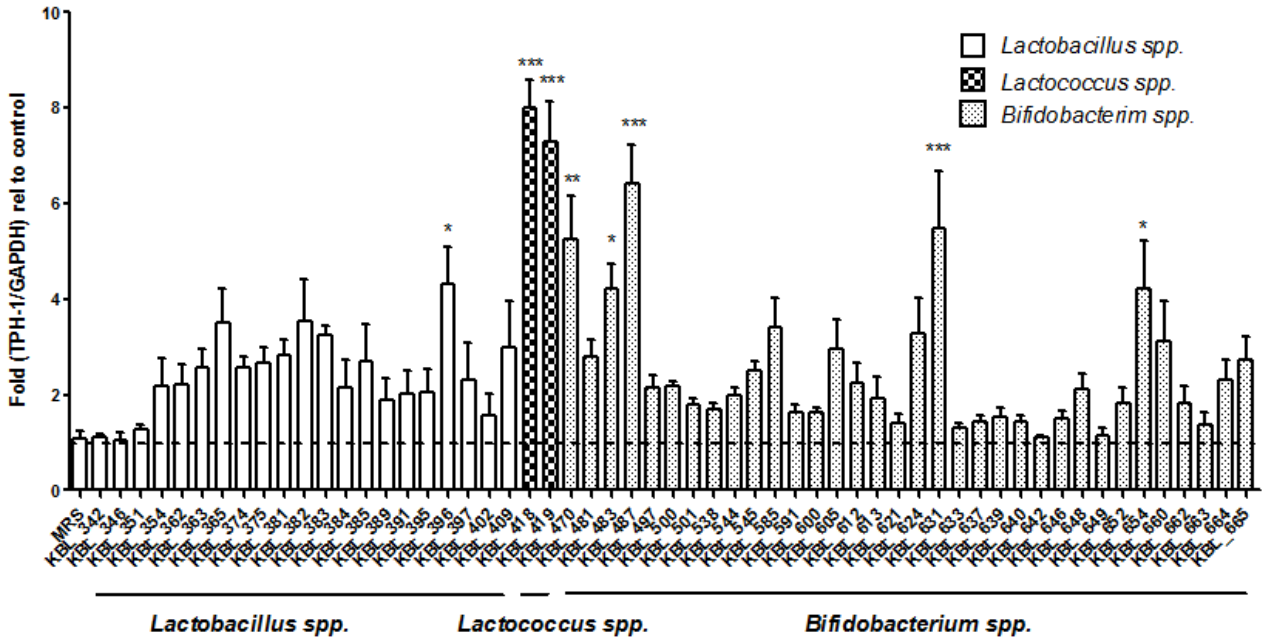

Figure 1. Lactic acid bacteria induce TPH-1 expression in RIN14B cell line

TPH-1 (Tryptophanhydroxylase-1) expression induced by CFCS (Cell Free Culture Supernatant) of *Lactobacillus spp.*, *Bifidobacterium spp.* and *Lactococcus spp.*. Fold expressions were measured by qRT-PCR and calculated by delta-delta C_t analysis. *p < 0.05, **p < 0.01, ***p < 0.001 compared to MRS medium control, One-way ANOVA followed by the Dunns test. Data are represented as mean ± SEM

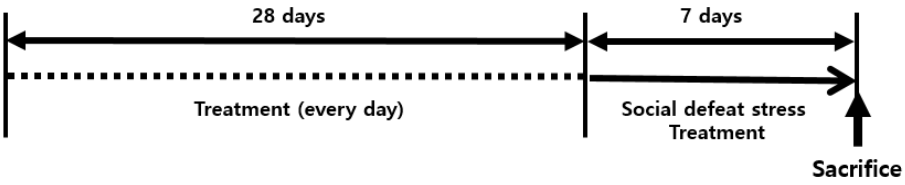

Figure 2. Schematic diagram of experimental approach

Lactobacillus plantarum was provided in the form of 'Lacto-ball' every day for 35 days. After 28 days of treatment, mice were exposed to social defeat stress (SDS) for 7 days and then sacrificed for further analysis. (N=6)

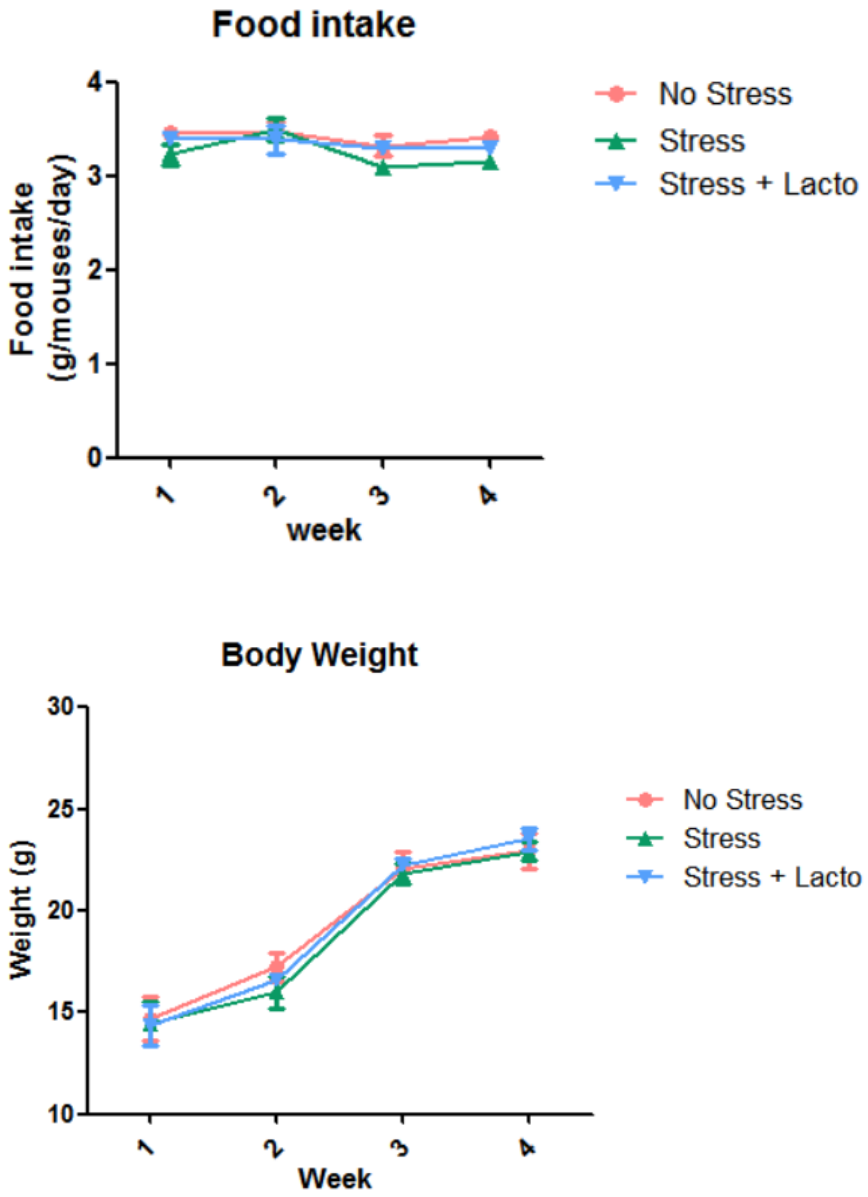

Figure 3. Food intake and Body weight among the mice of experimental groups

No significant difference in both food intake and body weight is observed among the experimental groups (N=6/group). Data are represented as mean ± SEM

2. **Social defeat stress alters the gut microbiome**

When systemic level of serotonin is dysregulated, various mental illness arises. In particular, the link between serotonin level and depression has been established [15]. Considering the role of serotonin in depression and the fact that depression can be induced by stress exposure, we design an experiment to see if lactic acid bacteria can prevent the depression which resulting from a social defeat stress (Figure 2.). Social stress is a risk factor for common psychopathologies such as depression [29]. To investigate the social stress-induced shift in gut microbiome, metabolic function and host's gene expression pattern, we examined the immunogenic, serotonergic effects of the social defeat stress model of depression [30].

