보건학석사 학위논문

# Effects of *Lactobacillus plantarum* and *Lactobacillus paracasei* for the prevention and alleviation of zymosan-induced Irritable Bowel Syndrome in mice

락토바실러스 플란타럼과 락토바실러스 파라카제이 혼합 균주의 과민성 장 증후군 예방 및 증상 완화 효과 연구

2019년 8월

서울대학교 보건대학원

환경보건학과 환경보건학 전공

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지도교수 고 광 표

이 논문을 보건학석사 학위논문으로 제출함

2019년 5월

서울대학교 보건대학원

환경보건학과 환경보건학 전공

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송림의 보건학석사학위논문을 인준함

2019년 8월

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# Abstract

# Effects of *Lactobacillus plantarum* and *Lactobacillus paracasei* for the prevention and alleviation of zymosan-induced Irritable Bowel Syndrome in mice

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Irritable bowel syndrome (IBS) tops list of functional gastrointestinal disorder with worldwide prevalence rates. Patients with IBS present with abdominal pain and altered bowel habits as well as emotional anxiety and depression. Nevertheless, no gold standard for the

treatment of IBS exists. However, it has become evident that altered gut microbiota composition and its functions are key pathophysiological factors along the gut–brain axis in IBS. Furthermore, a general decrease in *lactobacillus* and *Bifidobacterium* species abundance was identified in IBS patients. In this study, we aimed to evaluate the efficacy and effectiveness of mixed and single strain of *Lactobacillus plantarum* KBL396 and *Lactobacillus paracasei* KBL382 in a mouse model of IBS. IBS-like symptoms were induced in mice by intracolonic injection with zymosan suspension for 3 consecutive days. Mice were divided into groups that received either (1) phosphate buffer (PBS) + PBS (2) PBS + zymosan (3) amitriptyline (AMT) + zymosan (4) *L. plantarum* KBL396 + zymosan (5) *L. paracasei* KBL382 + zymosan (6) *L. plantarum* KBL396/ *L. paracasei* KBL382 + zymosan. Orally administered treatment was started 7 days prior to the zymosan injection and continued until they were sacrificed. We found that treatment of *L. plantarum* KBL396 and *L. paracasei* KBL382 altered inflammatory cytokines, BDNF, 5-HT, and SERT in the colon and brain at day 7 and day 14 after the zymosan injection. In addition, we also found that treatment of the selected Lactobacillus strains reduced anxiety-like behaviors in the open field and elevated plus maze. These

findings suggest that *L. plantarum* KBL396 and *L. paracasei* KBL382 may attenuate IBS-like symptoms and improve anxiety-like behaviors.

**Key words:** anxiety-like behavior, gut-brain axis, irritable bowel syndrome (IBS), Lactobacillus, microbiota, zymosan

**Student No. 2017-28157**

# CONTENTS

# List of Tables

# List of Figures

# I. Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder, affecting 7 to 21% of the world population [1]. Its characteristics of abdominal pain and altered bowel habits, including bloating and stool irregularities have a substantial impact on the lives of individual and society by impairing their quality of life as well as economic burden with medical coasts [2, 3]. However, despite its high prevalence and influences, the precise mechanism of pathogenesis and pathophysiology remains unclear with multifactorial etiology [4]. Moreover, because of its heterogeneous condition and absence of structural and biochemical abnormality, there is a lack of gold standard diagnostic criteria and medications to treat IBS [5].

IBS is also known as a disorder of the gut-brain axis. In patients with IBS, elevated psychological abnormalities, including higher level of anxiety and depression are observed [6]. Many studies have identified physiological and molecular modifications along with the gut-brain axis, which is correlated with gut microbiota and immune activation in IBS [7-10]. It also has been found that serotonin (5-HT) is an important neurotransmitter and involved in gastrointestinal motility

and sensitivity in bidirectional gut-brain interactions [11]. In patient with IBS, increased 5-HT and decreased serotonin transporter (SERT) has been observed in gastrointestinal tract [12-14]. Also, serotonin 3 receptors (5-HT3) that response to released serotonin from enterochromaffin (EC) cells in the wall of the gut are well known to transmit signals encoding important sensations such as nausea and pain to the central nervous system (CNS) [15-16]. 5-HT3 antagonists are used as a treatment of IBS, and have been shown to improve symptoms of non-constipated (NC) or diarrhea-predominant (D) IBS efficiently in both men and women [17]. In animal study, 5HT3 antagonists also reduce colorectal distension and visceral hypersensitivity induced by harmful gut stimuli [18]. Another vital pain modulator, brain-derived neurotrophic factor (BDNF), have been discovered and studied in IBS. BDNF, a member of the neurotrophin family of growth factors, is highly upregulated in colonic biopsies from patients with IBS [19]. Several studies have shown that increased expression of BDNF in colonic mucosa is associated with visceral hypersensitivity, which is an important hallmark characteristic of IBS, whereas the inhibition of BDNF reduced the abdominal pain degree [20-22].

Intestinal microbiota has been also recognized as one of pathophysiological factors of IBS. Current studies have suggested that intestinal microbiota plays critical roles and might contribute to the intestinal wall permeability, immune activation, and enteroendocrine signaling in gut-brain communication [23, 24]. In patients with IBS, the lower microbial diversity, especially decreased abundance of *Bifidobacterium* and *Lactobacillus* species and increased abundance of *Ruminococcus torques*, has been reported compared to healthy individuals [25-27]. Clinical trials have demonstrated that administration of probiotics could alleviate symptoms of IBS in patients [28]. Moreover, an animal study has shown that the combination of *Lactobacillus helveticus* and *Bifidobacterium longum* regulated hypothalamic-pituitary-adrenal (HPA) axis by glucocorticoid negative feedback and attenuated stress-induced visceral pain [29]. In addition, intestinal microbiota is associated with the decreased level of proinflammatory cytokines TNF-α, IL-1β, IFN-γ in IBS [30]. Although underlying pathophysiological mechanisms of the intestinal microbiota contributions to IBS are not fully understood, these findings have resulted in interest in the potential therapeutic role of manipulation of microbiota in IBS [31].

Our previous studies have found alleviating effects of *Lactobacillus paracasei* on dextran sulfate sodium (DSS)-induced colitis mice and *Lactobacillus plantarum* on stress-related anxiety and depression in animal model separately. In this study, we evaluated and compared the effects between two different *Lactobacillus* strains and their combination on behavioral anxiety and inflammatory response in zymosan-induced IBS mouse model.

## II. Materials and Methods

### 1. Animals

Seven-week-old male specific pathogen-free (SPF) C57BL/6 mice used in this study were purchased from Central Lab Animals Incorporated (Seoul, Korea). The performance and protocols of animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, Korea (Case Number: SNU-190110-2). Mice were housed under standard laboratory conditions under 12:12 light/dark cycle and 25 ± 2°C temperature and allowed free access to water and mice chow. Sixty-six mice were acclimated for 1 week before the test then randomly assigned into 6 experimental groups (11 mice per group in 3 cages). In order to revise the cage effect, we performed the testing by dividing the mice into three groups in a group. We also performed the second testing with same method, amount, and a pilot testing in a similar environment.

## 2. Bacteria preparation

*L. plantarum* KBL396 and *L. paracasei* KBL382 were obtained from fecal samples of healthy Korean Adults. A lyophilized combination of these two strains (*L. plantarum* KBL396 and *L. paracasei* KBL382), and individual strain *L. plantarum* KBL396 or *L. paracasei* KBL382 were prepared. Before administration, each single lyophilized strain was diluted in 10-fold and the number of colonies were counted by pour plate methods to measure their viability in the samples. These lyophilized bacteria were rehydrated in 1x phosphate-buffered saline (PBS) at a concentration of 109 colony-forming units (CFU) per milliliter. The bacterial solution was administered orally using plastic feeding needle at similar times each day for every 3 weeks in mice before and during the experimental procedure (7 days prior to the starting the zymosan injection until they were sacrificed).

