Original Article

# *Lactobacillus plantarum* B7 Improved *Salmonella* Typhimurium Developed Diarrhea in Mice

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## ABSTRACT

***AIM:*** To determine the effects of *Lactobacillus plantarum* B7 (*L. platarum* B7) reduces pro-inflammatory cytokines (TNF-α level, Interleukin-6level and CXCL1 level) and attenuates the physical symptoms of *S.* Typhimurium induced diarrhea in mice.

***METHODS:*** Male albino mice were randomly divided into 3 groups: control group (n=8), mice were fed with 1 mL of 0.85% saline by oral gavage feeding. Salmonella group (n=8), mice were fed with $3x10^9$ CFU of *S.* Typhimurium 1 mL suspended in 0.85% saline, and Salmonella +LP group (n=8), mice were fed with $1x10^9$ CFU of *L. plantarum* B7 suspended in 0.85% saline. After 2 hours, mice were fed with $3x10^9$ CFU of *S.* Typhimurium suspended in 1 mL of 0.85% saline for 3 consecutive days. All groups received a 3-day pre-treatment with streptomycin suspended in drinking water (5 mg/mL). The body weight of mice were measured and recorded daily. After 3 days, fresh specimens of feces were collected for stool culture and colony counts to assess S. Typhimurium infection. Blood samples were also collected to determine TNF-α level, Interleukin-6 and CXCL1 levels. Fecal characteristics and the percentage of fecal moisture content (%FMC) were measured.

***RESULTS:*** The quantitative of *S.* Typhimuriumin fecal specimens significantly decreased in the Salmonella + LP group compared with the Salmonella group (7.4.2±0.05 *vs* 8.86±0.02 CFU, $p<0.05$). The levels of TNF-α, IL-6 and CXCL1 significantly increased in the Salmonella group compared with the control group (128.59±12.82 vs. 53.49±8.90, 144.44±8.91 vs. 66.51±4.04, 96.09±10.81 vs. 32.32±4.54 pg/mL respectively, $p<0.05$) and significantly decreased in the Salmonella+LP group compared with the Salmonella group (36.15±9.22 vs. 128.59±12.82, 70.36±5.37 *vs.* 144.44±8.91, 35.40±2.77 *vs.* 96.09±10.81 pg/mL respectively, $p<0.05$). Fecal consistency was soft or loose in the Salmonella group, and was rod-shaped and dark in the Salmonella+LP group. Fecal moisture percentage (%FMC) significantly increased in the Salmonella group compared with the control group (43.24±2.05% *vs.* 14.19±1.57%, $p<0.05$), and significantly decreased in the Salmonella+LP group compared with the Salmonella group (24.65±2.08% *vs.* 43.24±2.05%, $p<0.05$).

***CONCLUSIONS:*** Oral administration of *L.plantarum* B7 can inhibit *S.* Typhimurium growth, decrease pro-inflammatory cytokine levels, attenuate inflammatory response and improve fecal moisture. *L.plantarum* B7 can prevent *S.* Typhimurium diarrhea in mice.