Since mice change their diet from weaning to feeding at 3 weeks old period, we treated 3 weeks old mice with *Lactobacillus plantarum* for 4 weeks to maximize the effect of the intervention. There were no significant differences in food intake and weight change due to the addition of bacteria in the food pellet (Figure 3.). After the treatment, mice were exposed to the social defeat stress for a week. Interestingly, the exposure to social defeat stress significantly altered the microbiome structure (Figure 4.) The beta diversity of gut microbiota of each sample was calculated via Principal Coordinates Analysis (PCoA). The result

was plotted on two dimensional space with PC1 (Principal Coordinate 1) and PC2 (principal Coordinate 2) as two-axis, each explains 15.1 % and 11.3 % of the variance. In particular, the abundance of *Prevotellaceae* (family), *Prevotella* (genus), *Proteobacteria* (phylum), *Burkholderiales* (order), *Sutterella* (genus), *Alcaligenaceae* (family), *Betaproteobacteria* (class), *Epsilonproteobacteria* (class), *Helicobacteraceae* (family), *Campylobacterales* (order), *Bilophila* (genus), *Peoptococcaceae* (family) was significantly higher in stress-exposed group and the abundance of *Desulfovibrio* (genus), *Coriobacteriaceae* (family), *Coribacteria* (class), *Actinobacteria* (phylum), *Adlercreutzia* (genus), *Coriobacteriales* (order), *Lactobacillus* (genus), *Lactobacillaceae* (family), *Bacilli* (class), *Lactobacillales* (order) was significantly higher in control group which did not exposed to social defeat stress (Figure 5.).

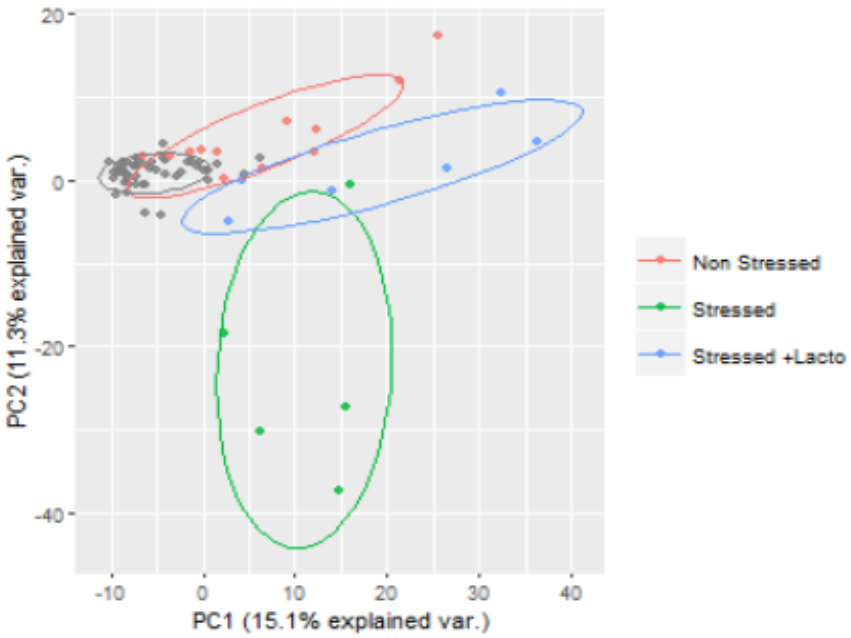

Figure 4. Principal Coordinate Analysis (PCoA) plot shows significant differences among the gut microbiome of Non-stressed group, Stressed group and Stressed, but *Lactobacillus plantarum* treated, group

The PCoA plot was generated based on genus level-relative operational taxonomic unit (OTU) data in Non-stressed group, Stressed group and Stressed, but *Lactobacillus plantarum* treated, group. Principal Coordinate 1 explains 15.1% of variance while Principal Coordinate 2 explains 11.3 % of variance. Gray plots represents data from non-stressed stool samples. Gray dots represent stool samples and colored dots represent cecum samples.

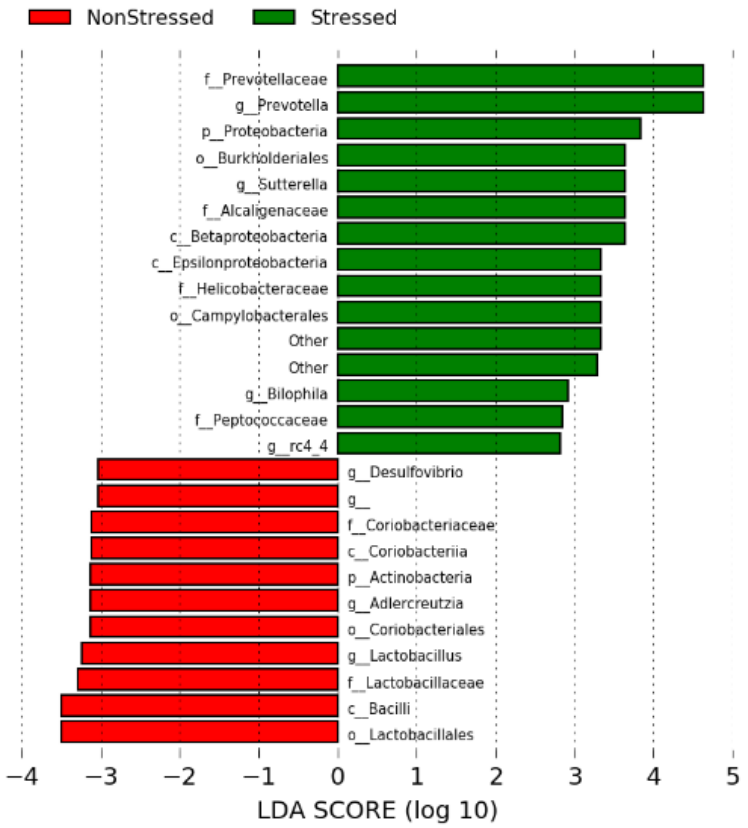

Figure 5. Significant bacterial components associated with stress exposure status. Characterization of significant abundance changes by the stress exposure was identified by LEfSe (LDA Effect Size). Statistical analysis was performed using the Kruskal-Wallis test (among classes) and Wilcoxon test (between subclasses) as a p value < 0.05 in both tests. The threshold logarithmic LDA score was set to 3.0.

3. **Treatment of *Lactobacillus plantarum* effectively attenuate the social stress induced dysbiosis of gut microbiome**

Social defeat stress can significantly shift the microbiota structure by affecting various bacteria at various levels of phylogeny (Figure 5.). Interestingly, this dysbiosis of gut microbiota was prevented through *Lactobacillus plantarum* treatment. PCoA analysis showed that stress exposure significantly shifted the gut microbiota structure, but this shift was effectively prevented by treating *Lactobacillus plantarum* (Figure 4, Figure 6). In particular, the abundances of bacteria within *Anaerosipes* spp., *Helicobateraceae, Mogibacteriaceae, Prevotella* spp., *Suterrella* spp., *Parabacteroides* spp. *and Christensenella* spp. that were increased in response to social defeat stress, were significantly reduced to the level of non-stressed control (Figure 7.). Given that the relative abundance of each bacteria, *Prevotella* spp., *Suterrella* spp. *and Parabacteroides* spp. outweighs that of other bacterial species, these bacteria may play a significant role in the pathogenesis of stress-related diseases. On the other hand, a bacterial abundance of *Lachnospiceae sp.* was reduced in response to social defeat stress and recovered by the treatment of *Lactobacillus plantarum* (Figure 7.).

4. **The metabolic-functional profiles of microbial communities were altered via social defeat stress exposure and the treatment of *Lactobacillus plantarum*.**

In accordance with changes in microbial communities due to social defeat stress, this study evaluated the metabolic functional profiles of each microbiota. The exposure of social defeat stress with or without the treatment of *L. plantarum* altered the metabolic functions (Figure 8.). Various metabolic pathways, including glycan Biosynthesis, tyrosine metabolism, fatty acid biosynthesis, glycan biosynthesis and metabolism, lipid biosynthesis protein, vitamin B6 metabolism and Carbohydrate digestion and absorption, were increased in response to social defeat stress, while metabolisms such as ascorbate and aldarate metabolism, flavonoid biosynthesis, linoleic acid metabolism, galactose metabolism and D- arginine and D- ornithine metabolism were reduced after the exposure to social defeat stress. These changes in metabolic function of microbiome did not observe in *Lactobacillus plantarum* treated group which implies that the treatment of *Lactobacillus plantarum* may prevent the stress-induced alteration of microbiota and thus prevents alterations in metabolic functions of microbiota.