### 3. Zymosan-induced IBS animal preparation and treatment

To induce IBS-like symptoms, a volume of 0.1 mL zymosan suspension (30 mg/mL in PBS; Sigma-Aldrich, St. Louis, MO, USA) was administered into the colons using a 22-gauge long stainless steel feeding needle. Mice were anesthetized by isoflurane inhalation during the performance. Either vehicle (PBS) or zymosan was given daily for 3 consecutive days (days 1, 2, and 3). Naïve group was subjected to the same procedure as those in zymosan-induced control group except intracolonic injection with 0.1 mL PBS. Three groups of mice were pretreated with mixed strains or single strain of two *Lactobacillus* spp. for 7day prior to injection with PBS or zymosan while other groups were pretreated with PBS. Mice were divided into 6 groups (n = 11 per group): (1) oral administration of PBS plus intracolonic injection with saline (2) oral administration of PBS plus intracolonic injection with zymosan (3) oral administration of amitriptyline (AMT; 30 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) plus intracolonic injection with zymosan (4) oral administration of *L. plantarum* KBL396 plus intracolonic injection with zymosan (5) oral administration of *L. paracasei* KBL382 plus intracolonic injection with zymosan (6) oral administration of combined two *Lacrobacillus* strains plus intracolonic

injection with zymosan. This procedure is shown in Table 1 and Figure 1.

**Table 1. Treatment groups for *in vivo* study.**

| Group | Oral administration (200 μL) | Intracolonic injection (0.1 mL) | Number of mice |
|---|---|---|---|
| 1 | Saline | Saline | 11 |
| 2 | Saline | Zymosan | 11 |
| 3 | Amitriptyline (30 mg/kg) | Zymosan | 11 |
| 4 | L. plantarum KBL396 ($4 \times 10^9$ CFU/day) | Zymosan | 11 |
| 5 | L. paracasei KBL382 ($4 \times 10^9$ CFU/day) | Zymosan | 11 |
| 6 | L. plantarum KBL396 + L. paracasei KBL382 ($4 \times 10^9$ CFU/day) | Zymosan | 11 |

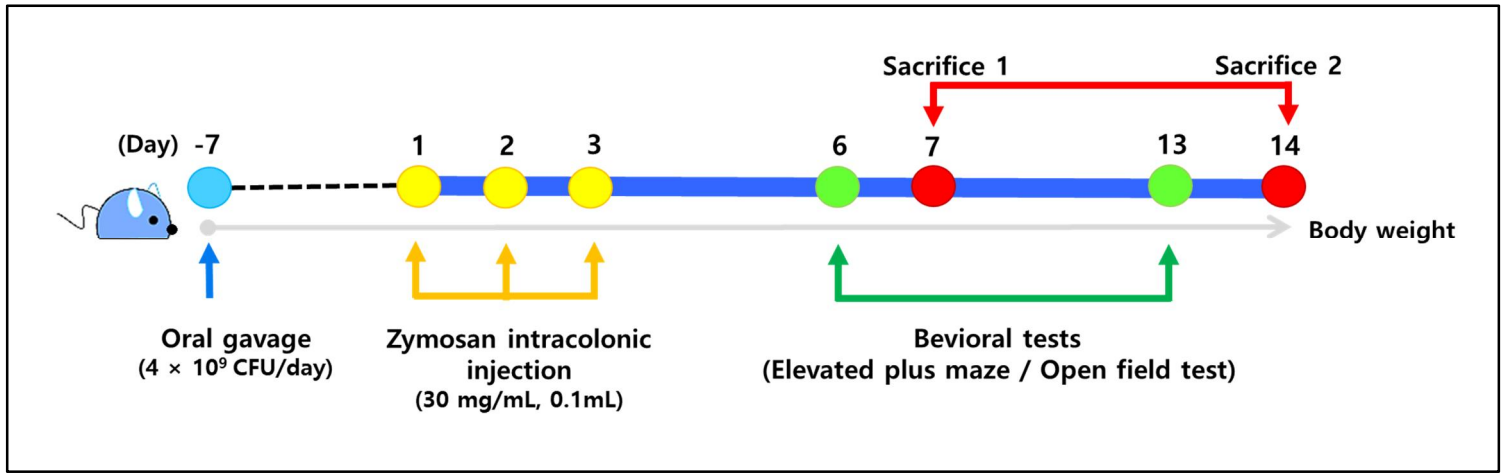



**Figure 1. Scheme of animal study.**

Oral administration was started 7 days prior to zymosan injection. Intracolonic injection of zymosan suspension was performed for 3 consecutive days. Behavioral tests were performed separately on day 6 and day 13 from the first day of zymosan injection. Mice were sacrificed the next day of behavioral testing.

**4. Quantitative real-time PCR (RT-qPCR)**

Total RNA from the distal colon, hippocampus, and prefrontal cortex was homogenized using Trizol solution. RNA was extracted with easy-spinTM Total RNA Extraction Kit (iNtRON biotechnology, Seongnam-si, Korea) by provided manufacturer's protocol. RNA was quantified and assessed purity using a NanoDrop ND2000 (Thermo Fisher Scientific, Illkirch, France). cDNA was synthesized from RNA samples with High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were performed with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). GAPDH was used as a housekeeping gene to normalize reverse transcription- quantitative polymerase chain reaction (RT-qPCR) data to the expression levels. Reactions were duplicated for each run and the expression level was calculated by relative quantity ($2^{-\Delta\Delta CT}$) method. Primer sequences used in this study were listed in Table 1.

**Table 2. RT-qPCR primers sequence used in this study.**

| Target | Sequence (from 5' to 3') | |
|---|---|---|
| **TNF-α (mouse)** | Forward | CAT CTT CTC AAA ATT CGA GTG ACA A |
| | Reverse | TGG GAG TAG ACA AGG TAC AAC CC |
| **IL-1β (mouse)** | Forward | GAA ATG CCA CCT TTT GAC AGT |
| | Reverse | CTG GAT GCT CTC ATC AGG ACA |
| **BDNF (mouse)** | Forward | GCG CCC ATG AAA GAA GTA AA |
| | Reverse | TCG TCA GAC CTC TCG AAC CT |
| **BDNF4 (mouse)** | Forward | CAG AGC AGC TGC CTT GAT GTT |
| | Reverse | GCC TTG TCC GTG GAC GTT TA |
| **SERT1 (mouse)** | Forward | CTT CAG CCC CGG ATG GTT |
| | Reverse | GTG GAC TCA TCA AAA AAC TGC AAA |
| **5HT3A (mouse)** | Forward | AAC AGC TAT GCA GAA ATG AAG TT |
| | Reverse | GGC TGA CTG CGT AGA ATA AAG G |
| **GAPDH (mouse)** | Forward | ATTGTCAGCAATGCATCCTG |
| | Reverse | ATGGACTGTGGTCATGAGCC |

**5. Enzyme-Linked Immunosorbent Assay (ELISA)**

After collecting blood samples, Prefrontal cortex and hippocampus were collected and stored at –80 °C until use. Brain tissues and mucosal samples were separately homogenized in RIPA buffer (1:10 w/v, Sigma-Aldrich) containing protease inhibitors. The protein concentration of each sample was determined using PierceTM BCA Protein assay kit (Thermo Fisher Scientific). All samples were assayed in duplicate. The protein levels of BDNF were measured using a commercially available ELISA kit (R&D, Minneapolis, MN, USA) according to the manufacturer's protocols.

Blood was obtained and kept at room temperature for 30 min for clotting. Clotted samples were centrifuged at 1,800 rpm for 5 min at 4°C, and then divided in aliquots and immediately stored at –80 °C until use. Concentration of protein of each sample were determined using Pierce$^{TM}$ BCA Protein assay kit (Thermo Fisher Scientific) and Serotonin Ultrasensitive ELISA kit (Eagle Biosciences, Amherst, NH, USA).