**Key words :** *Lactobacillus plantarum B7; Salmonella Typhimurium; Salmonella diarrhea*

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## Introduction

*Salmonella* Typhimurium is an enteropathogen in the family of *Enterobacteriacea.* It is a gram-negative, rod-shaped non-spore-forming, facultative anaerobic bacteria belonging to the species *Salmonella enteric.* Like other *Enterobacteriaceae, S.*Typhimurium produces acid on glucose fermentation, reduces nitrates, and does not produce cytochrome oxidase[1], and is a member of group-B of Salmonella based on sharing of O- antigens. The outer membranes of *S.* Typhimurium consist of lipopolysaccharides (LPS) or somatic-O which is the basic component serovars in the classification of *Salmonella* bacteria based on the somatic-O and flagella-H antigens as described by Kaufmann-White scheme[2]. S. Typhimurium is an important pathogen for human and warm- blooded animals. toxicity and ability to alter the host immune response and the inflammatory reaction[3]. Salmonella Typhimurium is a major cause of acute gastroenteritis and enterocolitis with or without bacteremia[4]. Common manifestations include frequent loose or watery stools diarrheal with excess luminal loss of water, electrolytes, fat, and other substances, and total stool passage of more than 200 g stool per day[5,6]. Inflammation damages to the intestinal mucosal lining especially the brush border leads to water and electrolyte leakages as well as decreased reabsorption of these substances. Nausea, vomiting, abdominal pain, fever and muscle weakness commonly occur 12-72 hrs after the onset of diarrhea[7]. Symptom usually last 4 to 7 days in most patients, and recovery may follow ever without treatment. In some cases, diarrhea can be severe with passage of bloody stool from severe mucosal damage. *S*. Typhimurium septicemia can also develop which is potentially fatal.

Worldwide, *S.* Typhimurium infection is indeed a common cause of death and the second most common cause of infantile mortality[8]. In 2009, WHO announced that diarrhea was the cause of 1.1 million deaths in children 5 years old and over, and 1.5 million deaths in children under 5 years old[9]. Currently, there are approximately 94 million worldwide cases of Salmonella inflammatory diarrhea, causing around 150,000 deaths[10,11]. Treatment of *Salmonella* diarrhea comprises antibiotics and intestinal anti-inflammatory agents. Intestinal anti-inflammatory agents are used to reduce inflammatory response and improve mucosal barrier function of the intestine. Ampicillin, cefotaxime, chloramphenicol and ciprofloxacin are antibiotics commonly used in the treatment of *Salmonella* infection[12]. Adverse effects of antibiotic and intestinal anti-inflammatory agents include nausea, vomiting, stomach cramps and allergic reactions. Disruption of normal gut flora may lead to drug interaction ever tendon and kidney damages[13]. The issue of antibiotic-resistant *Salmonellosis* can create further problems [14].

Probiotics have recently provided an alternative treatment approach for *Salmonella* diarrhea. Probiotics are live natural microorganisms which, when administered in adequate amounts, confer a health benefit on the host[15]. They are present in the normal human digestive tract and help maintain the balance of normal intestinal flora[16]. There are many strains of probiotics, including *Lactobacillus rhamnosus* GG, *Lactobacillu reuteri, Lactobacillus casei, Lactobacillus acidophilus* CL1285, *Escherichia coli* strain Nissle 1917, certain bifidobacteria and enterococci (Enterococcus faecium SF68) and certain yeasts such as *Saccharomyces boulardii.* Probiotics can inhibit growth and metabolic activity of pathogenic enteric bacteria (e.g. *Salmonella, Shigella, E. coli,* or *Vibrio cholerae*)[17]. The mechanisms involved in the probiotic treatment and prevention of diarrhea include protection of the intestinal epithelial barrier function, regulation of the intestinal microbial environment, and modifications of natural commensal probiotic bacteria to enhance diarrhea prevention. *Lactobacillus plantarum* is a gram-positive bacteria of the *Lactobacillaceae* family found in human gastrointestinal tract as well as female reproductive system[18]. *Lactobacillus* is used in food industry for fermentating food and beverages such as yogurt, cheese, pickles, beer, wine, cider, etc. *Lactobacillus plantarum* is mostly used in medicine as biotherapeutics for the prevention and treatment of various gastrointestinal disorders, including *Salmonella* infection and diarrhea[19-21]. Certain Some strains of *Lactobacillus plantarum* can inhibit growth of pathogenic bacteria[22], prevent bacterial adhesion to enterocytes, prevent invasion of enteropathogens into intestinal epithelial cells[23], induce confer anti-inflammatory and immunomodulatory activities resulting in reduction of inflammatory response[24,25], and enhance the intestinal barrier function to prevent diarrhea[26]. *L. plantarum* has also been used to reduce allergenicity from soy flour[27].