Figure 6. Heatmap of intestinal microbes shows significant among in stressed vs non-stressed and stressed vs stressed w/ *lactobacillus* treated groups.

Whether each sample was exposed to stress or treated with *Lactobacillus plantarum* was annotated at top. Heatmap was generated using log transformd-genus level-relative operational taxonomic unit (OTU) data.

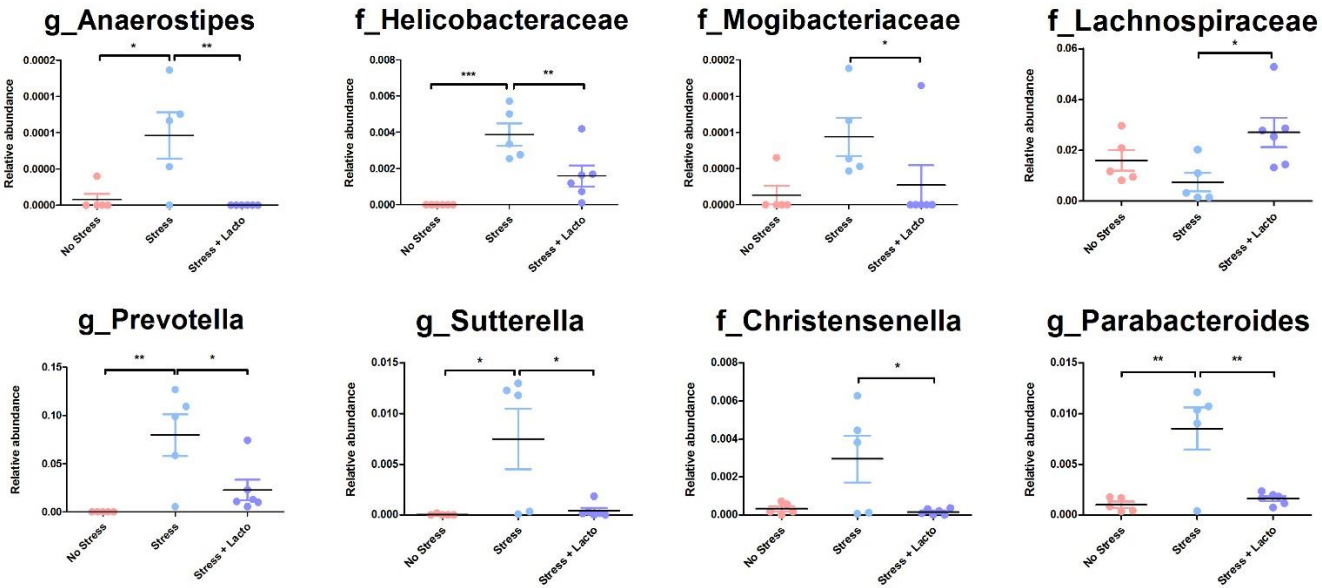

Figure 7. Bacterial abundance which are significantly different among experimental groups.

To identify which bacterial components account for the microbial alterations, LEfSe analysis was performed and individual bacterial genus was plotted. For LEfSe analysis, the Kruskal-Wallis and Wilcoxon test were used and threshold of logarithmic LDA score was set to 3.0. *p < 0.05, **p < 0.01, ***p < 0.001.

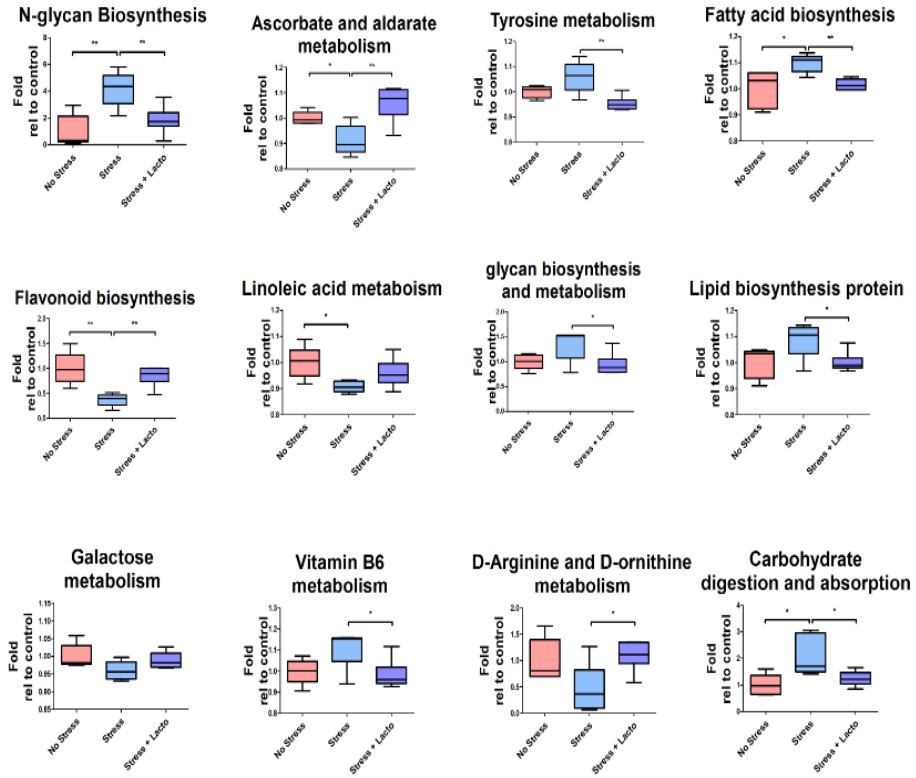

Figure 8. Metabolic functional difference among experimental groups.

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analysis was performed to predict metagenome using KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Among the resulting predicted metabolisms, significantly differed metabolic pathways were plotted as **Box-whisker plot.** Two-way ANOVA followed by Bonferroni post hoc. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5. Social defeat stress affects immune response and serotonergic response in the gut and the brain

To investigate the effects of social defeat stress on mice, we evaluated mRNA expressions of cytokines and various genes within the immunogenic and serotonergic responses of different tissues. The systemic immune response was evaluated from the mesenteric lymph node(MLN) since mesenteric lymph node has been reported as 'the center of the immune response' [31]. mRNA expression of pro- and inti-inflammatory cytokines including, TNF-a (tumor necrosis factor alpha), NFkB, Interleukin-1-beta (IL-1β), Interleukin-18, Interlukin-6 and Interleukin-10, and transcription factors, including FOXP3, RORγt, GATA3 and T-bet, were measured in the MLN. Interestingly, an expression of IL-1β was significantly increased in response to the stress exposure and reduced to normal levels in treatment group (Figure 11.). Transcription factors including FOXP3, GATA3 and RORγt were suppressed via stress exposure while T-bet was upregulated in response to stress exposure. This implies that the stress exposure may down-regulate T_{reg}, T_H2 and T_H17 mediated immune response and up-regulate the T_H1 mediated immune response since FOXP3, GATA3, RORγt and

T-bet facilitate T_{reg}, T_H2, T_H17 and T_H1 differentiation, respectively.

The intestinal immune response was evaluated from the Peyer's patch (PPs) [24]. Unlike the mRNA expression patterns in the MLN, there was no significant increase in IL-1β expression and no significant decrease in FOXP3, GATA3 and RORγt expression at Peyer's patch. However, Interleukin-18 and T-bet was significantly overexpressed in Peyer's patch.