## 6. Behavioral Testing

Two classical behavior tests of anxiety, the open-field (OFT) and elevated plus maze (EPM), were performed and automatically analyzed using a video tracking system (SMART 3.0 PanLab software, Harvard Apparatus, Cambridge, MA, USA). The elevated plus maze is one of the most commonly used tests to measure anxiety-like behavior in the rodent model. Behavioral tests were performed separately on day 6 and day 13 from the first day of zymosan injection. Before starting any behavioral test, mice were habituated to the procedure room for 30 min. During the test trials, two experimenters and the computer were behind a curtain to separate from the mice in the testing area. The testing area was illuminated with dim and indirect room light. The same conditions were used for both tests.

**7. Open field test**

The open field maze is a white polystyrene box (44 cm × 44 cm × 60 cm) with an enclosed wall. In this study, a multiple unit open field maze with four activity arenas were used and analyzed at one time. To remove scent clues, each box was empty and wiped with 95% ethanol after each test. The ethanol was allowed to evaporate and then each mouse was placed in the center of the box. The test was run for 5 min. Follows were analyzed using a video tracking system (SMART 3.0 PanLab software, Harvard Apparatus, Cambridge, MA, USA): (1) total distance traveled (2) distance traveled in the central zone (3) time spent in the central zone versus total time (4) number of entries in central zone.

## 8. Elevated plus maze

The elevated plus maze is a plus-shaped apparatus consisted of two open arms and two closed arms in line. The maze was raised 50 cm above from the floor. At start of each test, individual mice were placed on the center facing an open arm. Mice were allowed to freely travel on the maze for 5 min while the frequency and time spent in open and closed arms is recorded. experimental area of the maze was cleaned with 95% ethanol every time before subsequent tests.

## 9. Statistical analysis

All values are expressed as means ± standard error of the mean (SEM). GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) was used to evaluate analysis and graph all data. One-tailed Mann-Whitney U-test and one or two-way ANOVAs were performed with GraphPad Software. Differences in means and scopes were evaluated by t-tests and Fisher's exact tests. Differences were considered significant when $P < 0.05$. Statistical significance was presented as '*' $P < 0.05$, '**' $P < 0.01$, '***' $P < 0.001$.

## III. Results

### 1. Pretreatment of *L. plantarum* KBL396 and *L. paracasei* KBL382 has an impact on phenotypic characteristics in zymosan-induced mice

Previous studies have reported that zymosan-induced IBS mouse model is characterized by significant loss of body weight and a low-grade systemic toxicity [32]. To evaluate the effect of administration of two *Lactobacillus* strains in colon length, we measured the length of the colon of each mouse from the proximal colon to the anus. On day 7, injection of zymosan was associated with significantly decreased colon length while administration of two *Lactobacillus* strains prevented decreased colon length (Fig. 2A). The results of measured colon length on day 14 was similar to day 7 (Fig. 2B). We also monitored weight and its changes in mice for 14 days after zymosan or normal saline injection. We found that oral administration of *Lactobacillus* strains effect in body weight, decreased the loss of body weight caused by zymosan (Fig. 2C). Administration of *L. plantarum* KBL396 significantly improved the body weights compared to control. However, AMT administration exhibited the accelerated loss of body weight and associated with the lowest body

weight gain among groups (Fig. 2C). Overall, administration of these two *Lactobacillus* strains attenuated the loss of weight and decreased colon lengths.

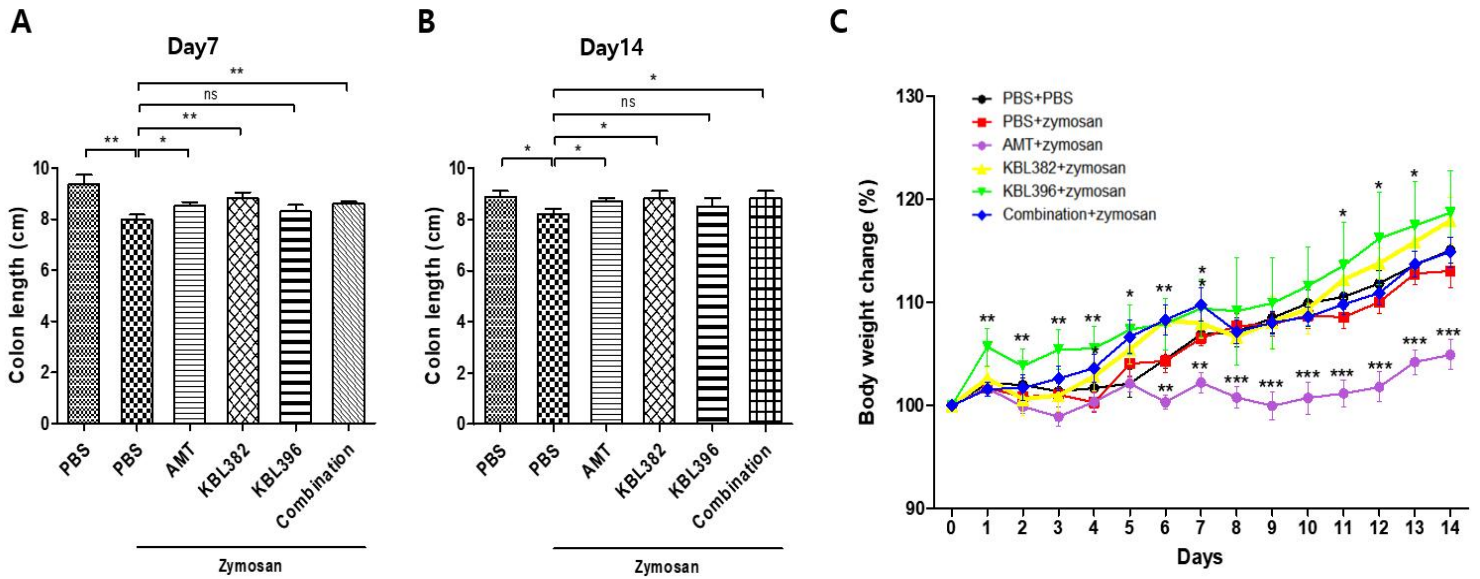

A
Day7
Colon length (cm)
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
B
Day14
Colon length (cm)
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
C
PBS+PBS
PBS+zymosan
AMT+zymosan
KBL382+zymosan
KBL396+zymosan
Combination+zymosan
Body weight change (%)
Days

**Figure 2. Effects of *L. plantarum* KBL396 and *L. paracasei* KBL382 pretreatment on phenotypic characteristics in zymosan-induced IBS mice.**

(A) The colon length of each group was measured on day 7 and (B) day 14 separately after they were sacrificed. (C) The bodyweights of six groups (see Table 1 for groups) of mice (n = 11/group) were measured on day 0 before zymosan injection and every day until study is over. Statistical analysis was performed using a one-tailed unpaired t test. Data are presented as mean ± standard error of mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

## 2. *L. plantarum* KBL396 and *L. paracasei* KBL382 treatment changes dysregulated cytokine response in IBS-like animal model

To evaluate the role of *L. plantarum* KBL396 and *L. paracasei* KBL382 in the immune response caused by zymosan, we measured the level of proinflammatory cytokines (IL-1β and TNF-α) in the colon using RT-qPCR. Mice with IBS-like symptoms by zymosan injection displayed increased level of proinflammatory cytokine TNF-α compared to normal saline injected mouse on days 7 and 14 (Fig. 3). However, expression of IL-1β showed no difference between saline intracolonic injected group and zymosan intracolonic injected group with PBS oral administration on day 14 (Fig. 3D). In contrast to the saline injected mice, AMT and single strain administration group with zymosan showed significant differences in IL-1β level on both days compared to the control group (Fig. 3B & 3D). In addition, AMT and single strain administration group with zymosan attenuated the levels of TNF-α on day 14 (Fig. 3C). The combination group of two *Lactobacillus* strains also suppressed TNF-α and IL-1β at day 7. (Fig. 3A & 3B). These results show that *Lactobacillus* strains can show the alleviation effect in mice with zymosan-induced IBS symptoms like low-grade inflammation.