The aims of the present study were determine the protective effects of *Lactobacillus plantarum* B7 on

Salmonella Typhimurium against the development of diarrhea, to investigate the mechanism of inflammatory response, and to observe the physical symptoms of *Salmonella* Typhimurium infection in mice.

## Materials and Methods

### Ethics

The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All experiments and procedures were carried out in laboratory mice and were conducted in accordance with the guidelines for study in experimental animals issued by the National Research Council of Thailand (1999).

### Animal preparation

Male albino mice weighing 20-25 grams were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornpathom, Thailand. The mice were kept at a controlled room temperature of 25 ± 1˚C with 12:12 hour light-dark cycle. All animals received proper care in accordance with guidelines laid down by the Ethical Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

### Bacterial preparations

*Salmonella* Typhimurium ATCC 13311 was grown on Salmonella-Shigella agar (SS agar) (Oxoid, Basingstoke, United Kingdom). The plates were incubated at 37˚C under aerobic conditions for 24 hours.

*Lactobacillus plantarum* B7 was isolated from a dyspeptic Thai patient at King Chulalongkorn Memorial Hospital, and was stored in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom) with 20% glycerol at -80˚C. This strain was recovered from a frozen stock and cultivated twice on MRS agar anaerobically (10% $CO_2$, 10% $H_2$ and 80% $N_2$) in an anaerobic jar at 37˚C for 48 hours.

### Experimental protocol

Albino male mice were randomly divided into 3 groups. Group 1 (Control group, n=8): The mice were fed with 1 mL 0.85% saline by oral gavage feeding once daily for 2 days, and there after housed with free access to water and standard food. Group 2 (*Salmonella* group or S group, n=8): The mice were fed with $3x10^9$ CFU *S.* Typhimurium 1 mL suspended in 0.85% saline by oral gavage feeding once daily for 2 days, and there after housed with free access to water and standard food. Group 3 (*Salmonella* +LP group n=8): The mice were fed with $1x10^9$ CFU *L. plantarum* B7 suspended in 1 mL 0.85% saline by oral gavage feeding. Two hours after treatment with *L.plantarum* B7 2 hour, the mice were fed again with $3x10^9$ CFU *S.* Typhimurium suspended in 1 mL 0.85% saline by oral gavage feeding daily for 2 days, and there after housed with free access to water and standard food.

All experimented mice were pre-treated with streptomycin suspended in drinking water (5 mg/mL) daily for 3 days, followed by treatment with $3 \times 10^9$ CFU *S.* Typhimurium 1 mL or $1 \times 10^9$ CFU *L.plantarum* B7 suspended in 1 mL 0.85% saline by oral gavage feeding.

The body weight and physical symptoms including activities and fecal moisture content of each animal were recorded daily. After treatment with $3x10^9$ CFU *S.* Typhimurium 1 mL or $1 \times 10^9$ CFU *L.plantarum* B7 daily for 2 days, fresh fecal specimens were collected to search for *S.* Typhimurium infection by stool culturing with colony counting and measurement of fecal moisture. The mice were finally sacrificed with a lethal dose of intraperitoneal thiopental sodium injection. Blood samples were collected from cardiac puncture to determine TNF-α level, IL-6 level and CXCL1 level in the serum, using enzyme-linked immunosor-bent assay (ELISA) method.

### Determination of *Salmonella* Typhimurium in feces: Stool culture

Fresh fecal sample (1 gram) was homogenized in phosphate buffer saline (PBS) pH 7.4 400 µL and serial dilutions ($10^{-1}$-$10^{-7}$) were prepared. A suspension of 100 µL was plated on SS agar by a spreader technique and incubated at 37˚C for 24 hours. of approximately 30 colonies Salmonella Typhimurium were counted to confirm that the selected colonies were truly *Salmonella* Typhimurium. A single colony from the SS agar plate was inoculated onto TSI slant agar and incubated at 37˚C for 24 hours. The appearance of colonies on TSI agar test was determined and *Salmonella* Typhimurium confirmed by serological test using *Salmonella* group B antibodies.