Messenger RNA (mRNA) expressions in Colon, Dorsal Raphe Nucleus (DRN), Hippocampus and Nucleus Accumbens (NAc) were also measured to evaluate serotonergic and immunogenic response in these regions (Figure 10.). The colon has been known to be responsible for most of the serotonin (5-HT, 5-hydroxytrypamine) biosynthesis[6]. In the brain, serotonin is produced in the Dorsal Raphe Nucleus (DRN) and then spread to Hippocampus and Nucleus Accumbens (NAc) [32]. Interestingly, colonic serotonin biosynthesis was decreased via down-regulated TPH-1 (Tryptophan hydroxylase -1) expression and up-regulated IDO expression. Tryptophan hydroxylase-1 is a rate limiting enzyme of serotonin biosynthesis pathway and IDO metabolize tryptophan, precursor of serotonin, into kynurenine and thus competitively inhibit serotonin biosynthesis. In addition, serotonin

transporter (slc6a4) was decreased after the stress exposure which implies colonic serotonin reuptake is suppressed which is possibly due to the decrease in serotonin biosynthesis.

In the brain, there was a tendency toward significant increase in TPH-2 expression and decrease in IDO expression in Dorsal Raphe Nucleus, but were not statistically significant. Interestingly, this increased serotonin biosynthesis in the brain is directly opposite to that of colon, however the link is unknown. In addition, we observed that serotonin receptor 2C (5-HT_{2C}) was significantly increased in both hippocampus and NAc, two important destinations of serotonin projection pathway in the brain. This increased serotonin receptor may in response to decreased serotonin biosynthesis in the brain but the link is not known. More importantly, we observed an increase in interleukin – 1 beta in hippocampus which is in accordance with previous report that hippocampal IL-1β plays critical role in etiology of chronic stress- induced depression [33].

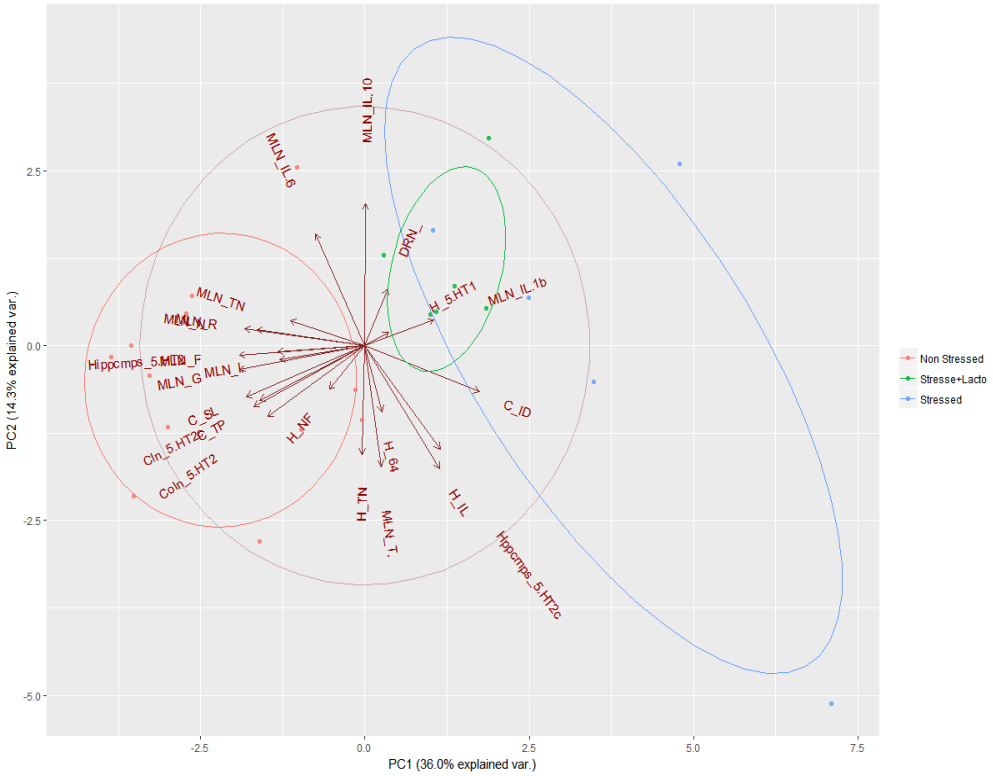

Figure 9. Principle Coordinate Analysis (PCoA) plot shows significant differences among the mRNA expression patterns of non-stressed group, stressed group and Stressed, but *L. plantarum* treated, group

The PCoA plot was generated based on genus level-relative operational taxonomic unit (OTU) data in Non-stressed group, Stressed group and Stressed, but *Lactobacillus plantarum* treated, group. Principal Coordinate 1 explains 36.0% of variance while Principal Coordinate 2 explains 14.3 % of variance.

6. **Treatment of *L. plantarum* prevents the effects of social defeat stress on host's immunogenic and serotonergic response in the gut and the brain**

We also evaluated the effect of *L. plantarum* treatment (Lp) on the immunogenic and serotonergic response to social defeat stress. PCoA plot shows that the exposure to social defeat stress significantly alter the gene expression patterns of the host which was prevented by Lp (Figure 9.).

The decreased colonic serotonin biosynthesis was partly prevented in Lp, in particular, the reduced TPH-1 expression was significantly increased and the increased IDO expression was decreased by Lp. Furthermore, reduced slc6a4 expression was also increased in Lp. Even though Lp could not fully prevent the gene expression patterns of enzymes involved in serotonin biosynthesis, the level of serum serotonin was back to normal in Lp group (Figure 11-12).

In terms of systemic immune response, the increased expression of IL-1β and T-bet in MLN were fully recovered (Figure 11.A). While the decreased expression of FOXP3, GATA3 and RORγt was partly recovered. In the Peyer's patch, the increased IL-18 expression was not

recovered while T-bet expression was reduced to normal level. This implies that social defeat stress may provoke T_h1 helper cells mediated immune response, which is immunity against bacteria and protozoa, via increasing T-bet expression in both MLN and PPs, and thus affects the gut microbiota structure (Figure 11.).

Furthermore, Lp attenuated the social defeat stress induced immune response in hippocampus. IL-1β expression which was increased in response to the stress exposure, was fully recovered through the treatment (Figure 10.B). In addition, increased expression of hippocampal serotonin receptor 2C (5-HT_{2C}) was also reduced to normal level via Lp.

According to previous studies [33-35], hippocampal interleukin – 1 plays significant role in chronic stress induced depression and its knockout effectively prevented the stress induced depression [36]. This implies that Lp may prevent the disease onset of stress induced depression via regulation of hippocampal IL-1β expression.