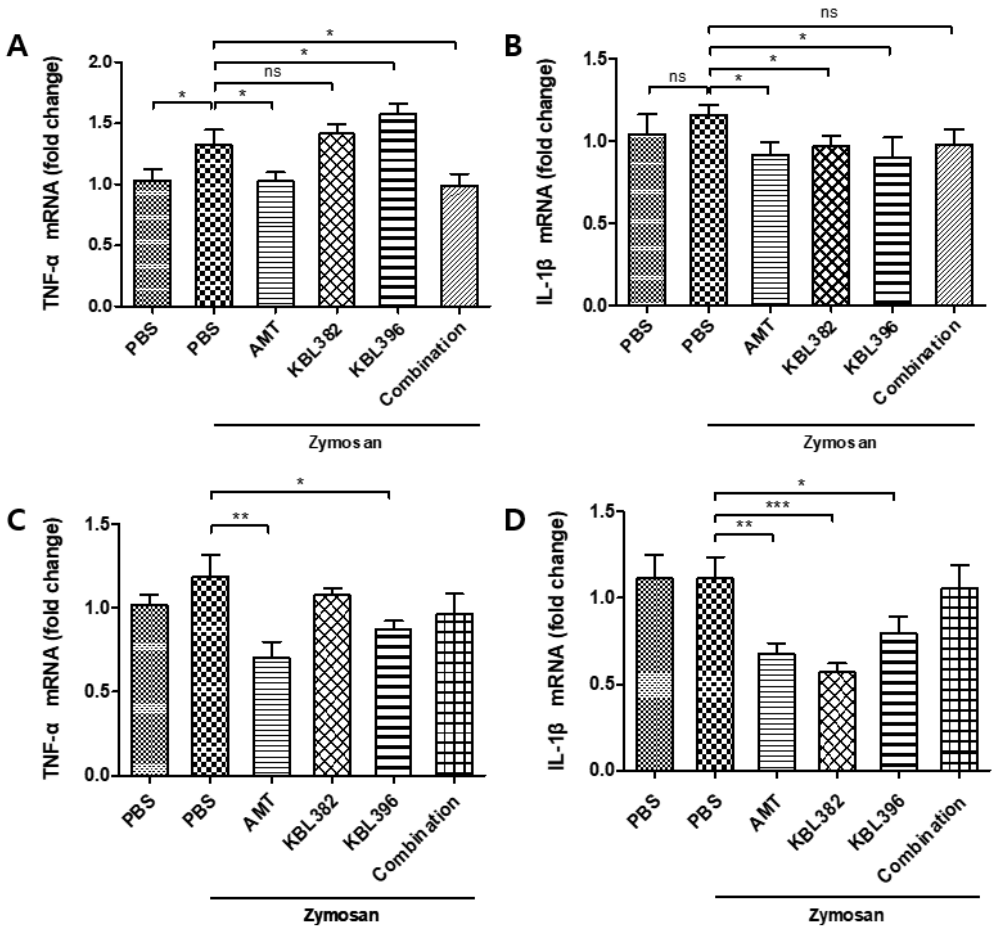

A
TNF-α mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
*
*
ns
*
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
B
IL-1β mRNA (fold change)
1.5
1.0
0.5
0.0
ns
ns
*
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
C
TNF-α mRNA (fold change)
1.5
1.0
0.5
0.0
**
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
D
IL-1β mRNA (fold change)
1.5
1.0
0.5
0.0
**
***
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan

**Figure 3. Effects of *L. plantarum* KBL396 and *L. paracasei* KBL382 pretreatment on different levels of inflammatory cytokines in the colon.**

Relative expression levels of proinflammatory cytokines were analyzed by RT-qPCR in zymosan-induced IBS mice and saline-treated mice. The levels of (A) TNF-α and (B) IL-1β in the colon were determined at day 7. The levels of (C) TNF-α and (D) IL-1β expression were determined at day 14. Values represent the mean of at least 8 animals ±SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

### 3. Colonic mucosal BDNF, serotonin, and serotonin transporter level are affected by administration of *L. plantarum* KBL396 and *L. paracasei* KBL382 in mice

Recent study has shown that brain-derived neurotrophic factor (BDNF) is modulated by gut microbiota in the CNS [33] and increased expression of BDNF in colonic mucosa has been found in IBS patients [19-22]. To determine the role of *L. plantarum* KBL396 and *L. paracasei* KBL382 in the mucosal BDNF level, relative expression levels of BDNF in the distal colon were measured by RT-qPCR at days 7 and 14. BDNF expressions in the colon were significantly increased in the zymosan injected control mice compared to normal saline injected mice. Moreover, all three different treatment groups with *Lactobacillus* strains showed a significant decrease in the mucosal BDNF level, while AMT treated mice showed similar BDNF mRNA level to control mice in the colon on day 7 (Fig. 4A). This trend continued until day 14 except the AMT treated group which showed significant decrease in levels of BDNF in colonic mucosa (Fig. 4D). In patients with IBS, it has been reported that 5HT3 antagonists can reduce the visceral hypersensitive, which is a hallmark characteristic of IBS, by preventing the activation of 5HT3 receptor [34]. In this study,

only a slight difference was observed for 5HT3A mRNA level on day 7 in colon (Fig. 4B). However, it was significantly increased in AMT and *L. plantarum* KBL396 treated mice on day 14 (Fig. 4E). On the other hand, control mice showed the decreased level of SERT1 expression compared to other different treated groups on both days (Fig. 4C & 4F). In addition, three groups administered with *Lactobacillus* showed a significantly higher level of SERT1 compared to control group (Fig. 4F). Notably, the lowest level of mucosal BDNF was seen in the group treated with the combination of the two strains at day 7 and the highest level of SERT1 at day 14 in colonic mucosa.