The number of *Salmonella* Typhimurium in each sample was calculated with following equation:

$$\text{Number of bacteria / mL (CFU/mL)} = \frac{\text{Number of colonies on plate} \times \text{reciprocal of dilution sample}}{\text{Volume of sample}}$$

### Determination of serum cytokine levels

Blood samples were collected via cardiac puncture and allowed to clot over 2 hours at room temperature before 20-minute centrifuging at approximately 1000 x g. The serum was then removed and stored at -80˚C for further determination of TNF-α, IL-6 and CXCL1 levels using an enzyme-linked immunosorbent assay (ELISA), ELISA kit.

### Determination the fecal moisture content

The percentage of water in the fecal sample was determined by drying the sample to a constant weight using a microwave oven drying. The weight of fresh fecal sample was recorded as the "wet weight of sample". The wet sample was then dried at 100˚C for 15 minutes, using hot air oven, followed by cooling. The weight of a cooled sample was recorded as the " dry weight of sample"[28-29].

The percentage of moisture content of the sample was calculated using the equation:

$$\% \text{ Moisture in the sample} = \frac{A - B}{B} \times 100$$

A = Weight of wet sample (grams)
B = Weight of dry sample (grams)

### Statistical analysis

Descriptive statistics was used in this study. The data were presented as mean and standard deviation (SD). Comparisons between groups of animals were using one-way analysis of variance (one-way ANOVA) and Tukey post-hoc comparisons. Differences at $p<0.05$ were considered statistically significant.

## RESULTS

### Concentration of *S.* Typhimurium in fecal specimen

*S.* Typhimurium concentration in 1 gram of fecal specimen significantly increased in the Salmonella group compared with the Control group (8.86±0.02 vs. 0.00±0.00 CFU, $p< 0.05$), but decreased significantly in the Salmonella+LP group compared with the Salmonella group (7.4.2±0.05 *vs.* 8.86±0.02 CFU, $p<0.05$), as shown in Figure 1.

### Serum CXCL1 level

Serum levels of CXCL1 were presented in Figure 2. CXCL1 levels were significantly increased in the Salmonella group compared with the control group (96.09±10.81 *vs.* 32.32±4.54 pg/mL, $p<0.05$). In contrast, administration of *L. plamtarum* B7 significantly decreased the level of serum CXCL1 when compared with the Salmonella group (35.40±2.77 vs. 96.09±10.81 pg/mL, $p<0.05$)

### Serum TNF-α level

As shown in Figure 3, serum TNF-α levels in the Salmonella group were significantly increased compared with the Control group (128.59±12.82 vs. 53.49±8.90 pg/mL, $p<0.05$). After administration of *L.plantarum* B7 in the Salmonella+LP group, serum TNF-α level were, however, significantly decreased compared with the Salmonella group (36.15±9.22 vs. 128.59±12.82 pg/mL, $p<0.05$)

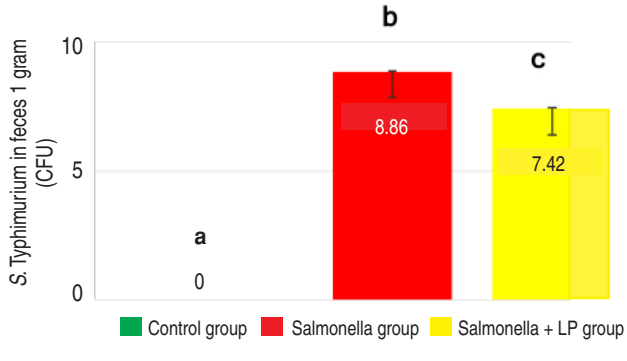


**Figure 1.** Quantitation of *S.* Typhimurium in 1 gram of fecal specimen (CFU) (mean ± SD).
Control group (n = 8): mice fed with 0.85% saline; Salmonella group (n = 8): mice fed with S. Typhimurium $3 \times 10^9$ CFU/mL Salmonella +LP group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL and L. plantarum B7 $1 \times 10^9$ CFU/mL.
[ab]Superscript letters indicate significant differences ($p < 0.05$).