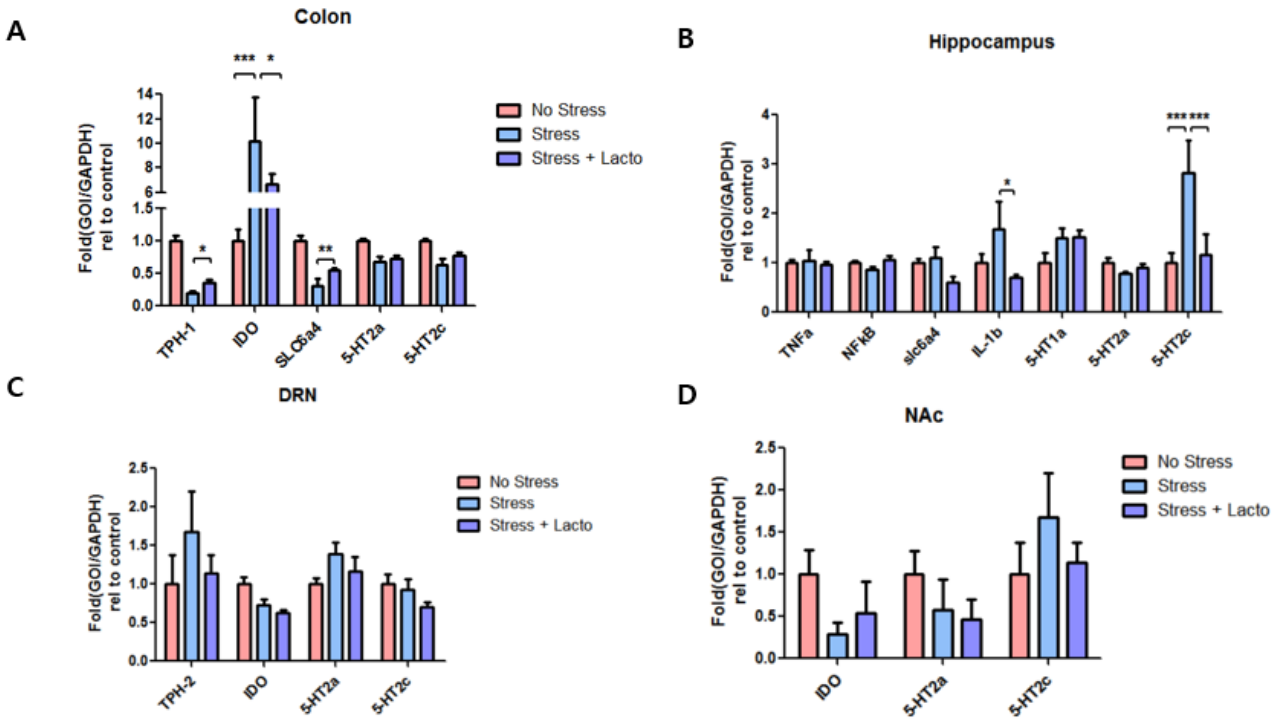

Figure 10. mRNA expression pattern in A) Colon, B) Dorsal Raphe Nucleus (DRN) C) Hippocampus and D) Nucleus Accumbens (NAc)

Two-way ANOVA followed by Bonferroni post hoc. *p < 0.05, **p < 0.01, ***p < 0.001, Data are represented as mean ± SEM

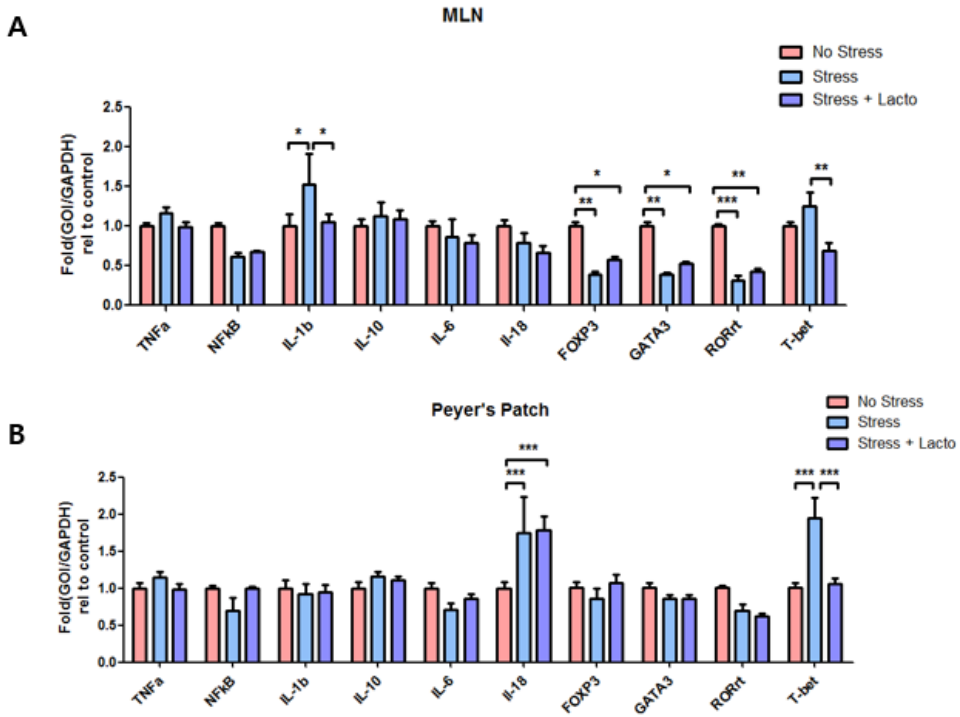

Figure 11. mRNA expression pattern in A) mesenteric lymph node(MLN) and B) Peyer's patch(PP)

Two-way ANOVA followed by Bonferroni post hoc. *p < 0.05, **p < 0.01, ***p < 0.001, Data are represented as mean ± SEM

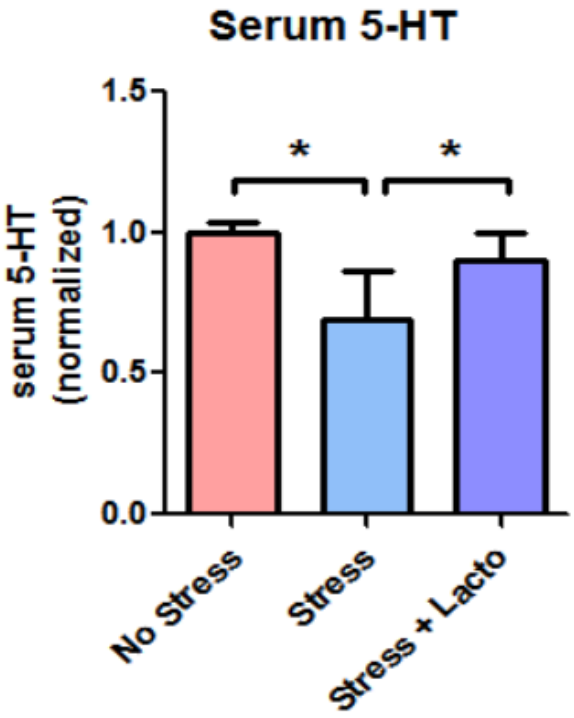

Figure 12. Serum serotonin level. Amount of serum serotonin in each group was normalized to the level of the control group (No Stress) Two-way ANOVA followed by Bonferroni post hoc. *p < 0.05, **p < 0.01, ***p < 0.001, Data are represented as mean ± SEM

IV. Discussion

It is well known that the activation of sympathetic nervous system (SNS) and HPA axis induced by a stressor affects many aspects of gastrointestinal (GI) tract functions such as GI motility, mucous secretion and intestinal immune responses. In this regard, Stressor may affect the microbiome by modulating intestinal microenvironment. We investigated the effect of social defeat stress on the gut microbiota, their metabolic functions and host's responses.

It has been reported that coping is the major determinant of stress resilience, and serotonin transmission in the brain plays important role in coping strategy [37]. In addition, it is recently reported that gut microbiota plays a significant role in host serotonin biosynthesis [6]. Thus, we wanted to know whether probiotics can improve stress resilience via either inducing host serotonin biosynthesis or preventing the stress-induced dysbiosis of gut microbiota.

We found that each of lactic acid bacteria strains used in this study has different TPH-1 inducing ability and choose *Lactobacillus plantarum* KBL396 for further *in vivo* experiment.

In response to the social defeat stress, gut microbiota structure was

significantly altered, resulting further alterations in metabolic functions of microbial community. In particular, the abundances of bacteria *within Anaerosipes sp., Helicobateraceae, Mogibacteriaceae, Prevotella sp., Suterrella sp., Parabacteroides sp. and Christensenella sp*. that were increased in response to social defeat stress. This alteration of microbiota is potentially via Enteric Nervous System (ENS; complex neuronal network with multiple neurotransmitters and neuromodulators including 5-HT, acetylcholine) activation, hypothalamic—pituitary-adrenal (HPA) axis and activated immune response, however, further study is needed to elicit exact mechanism of such alteration. Here, the treatment of *Lactobacillus* (Lp) could effectively alleviated such stress induced-dysbiosis in the gut microbiome, increased abundances of the fore-mentioned bacterial species.

In addition, metabolisms such as 'Glycan biosynthesis', 'Lipid biosynthesis' and 'Carbohydrate digestion and absorption' were significantly increased due to these changes in gut microbiota and fully normalized via Lp. Lipoglycans, also known as Lipopolysaccharides (LPS), have been verified to elicit several symptoms of depression through an activation of indoleamine-2,3-dioxygenase (IDO) which caused by LPS induced pro-inflammatory cytokines. An activation of

IDO results in decreased tryptophan levels and increased production of kynurenine, and thus less serotonin biosynthesis [38, 39]. Since serotonin plays a significant role in etiology of various mental illness such as depression[15] and anxiety[16], microbial dysbiosis due to social defeat stress may deteriorate the symptoms of stress-induced depression via producing more lipoglycans.