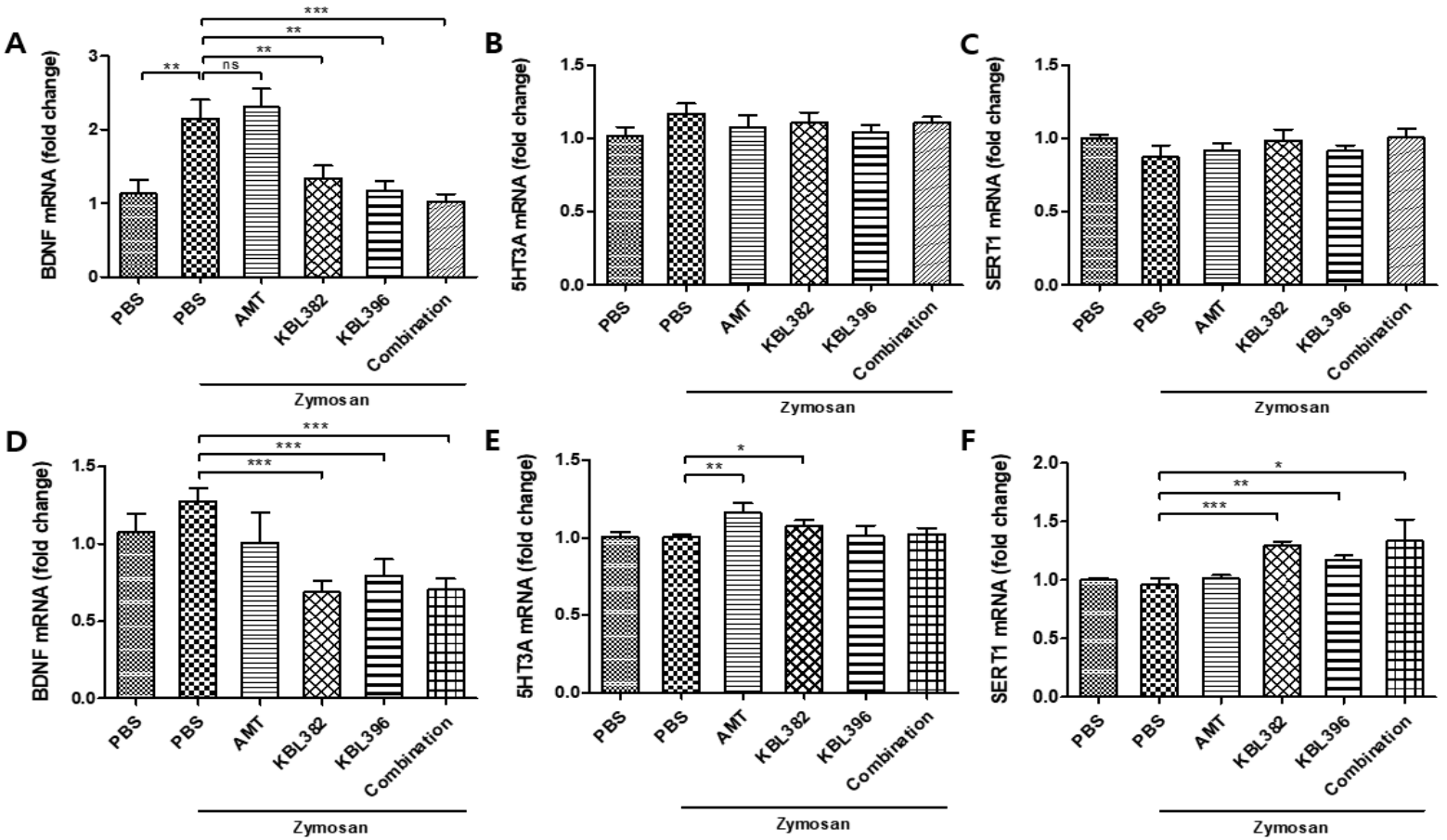

A
BDNF mRNA (fold change)
B
5HT3A mRNA (fold change)
C
SERT1 mRNA (fold change)
D
BDNF mRNA (fold change)
E
5HT3A mRNA (fold change)
F
SERT1 mRNA (fold change)
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
ns

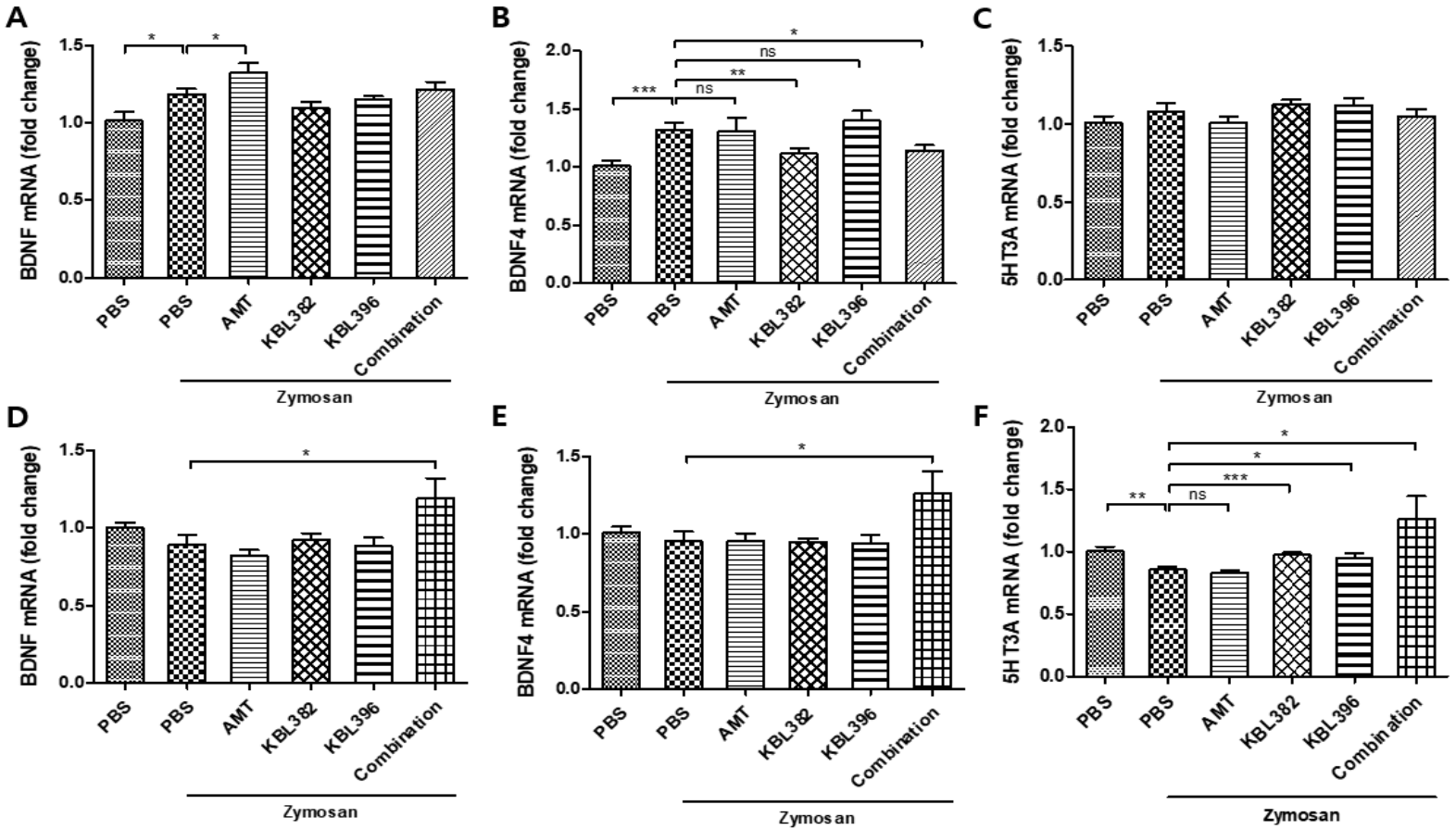
**Figure 4. Different levels of Brain-derived neurotrophic factor (BDNF), serotonin receptor (5HT3A), and serotonin transporter (SERT1) in the colon of six groups of mice under different treatments.**

Relative expression levels of mRNA in the distal colon were determined by RT-qPCR at days 7 and 14. (A) Total BDNF, (B) 5HT3A, and (C) SERT1 levels were measured on day 7. (D) Total BDNF, (E) 5HT3A, and (F) SERT1 levels were measured on day 14. Statistical analysis was performed using t test and values represent the mean of at least 8 animals ±SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

**4. *L. plantarum* KBL396 and *L. paracasei* KBL382 treatment effect on changes in the expression of BDNF and serotonin level in the hippocampus and prefrontal cortex**

Recent study has shown that administration of probiotics can modulate levels of BDNF in the brain [35, 36]. To investigate the effects of selected *Lactobacillus* strains and the combination of these two strains on BDNF in IBS-like mice, BDNF total variant and BDNF exon IV expression in the hippocampus and prefrontal cortex were measured by reverse transcription polymerase chain reaction (RT-PCR) at days 7 and 14. Zymosan injected control mice had a significant increase in both BDNF total variants and BDNF exon IV compared to normal saline injected mice on day 7, while there was no significant differences between two groups on day 14 (Fig. 5). The combination of two *Lactobacillus* strains increased both BDNF total variants and BDNF exon IV significantly on day 14 (Fig. 5D and 5E), while decreased BDNF exon IV on day 7 in zymosan-induced mice (Fig. 5B). Depression is associated with IBS in bidirectional gut-brain communication [37] and it is well known that decreased levels of serotonin in the brain caused a decreased mood [38]. Zymosan injected control mice showed a significant decrease in 5HT3A compared to

other treatment groups, except AMT treated mice, on day 14 (Fig. 5F). Although there were no significant changes in 5HT3A between groups (Fig. 5C), three groups administered with *Lactobacillus* showed a higher level of 5HT3A tendency than zymosan injected control group. The results obtained showed that *L. plantarum* KBL396 and *L. paracasei* KBL382 influences brain chemistry in zymosan-induced IBS animal model.