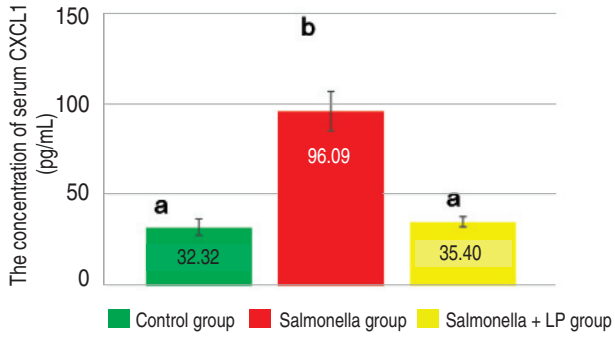


**Figure 2.** Serum levels of CXCL1 in all three groups (mean ± SD)
Control group (n = 8): mice fed 0.85% saline; Salmonella group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL; Salmonella +LP group (n = 8): mice fed with *S.* Typhimurium $1 \times 10^9$ CFU/mL and *L. plantarum* B7 $1 \times 10^9$ CFU/ml.
[ab]Superscript letters indicate significant differences ($p < 0.05$).

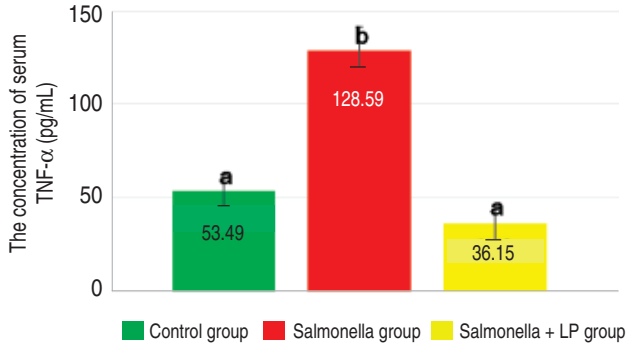


**Figure 3.** Serum levels of TNF-α in all the groups (mean ± SD)
Control group (n = 8): mice fed 0.85% saline; Salmonella group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL; *Salmonella* +LP group (n = 8): mice fed with *S.* Typhimurium $1 \times 10^9$ CFU/mL and *L. plantarum* B7 $1 \times 10^9$ CFU/mL.
[ab]Superscript letters indicate significant differences ($p < 0.05$).

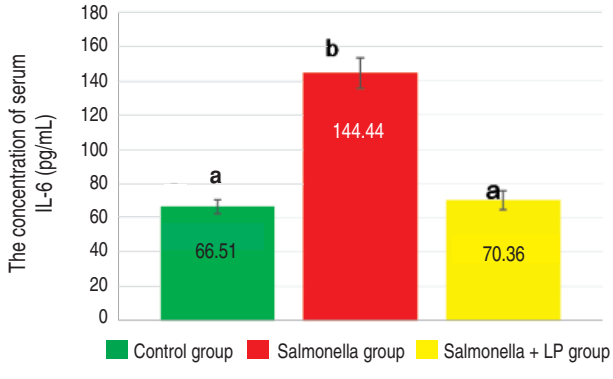


**Figure 4.** Serum levels of IL-6 in all three groups (mean ± SD)
Control group (n = 8): mice fed 0.85% saline; Salmonella group (n = 8): mice fed with S. Typhimurium $3 \times 10^9$ CFU/mL; Salmonella +LP group (n = 8): mice fed with *S.* Typhimurium $1 \times 10^9$ CFU/mL and *L. plantarum* B7 $1 \times 10^9$ CFU/mL.
[ab]Superscript letters indicate significant differences ($p < 0.05$).