In this study, we could observe a reduced serotonin biosynthesis after stress exposure. This could be resulting from host's innate response to the stress or partial role of the microbiota on the host serotonin biosynthesis. Interestingly, Lp could effectively prevent the stress induced reduction in the host serotonin biosynthesis. The gene expression of two major enzymes which plays significant role in serotonin biosynthesis was significantly altered due to stress exposure and was partially prevented by Lp. However, a decrease in the level of serum serotonin was fully prevented by Lp which suggests that Lp may contribute to alleviate stress-induced mental illness, such as depression through prevention of alteration in serotonin biosynthesis of the host. However further investigation is needed to elicit molecular mechanism.

In addition, stress exposure provoke systemic Th1 (T helper cell 1) type immune response via increased expression of transcription factor T-bet

and increase the expression of pro inflammatory cytokine interleukin-1 beta (IL-1β) while the other types of immune responses, including Th2, Th17 and Treg mediated immune response, were significantly suppressed.

In the brain, there was a tendency toward significant increase in TPH-2 expression and decrease in IDO expression in Dorsal Raphe Nucleus, but were not statistically significant. Interestingly, this increased serotonin biosynthesis in the brain is directly opposite to that of colon, however the link is unknown. Additionally, it has been reported that higher TPH2 expression in depressed suicides may reflect a homeostatic response to deficient 5-HT levels[40]. Furthermore, we observed that serotonin receptor 2C (5-HT_{2C}) was significantly increased in both hippocampus and NAc, two important destinations of serotonin projection pathway in the brain. This increased serotonin receptor may in response to decreased serotonin biosynthesis in the brain but the link is not known. More importantly, we observed an increase in IL-1β in hippocampus which is in accordance with previous report that hippocampal IL-1β plays critical role in etiology of chronic stress- induced depression [33].

Lp could effectively prevent the microbial dysbiosis through the

inhibition of the fore-mentioned 'psycho-pathogenic' bacteria. Accordingly, a glycan production by these bacteria was also inhibited, which may results in a reduced activation of pro-inflammatory cytokines IL-1β. Followed by this reduced activation of pro-inflammatory cytokine IL-1β, stress-induced activation of IDO was significantly reduced as previously reported [41] while decreased TPH-1 expression was significantly increased. Eventhough actual link between the member of this potential pathway are only partly shown here, it is possible that the exposure to social defeat stress alter the microbiota and their metabolic functions in a way that provokes pro-inflammatory responses in the gut, which promotes kynurenine biosynthesis through the activation of IDO and inhibits serotonin biosynthesis by suppressing TPH-1 expression, and ultimately contribute to the development and deterioration of depression due to decrease in systemic serotonin level.

From these findings, we suggest the idea that many of the harmful outcomes of stress exposure maybe due to the dysbiosis of gut microbiota and alteration in microbial metabolic functions, which may prevented via consumption of *Lactobacillus* spp.

v. References

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VI. Supporting Information

Supplementary figure 1. Overall schemata of Stress-Gut-Brain-Microbiota Axis

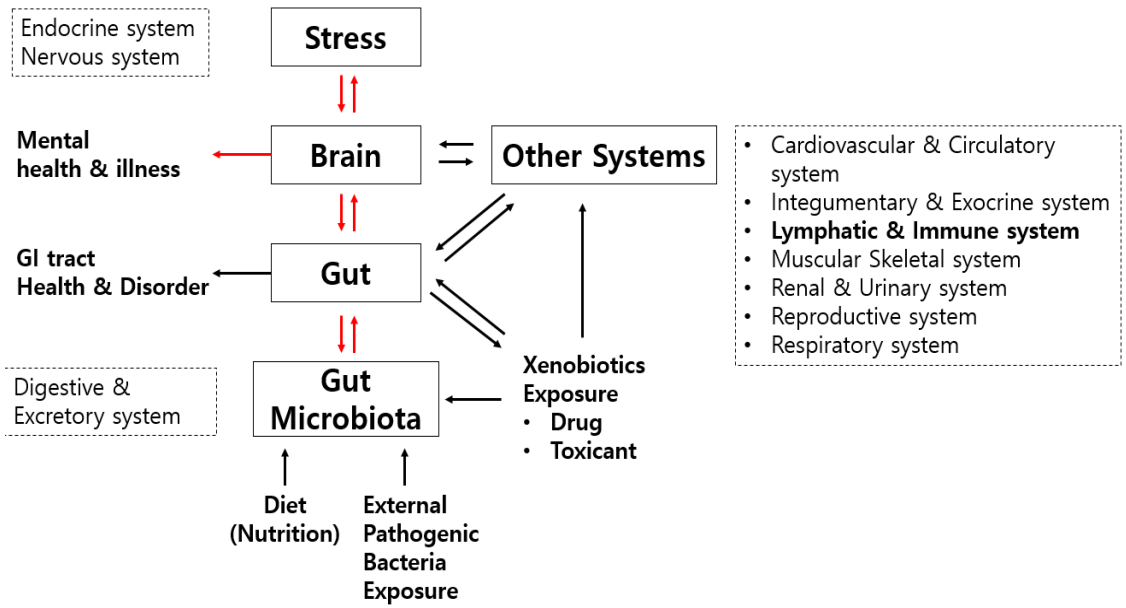
Supplementary figure 2. Changes of the relative abundance of Lactobacillus spp. in response to stress exposure and Lactobacillus plantarum treatment.

Supplementary figure 3. Food intake and Body weight

Supplementary figure 4. mRNA expression pattern in Colon and Hippocampus.

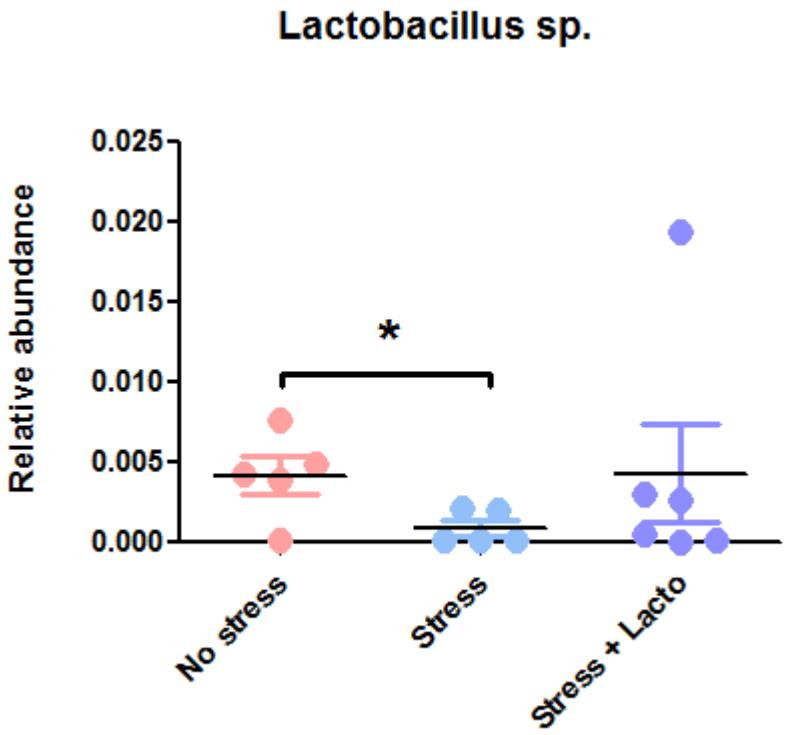
Supplementary figure 5. mRNA expression pattern in MLN and PP

Supplementary figure 6. Significant bacterial components associated with stress exposure status and Lactobacillus treatment.