A
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1.5
1.0
0.5
0.0
*
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
B
BDNF4 mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
***
ns
**
ns
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
C
5HT3A mRNA (fold change)
1.5
1.0
0.5
0.0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
D
BDNF mRNA (fold change)
1.5
1.0
0.5
0.0
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
E
BDNF4 mRNA (fold change)
1.5
1.0
0.5
0.0
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
F
5HT3A mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
**
ns
***
*
*
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan

**Figure 5. Different levels of Brain-derived neurotrophic factor (BDNF), BDNF4, and serotonin receptor (5HT3A) in the hippocampus (HPC) and prefrontal cortex (PFC) of six groups of mice under different treatments.**

Relative expression levels of mRNA in the brain region were determined by RT-qPCR at days 7 and 14. (A) Total BDNF, (B) BDNF4, and (C) 5HT3A levels were measured on day 7. (D) Total BDNF, (E)BDNF4, and (F) 5HT3A levels were measured on day 14. Statistical analysis was performed using t test and values represent the mean of at least 8 animals ±SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

## 5. Administration of *L. plantarum* KBL396 and *L. paracasei* KBL382 effects on anxiety-like behavior in the open filed test

Several studies have found that the administration of the combination of probiotic gut bacteria have an impact on brain functions as well as anxiety [39, 40]. In the open field test, the lower number of entries into the central zone was observed in the combination group compared to the control group, while the travel distance within and the time spent in central zone were increased slightly on day 6 (Fig. 6). However, no significant change was observed amongst the two groups on day 13. A higher number of entries into the central zone was observed on both days in the AMT group. There was no statistically significant difference between *L. paracasei* KBL382 treated and control group except *L. paracasei* KBL382 treated mice spent more time in the central zone on day 6 (Fig. 6E). Finally, administration of *L. plantarum* KBL396 has a beneficial impact on anxiety-like behavior on day 13.

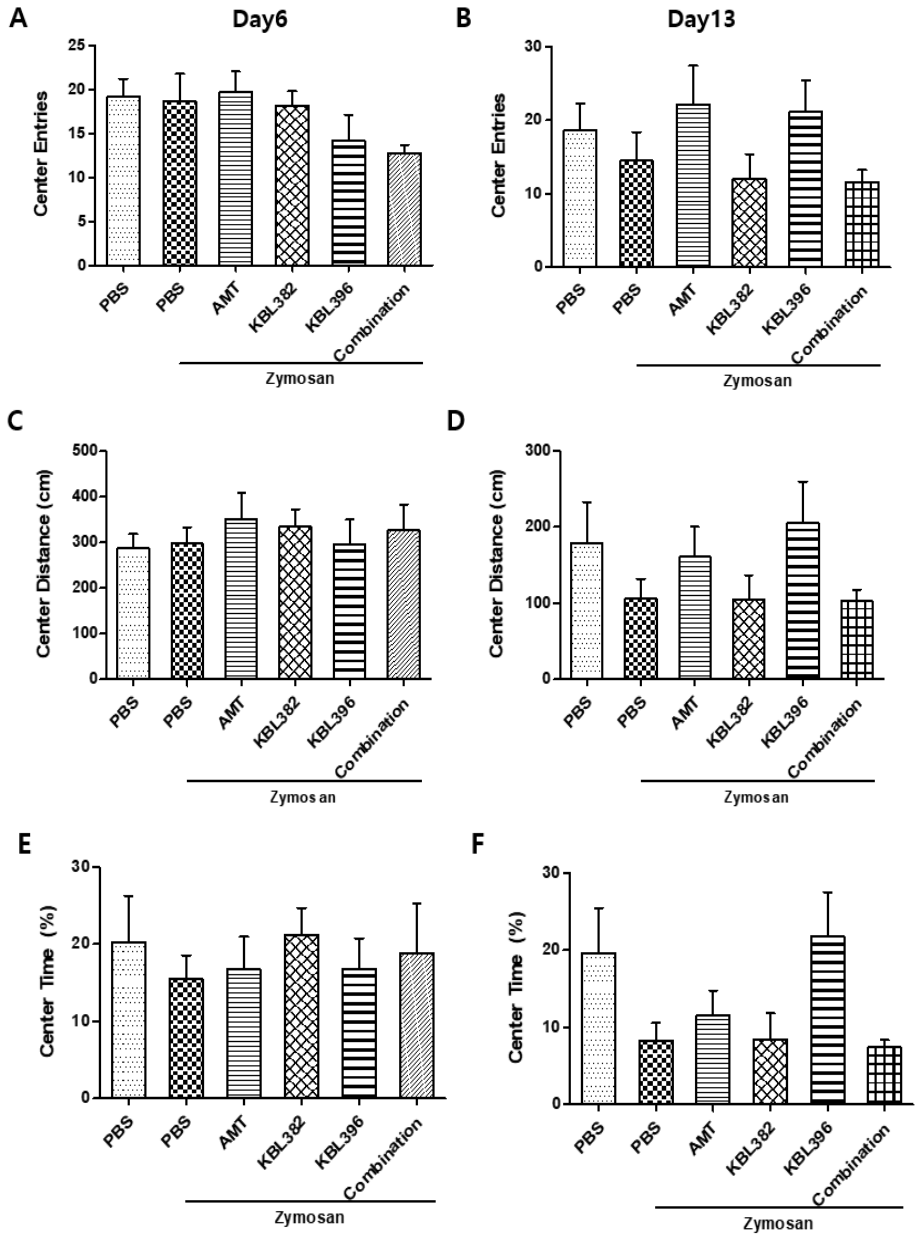
A
Day6
Center Entries
25
20
15
10
5
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
B
Day13
Center Entries
30
20
10
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
C
Center Distance (cm)
500
400
300
200
100
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
D
Center Distance (cm)
300
200
100
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
E
Center Time (%)
30
20
10
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
F
Center Time (%)
30
20
10
0
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan

**Figure 6. Effects of *L. plantarum* KBL396 and *L. paracasei* KBL382 treatment on behavioral assessment of zymosan-treated mice in the open field (OFT).**

The number of entries into the central zone was counted for 5 min in the open field test on (A) day 6 and (B) day 13. The distance traveled in the central zone was measured for 5 min in the open field test on (C) day 6 and (D) day 13. The percentage of relative stay in the central zone was measured for 5 min in the open field test on (E) day 6 and (F) 13. Statistical analysis was performed using one-way ANOVA and data are presented as mean ± standard error of mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.00$

## 6. Administration of *L. plantarum* KBL396 and *L. paracasei* KBL382 effects on anxiety-like behavior in the elevated plus maze test

In the elevated plus maze test, mice administered with the combination of two *Lactobacillus* strains showed increased the distance travelled in and time spent on the open arms in comparison with control mice injected with zymosan, although no significant differences were found in the number of entries into the open arms between two groups on days 6 and 13 (Fig. 7). Mice administered with *L. plantarum* KBL396 showed the higher number of entries into the open arms day 6 and 13 (Fig. 7A & 7B). Also, the increased travel distance and time spent on the open arms were observe in this group compared to control mice injected with zymosan on day 13 (Fig. 7D & 7F). Mice administered with *L. paracasei* KBL382 showed the higher number of entries, increased the distance travelled, and more time spent on the open arms compared to control mice injected with zymosan on day 6, while no significant differences were observed on day 13 in comparison with control mice injected with zymosan.