## Serum IL-6 level

The levels of serum IL-6 in all groups were presented in Figure 4. IL-6 levels in the Salmonella group significantly increased compared with the Control group (144.44±8.91 vs. 66.51±4.04 pg/mL, $p<0.05$). However, IL-6 levels in the Salmonella+LP group significantly decreased compared with the Salmonella group (70.36±5.37 *vs.* 144.44±8.91 pg/mL, $p<0.05$)

## Fecal characters

Fecal characteristics in all groups are shown in Finger 5. In the control group, the feces was rod-shaped, dark and trifling or without saw-dust appearance on the surface. In the Salmonella group, after feeding with *S.* Typhimurium the feces appeared soft, loose, less dark and with "saw dust" covering. In the Salmonella+LP group, the feces was have the rod-shaped, dark and with a little "saw dust" on the surface.

## Fecal mositure content

The percentage of fecal moisture content (%FMC) in all groups was presented in Figure 6. In the Salmonella group, %FMC significantly increased compared with the Control group (43.24±2.05% vs. 14.19± 1.57%, $p<0.05$). In the Salmonella+LP group, %FMC significantly decreased compared with the Salmonella group (24.65±2.08% vs. 43.24±2.05%, $p<0.05$).

## Discussion

*Salmonella* Typhimurium commonly causes acute


**Figure 5.** Fecal characteristics in all three groups
Control group (n = 8): mice fed with normal diet plus vehicle; Salmonella group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL; Salmonella +LP group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL and *L. plantarum* B7 $1 \times 10^8$ CFU/mL.

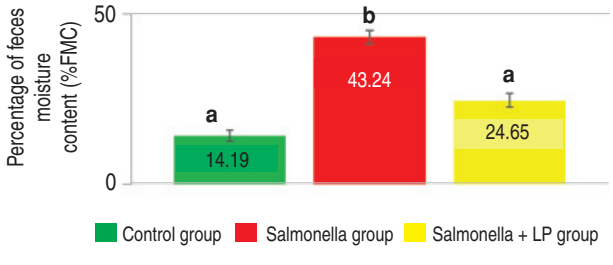


**Figure 6.** Percentage of fecal moisture content in all three groups (mean ± SD)
Control group (n = 8): mice fed 0.85% saline; Salmonella group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL; Salmonella +LP group (n = 8): mice fed with *S.* Typhimurium $3 \times 10^9$ CFU/mL and *L.plantarum* B7 $1 \times 10^9$ CFU/mL.
[ab]Superscript letters indicate significant differences ($p < 0.05$).

gastroenteritis and diarrhea. Various aspects of *S.* Typhimurium diarrhea has remained poorly understood, and pathophysiological and epidemiological studies are lacking. In the present study, a model of S. Typhimurium study in mice was designed, in which pre-treatment with streptomycin suspended in drinking water (5 mg/mL) for 3 days was given[30]. The idea was to eliminate other pathogens in the GI tract thus increasing susceptibility to S. Typhimurium. *Lactobacillus plantarum* B7, a probiotic with antagonistic activity against pathogenic bacteria was chosen. *L. plantarum* B7 has an anti-inflammatory property and can reduce pro-inflammatory cytokines (TNF-α, IL-6, CXCL1)[31-34]. It also possesses anti-pathogenic properties; inhibiting growth of as well as reducing pathogenic bacteria (*S.* Typhimurium), according to previous studies[34-37].

In conclusion: Oral administration of *L.plantarum* B7 can inhibit or reduce *S.* Typhimurium growth and colonies, decrease serum pro-inflammatory cytokines (TNF-α, IL-6, CXCL1), attenuate inflammatory response and improve fecal moisture content. These properties can help prevent *S.* Typhimurium diarrhea in mice.

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