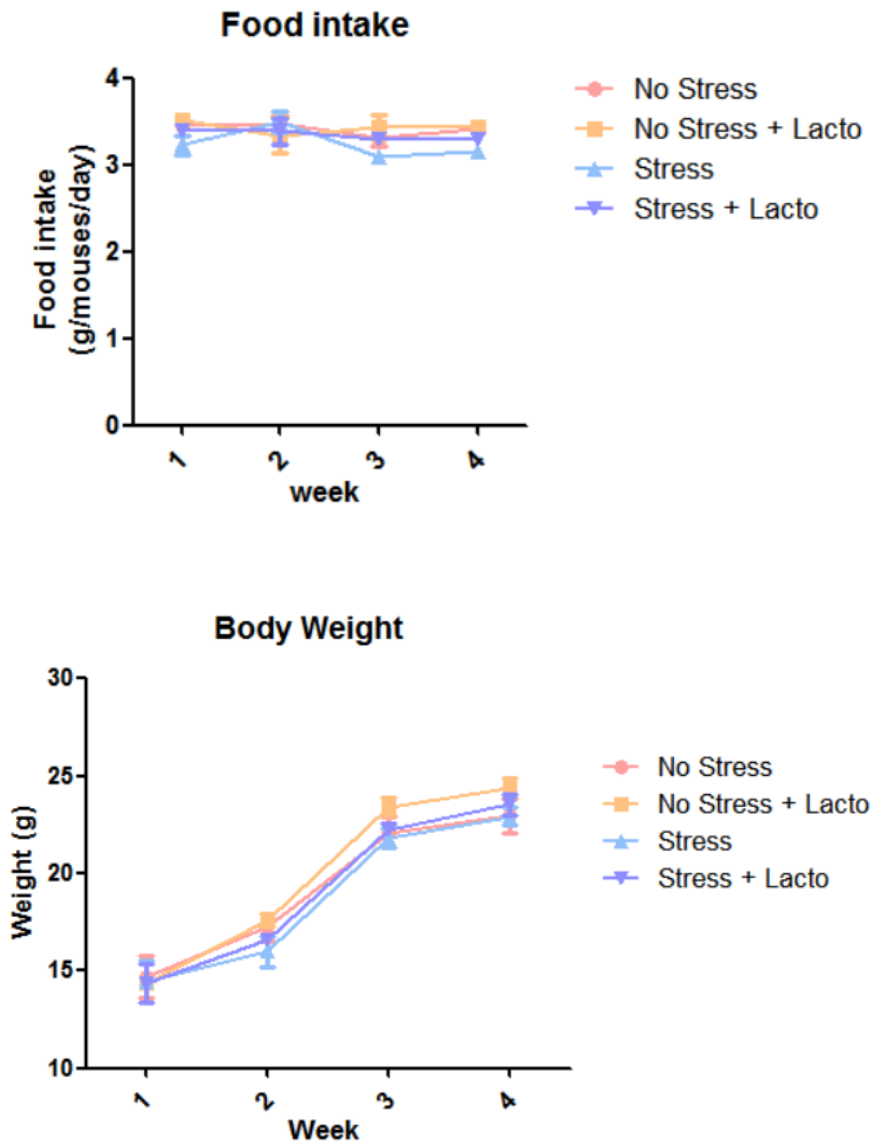


Supplementary figure 1. Overall schemata of Stress-Gut-Brain-Microbiota Axis

The Brain, Gut and Gut microbiota are interconnected via various pathways and an exposure to stress can affect not only brain and mental health, but also GI tract, Gut microbiota and other systems through nervous and endocrine system. Gut microbiota may reversely affect the brain and mental health through number of pathway, such as immunomodulation and microbial metabolism of nutrients and xenobiotics, and host's response to the stress exposure.

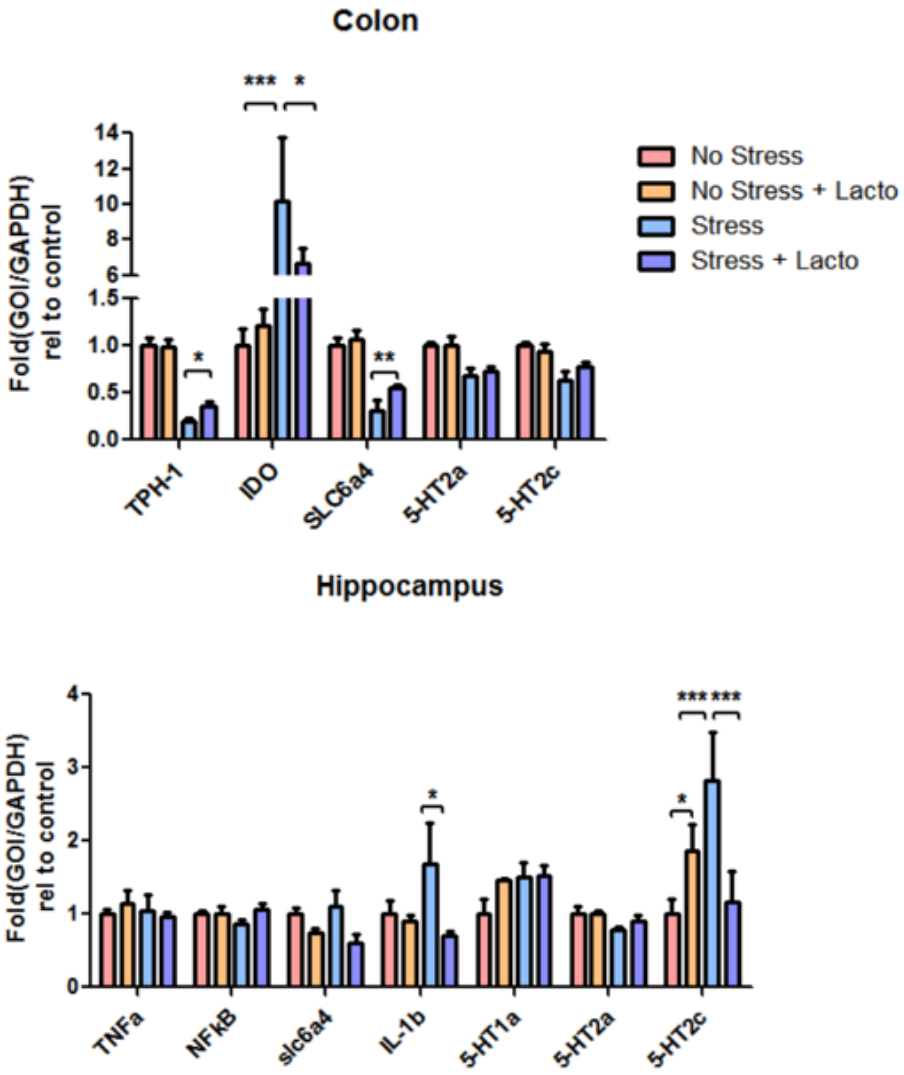


Supplementary figure 2. Changes of the relative abundance of Lactobacillus spp. in response to stress exposure and Lactobacillus plantarum treatment.



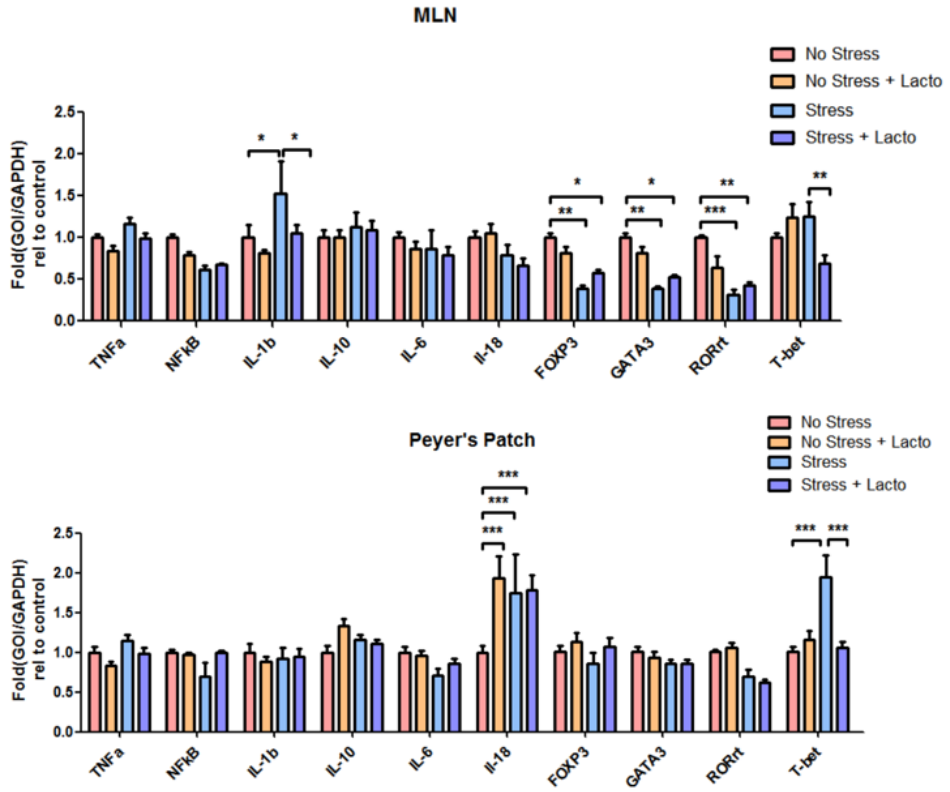
Supplementary figure 3. Food intake and Body weight

Food intake and Body weight does not different among experimental groups.