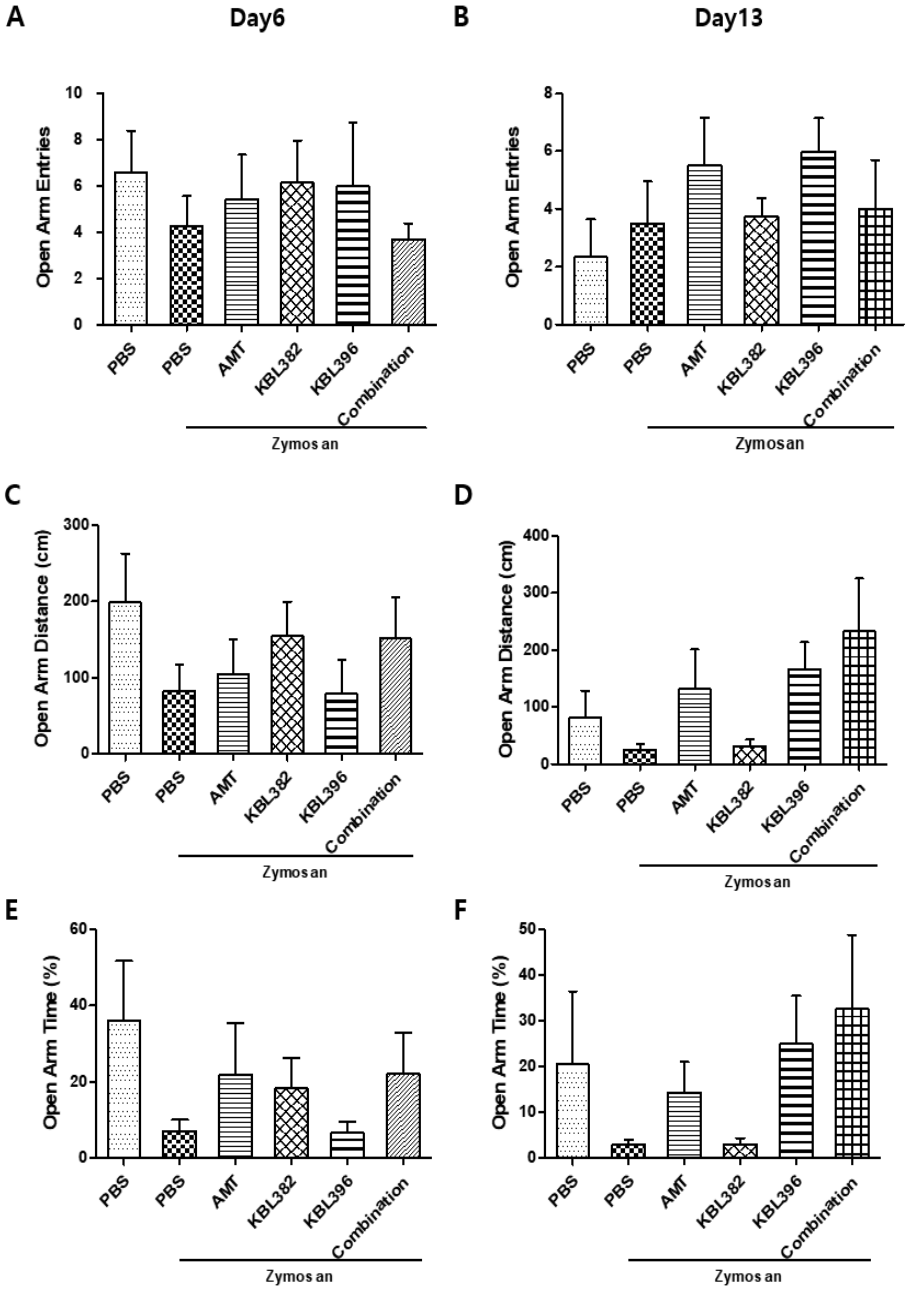
A
Day6
Open Arm Entries
PBS
PBS
AMT
KBL382
KBL396
Combination
Zymosan
B
Day13
Open Arm Entries
C
Open Arm Distance (cm)
D
Open Arm Distance (cm)
E
Open Arm Time (%)
F
Open Arm Time (%)

**Figure 7. Effects of *L. plantarum* KBL396 and *L. paracasei* KBL382 treatment on behavioral assessment of zymosan-treated mice in elevated plus maze (EPM).**

The number of entries into open arms was scored for 5 min in the elevated plus maze on (A) day 6 and (B) day 13. The distance traveled in the open arms was measured for 5 min in the elevated plus maze on (C) day 6 and (D) 13. The percentage of relative stay in the open arms was measured for 5 min in the elevated plus maze on (E) day 6 and (F) day 13. Statistical analysis was performed using one-way ANOVA and data are presented as mean ± standard error of mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

## IV. Discussion

In the present study, we examined the administration of two selected *Lactobacillus* strains and its beneficial effects in zymosan-induced IBS mice. Our results included three major findings: (1) decreased expression of proinflammatory cytokines TNF-α and IL-1β in the colons of mice administered with *Lactobacillus* strains demonstrated that probiotic gut bacteria modulate the immune response and improve low-grade inflammation in IBS. (2) Decreased level of BDNF and increased level of SERT1 expression in the colons of mice administered with *Lactobacillus* strains showed treatment of *L. plantarum* KBL396 and *L. paracasei* KBL382 help alleviate IBS symptoms, such as visceral hypersensitivity. (3) Increased mRNA expression of 5HT3A in brain and reduced anxiety-like behavior in the elevated plus maze test in mice administered with the combination of two *Lactobacillus* strains indicate that treatment of the combination of *L. plantarum* KBL396 and *L. paracasei* KBL382 has an impact on brain chemistry and psychological features of IBS in bidirectional interactions within the brain-gut-microbiota axis. The findings of this study suggest that probiotic gut microbiota, especially *Lactobacillus plantarum* KBL396 and *Lactobacillus paracasei* KBL382, can play an

important role in improving symptoms and psychiatric morbidity associated with IBS.

Some evidence studies have reported that administration of combined probiotic *Lactobacillus* and *Bifidobacterium* spp. have significant beneficial effects on abdominal pain, delayed colonic transit, and bloating [39 - 41]. Furthermore, recent study has shown that pretreatment of the combination of two probiotics *Bifidobacterium longum* R0175 and *Lactobacillus helveticus* R0052 has more efficacy in reducing the chronic stress-induced visceral hypersensitivity than pretreatment with single strain alone in mice [42]. Our previous work demonstrated that *Lactobacillus plantarum* KBL396 can prevent depressive-like behavior and diminish immune changes induced by chronic social defeat stress in mice. In addition, it was confirmed that treatment of *Lactobacillus paracasei* KBL382 can help reduce the signs and symptoms of inflammation in a DSS-induced colitis mouse model. These findings suggest that administration of combined *L. plantarum* KBL396 and *L. paracasei* KBL382 may have positive effects on IBS-like symptoms in mice since IBS is related with the dysfunctional gut-brain-microbiome axis. In this study, we demonstrated that pretreatment

of mixed strains of two *Lactobacillus* strains display reduced proinflammatory properties in zymosan-induced low grade inflammation in mice on days 7 and 14 (Fig. 3). In animal study, it has been reported that proinflammatory cytokines TNF-α can induce psychiatric-like symptoms [43]. Interestingly, mice pretreated with single strain of *Lactobacillus* showed increased TNF-α mRNA expression on day 7, while TNF-α level was decreased on day 14 in comparison with control mice. However, little is known about the specific mechanisms underlying the anti-inflammatory effects of intestinal microbiome. Therefore, further studies are necessary to fully understand this.

Numerous studies have shown that increased production of BDNF in colonic mucosa contributes to IBS-like colonic hypersensitivity [20, 21, 44]. In previous study, BDNF knockdown or tyrosine receptor kinase B (TrkB) inhibitor, also known as the high-affinity receptor of BDNF inhibitor, reduces visceral hypersensitivity in mice [21]. Our study demonstrates that mice treated with mixed and single strain of *Lactobacillus* exhibited decreased mucosal BDNF expression in colon (Fig. 4A & 4D). On the contrary, deceased SERT

expression has been reported in IBS patients [45, 46]. The serotonin transporter terminates serotonergic signaling of serotonin by sodium- and chloride- dependent reuptake [47]. In this study, we convincedly demonstrated that level of SERT expression increases significantly in mice that administered with mixed and single strain of *L. plantarum* KBL396 and *L. paracasei* KBL382 on day 14 (Fig. 4F). However, there was no statistically significant change in the expression of 5HT3A between the groups (Fig. 4B &4E). Although no significant differences were seen in level of 5HT3A mRNA in the colon, we found statistically significant changes in 5HT3A levels in brain (Fig. 5F). Indeed, it has been suggested that gut microbiota can influence brain chemistry. In brain, serotonin (5-HT) plays an important role in the mood and its activation shows negative correlation with anxiety and depression [48]. It is also well recognized that the 5-HT3 receptor antagonists are used for the treatment of IBS symptoms as the 5HT3A polymorphism is associated with IBS [49]. Our results showed a significant increase in level of 5HT3A expression in the brain of mice that treated with mixed and single strain of *L. plantarum* KBL396 and *L. paracasei* KBL382 on day 14 (Fig. 5D). It was also observed that pretreatment of the combination of the two *Lactobacillus* strains produced significant

increase in BDNF and BDNF exon IV expression in the brain (Fig. 5D & 5E). Interestingly, it has been reported that oral administration of probiotics increases BDNF expression in brain and shows a significant anxiolytic effect [50].