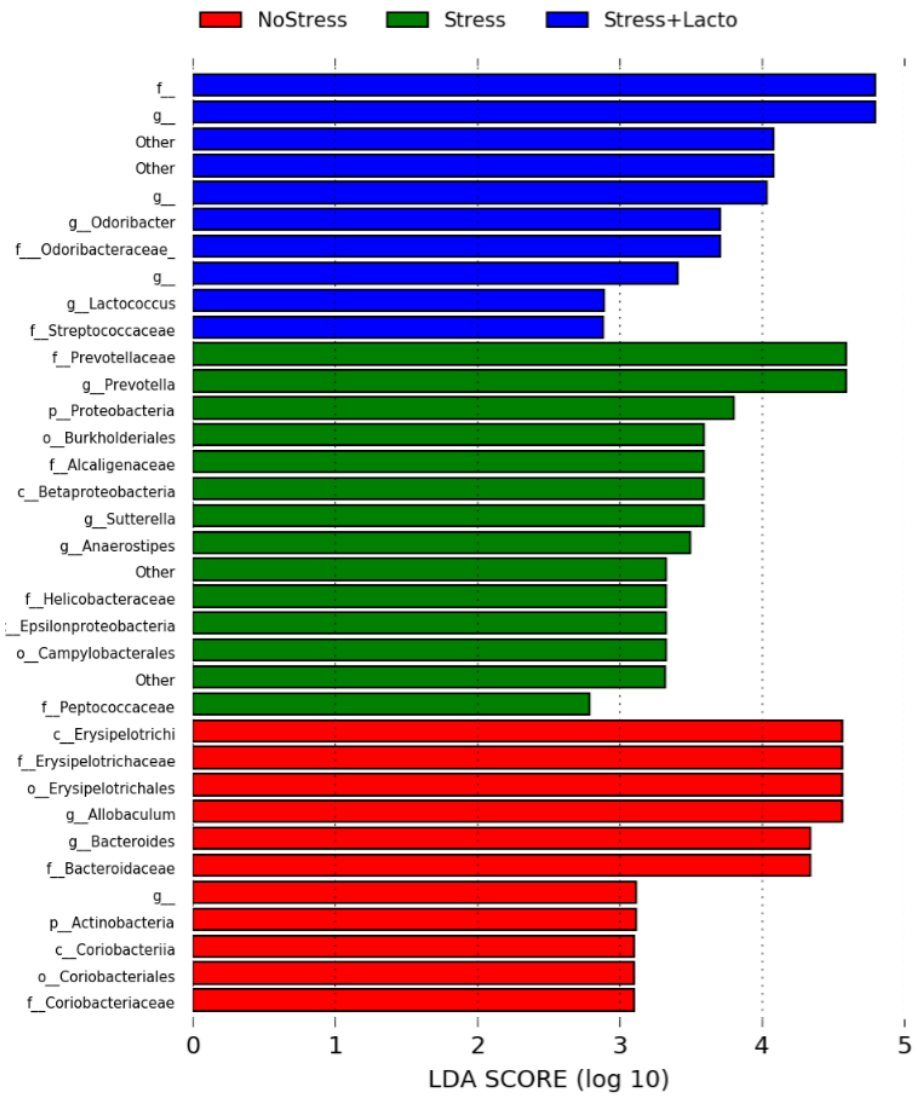
Supplementary figure 4. mRNA expression pattern in Colon and Hippocampus.

There were no major difference in mRNA expressions between No Stress and No Streee + Lacto groups. Two-way ANOVA followed by Bonferroni post hoc. *p < 0.05, **p < 0.01, ***p < 0.001, Data are represented as mean ± SEM



Supplementary figure 5. mRNA expression pattern in MLN and PP

There were no major difference in mRNA expressions between No Stress and No Streee + Lacto groups. Two-way ANOVA followed by Bonferroni post hoc. *p < 0.05, **p < 0.01, ***p < 0.001, Data are represented as mean ± SEM



Supplementary figure 6. **Significant bacterial components associated with stress exposure status and Lactobacillus treatment.**

Characterization of significant abundance changes by the stress exposure was identified by LEfSe (LDA Effect Size). Statistical analysis was performed using the Kruskal-Wallis test (among classes) and Wilcoxon test (between subclasses) as a p value < 0.05 in both tests. The threshold logarithmic LDA score was set to 3.0.

국문초록

락토바실러스 플란타룸이 스트레스에 의한 장내 미생물총 불균형의 완화에 미치는 효과

서울대학교 보건대학원
환경보건학과 환경보건미생물학 전공
최 용 빈

지도교수 고 광 표

급격한 사회변화와 경쟁심화로 인해 전체 국민의 상당수가 정신적 문제를 겪고 있으며, 특히 과도한 스트레스로 인한 우울증으로 고통받는 인구가 크게 증가하고 있다. 우울증의 발병 기전에 있어 모노아민 계열 신경전달 물질인 세로토닌이 중요하게 작용한다는 점과 장내미생물이 숙주의 세로토닌 생합성을 조절한다는 기존 보고에 근거하여, 본 연구는 유산균의 숙주 세로토닌 생합성 촉진능을 확인함과 동시에, 유산균에 의한 세로토닌 생합성 촉진을 통해 스트레스 노출에 따른 생리학적 불균형이 예방, 완화될 수 있는 지를 확인하고자 수행되었다.

본 연구에서는 스트레스 노출에 의한 장내미생물총 구조의 급격한 변화와 이에 따른 미생물총의 물질대사능력의 변화, 숙주의 생리적 변화와 이러한 불균형이 유산균의 투여에 따라 예방될 수 있음을 확인하였다. 구체적으로, 스트레스 노출에 따라 *Anaerosipes sp., Helicobateraceae, Mogibacteriaceae, Prevotella sp., Suterrella sp., Christensenella sp., Parabacteroides sp.* 등의 속(genus)이 유의하게 증가하였으며, 이에 따라 장내미생물총의 glycan 생합성 경로, 탄수화물 대사 및 흡수에 관한 물질대사 경로가 유의하게 증가하였음을 확인하였다. 이와 더불어 스트레스 노출에 의해 Th1 계열 면역반응을 통해 IL-1β 발현의 증가와 더불어 숙주의 세로토닌 생합성이 현저히 감소하였는데, 이는 glycan 생합성의 증가에 따른 LPS의 증가가 IL-1β 등의 proinflammatory cytokine을 촉진하여 TPH-1의 억제와 IDO의 촉진을 통해 세로토닌 생합성을 억제하는 것으로 보인다.

더불어 유산균의 투여는 이러한 장내미생물총의 변화와 이에 따른 장내미생물총 물질대사능의 변화를 효과적으로 예방하였으며, 숙주의 면역반응의 변화와 세로토닌 생합성의 저해 또한 상당부분 예방됨을 확인하였다. 따라서 본 연구에서는 스트레스에 따른 정신질환의 발병기전 또는 악화기전에 관여 할 것으로 보이는 미생물 및 대사경로를 제시하고, 이들 미생물을 통해 정신질환을 악화시키는 유해균으로써의 'psycho-pathogen'의 개념을 제시하고자 한다.

주요 단어: 장내미생물총, 스트레스, 세로토닌, 우울증, 프로바이오틱스, 유산균, 마이크로비옴

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