It is becoming increasingly evident that gut microbiota can influence brain function and behavior [51-53]. In several studies, pain and anxiety behaviors in zymosan-induced animal model of IBS have been reported [54-56]. In this study, we found that single strain of *L. plantarum* KBL396-treated mice experienced less anxiety compared to control mice, as measured by the number of entries, distance traveled, and time spent in the central zone in the open field test on day 13 (Fig. 6). Interestingly, mice treated with mixed and single strain of *L. plantarum* KBL396 and *L. paracasei* KBL382 display reduced anxiety-like behavior, as measured by the number of entries, distance traveled, and time spent in the open arms using elevated plus maze. (Fig. 7). These results collectively suggest that *L. plantarum* KBL396 and *L. paracasei* KBL382 have anxiolytic effects on anxiety-like behaviors. However, no statistically significant difference was observed between groups. In the same group there were deviations within the group of

mice, as a result there were too little number of mice to show the statistical significance of the tests. In order to show the statistical significance, a retest need to be performed with greater number of mice. Therefore, other environmental factors influencing the animal's behavior and movement need to be more carefully controlled to minimize variations of individual mice within the same group.

In summary, our study shows that treatment with either mixed or single strain of *L. plantarum* KBL396 and *L. paracasei* KBL382 reduces colonic inflammation and alters chemistry associated with visceral hypersensitivity in gut-brain axis in zymosan-induced mouse model of IBS. Our findings provide a base for further research on therapeutic solution for the treatment of IBS.

# V. References


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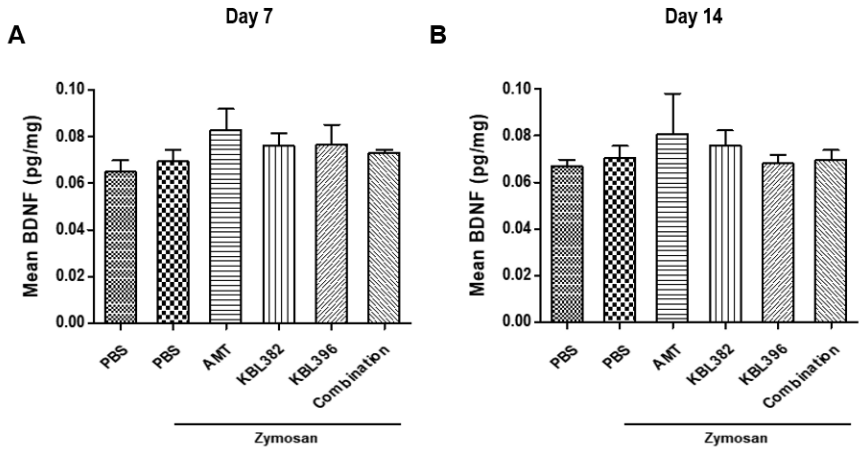
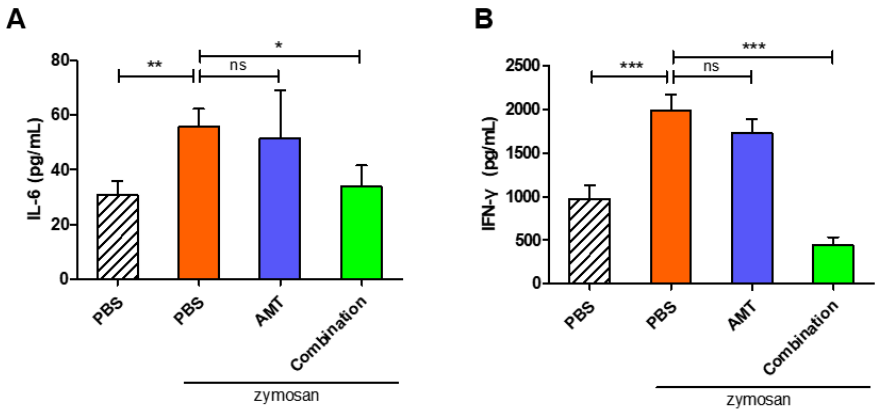
## [Appendix 1] Supplementary figure 1




**Supplementary Figure 1. Brain Bdnf level**

Mean BDNF in the brain region were determined by ELISA at (A) day 7 and (B) day 14. Statistical analysis was performed using t test and values represent the mean of at least 8 animals ±SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

## [Appendix 2] Abbreviation




**Supplementary Figure 2. Level of IL-6 and IFN-γ (colon) in serum**

Level of IL-6 and IFN-γ in serum were determined by ELISA at day 7. (A) IL-6 is downregulated in the combination group. (B) IFN-γ level is decreased in the combination group in serum. Statistical analysis was performed using t test and values represent the mean of at least 5 animals ±SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

**[Appendix 3] Abbreviation**

amitriptyline (AMT)

brain-derived neurotrophic factor (BDNF)

Central nervous system (CNS)

colony-forming units (CFU)

dextran sulfate sodium (DSS)

Elevated Plus Maze (EPM)

Enzyme-Linked Immunosorbent Assay (ELISA)

Irritable bowel syndrome (IBS)

Open Field Test (OFT)

phosphate-buffered saline (PBS)

serotonin transporter (SERT)

serotonin (5-HT)

# 국문초록

## 락토바실러스 플란타럼과 락토바실러스 파라카제이 혼합 균주의 과민성 장 증후군 예방 및 증상 완화 효과 연구

서울대학교 보건대학원

환경보건학과 환경보건학 전공

송림

지도교수 고 광 표

과민성 장 증후군은 가장 흔한 기능성 위장관 장애로 전세계적으로 유병률이 증가하고 있다. 과민성 장 증후군 환자에서는 복통과 배변 습관 변화 등의 이상 뿐만 아니라 불안장애와 우울증과 같은 정신과적 증상이 동반되는 것으로 알려져 있다. 그럼에도 불구하고 아직까지 확실한 치료법이 없어 단기적으로 증상을 개선시키는 치료밖에 없는 실정이다. 그러나 최근에는 과민성 장 증후군 환자에서 장내 미생물군총과 그 기능이 변화되는 것이 밝혀지면서 장내 미생물이 과민성 장 증후군에서 장-뇌 축을 따르는 중요한 병리 생리학적 요소임이 알려졌다. 특히, 과민성 장 증후군 환자에서 일반적으로 락토바실러스와 비피도박테리움 종이 감소하는 것으로 확인되었다. 본 연구에서는 과민성 장 증후군 마우스 모델에서 락토바실러스 플란타룸 KBL396과 락토바실러스 파라카제이 KBL382의 혼합 및 단일 균주의 효능 및 효과를 확인하고자 하였다. 마우스에 과민성 장 증후군과 같은 증상을 유도하기 위해 자이모산 현탁액을 3일간 연속적으로 항문 주사 하였다. 락토바실러스 플란타룸 KBL396과 락토바실러스 파라카제이 KBL382를 투여한 마우스에서 자이모산 주입

후 각각 7일차와 14일차에 대장과 뇌에서 염증성 사이토카인의 감소 및 뇌유래신경영양인자 및 신경전달물질의 mRNA 발현량이 변화되는 것을 확인하였다. 또한, 고가식 십자미로와 오픈 필드 평가를 통해 두 락토바실러스 균주를 혼합 또는 단일로 투여한 마우스에서 불안 장애를 나타내는 행동이 감소되는 것을 확인하였다. 이러한 결과는 락토바실러스 플란타룸 KBL396과 락토바실러스 파라카제이 KBL382가 마우스에서 과민성 장 증후군 증상을 완화시키고 불안 장애와 같은 행동을 개선할 수 있음을 보여주고 있다.

**주요 단어:** 불안장애 행동, 장-뇌 축, 과민성 장 증후군, 락토바실러스, 장내 미생물, 자이모산

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