sciendo ASIAN BIOMEDICINE DOI 10.1515/abm-2019-0022 — Asian Biomed (Res Rev News) 2018; 12(5):211–218

Original article

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Lactobacillus plantarum B7 attenuates *Salmonella typhimurium* infection in mice: preclinical study in vitro and in vivo

Siwaporn Wongsen[1], Duangporn Werawatganon[1,*], Somying Tumwasorn[2]

Abstract

Background: *Salmonella typhimurium* is a cause of gastroenteritis including diarrhea. *Lactobacillus plantarum* is a probiotic widely used to prevent and treat diarrhea.
Objectives: To determine the protective effects of *L. plantarum* B7 on diarrhea in mice induced by *S. typhimurium*.
Methods: Inhibition of *S. typhimurium* growth by *L. plantarum* B7 was determined using an agar spot method. Mice were divided into 3 groups (n = 8 each): a control group, an S group administered 3×10^9 CFU/mL *S. typhimurium*, and an S + LP group administered 1×10^9 CFU/mL *L. plantarum* B7 and 3×10^9 CFU/mL *S. typhimurium* daily for 3 days. Counts of *S. typhimurium* and percentage of fecal moisture content (%FMC) were determined from stool samples. Serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and CXCL1 were determined.
Results: *L. plantarum* B7 produced a clear zone on *S. typhimurium*. There were significantly less *S. typhimurium* in the feces from mice in the S+LP group than in the S group. Serum levels of TNF-α, IL-6, and CXCL1 in mice from the S group were significantly higher than levels in the S+LP and control groups. Feces from mice in the S group were soft and loose, whereas in the S+LP group they were hard and rod shaped. The %FMC in the S+LP group was significantly less than in the S group.
Conclusions: *L. plantarum* B7 can inhibit growth of *S. typhimurium*, decrease levels of proinflammatory cytokines, and attenuate symptoms of diarrhea induced in mice by *S. typhimurium*.

Keywords: bacteremia; diarrhea; gastroenteritis; *Lactobacillus plantarum*; *Salmonella typhimurium*

Salmonella typhimurium is an enteropathogen in the family Enterobacteriaceae, and a major cause of acute gastroenteritis and bacteremia [1]. In addition to diarrhea, common symptoms including nausea, vomiting, abdominal pain, fever, and weakness, appear 12–72 h after infection with *S. typhimurium* [2]. In most people, symptoms usually last 4–7 days and the infection does not require treatment. However, some patients the infection may progress with severe symptoms, which can be dangerous and life threatening. *S. typhimurium* can spread from intestines to the blood and eventually cause death without appropriate and timely antibiotic treatment [3]. Diarrhea induced by *Salmonella* is mostly treated with antibiotics and

***Correspondence to: Duangporn Werawatganon,** Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand, e-mail: Duangporn.w@chula.ac.th
[1]Alternative and Complementary Medicine for Gastrointestinal and Liver Diseases Research Unit, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

anti-inflammatory agents; however, the use of antibiotics and anti-inflammatory agents may have adverse effects and often leads to antibiotic resistance.

One alternative treatment for diarrhea induced by *Salmonella* is the use of probiotics. Probiotics are natural, live microorganisms; when administered in adequate amounts, they can promote health benefits for the host [4, 5]. Many strains of probiotics (e.g., *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri*, *Lactobacillus casei*, *Lactobacillus acidophilus* CL1285, *Escherichia coli* strain Nissle 1917, *bifidobacteria, enterococci* [*Enterococcus faecium* SF68], and *Saccharomyces boulardii*) can inhibit growth, metabolic activity, and adhesion of pathogenic enteric bacteria (*Salmonella*, *Shigella*, *E. coli*, or *Vibrio cholerae*) to intestinal epithelial cells [6]. Potential mechanisms of probiotics to prevent and treat of diarrhea include protection of intestinal epithelial function and regulation and modifications of the intestinal microbial environment. *Lactobacillus plantarum* is a gram-positive bacterium in the Lactobacillaceae family. Normal flora are found in the human gastrointestinal (GI) tract and the reproductive system [7]. In the food industry, *L. plantarum* has fermentative properties and has benefits in food and beverage production including for yogurt, cheese, pickles, beer, wine, and cider. In medicine, *L. plantarum* is used mostly as a probiotic and biotherapeutic agent to prevent and treat GI disease and diarrhea [8–10]. Some strains of *L. plantarum* inhibit pathogen growth [11], prevent adhesion and invasion of enteropathogens to intestinal epithelial cells [12], act as an anti-inflammatory and regulate immunomodulatory activities to reduce inflammatory responses [13, 14], which enhances intestinal function to prevent diarrhea [15] and reduces allergenicity from soy flour [16].

The present study aims to determine the protective effects of *L. plantarum* B7 on diarrhea in mice associated with *S. typhimurium* and to illustrate the role of inflammatory response of *S. typhimurium* infection in mice.

Materials and methods

Assay of inhibitory activity in vitro: preparation of bacteria

S. typhimurium (*Salmonella enterica* serovar Typhimurium) ATCC 13311 were grown on tryptic soy agar (TSA) at 37°C in aerobic conditions for 24 h and adjusted to final concentration of 1×10^7 CFU/mL. The frozen cultures of *L. plantarum* B7 were precultivated on de Man, Rogosa and Sharpe (MRS) agar at 37°C for 48 h in anaerobic conditions. Then, a single colony was isolated and subcultured on MRS broth 2 times in 96-well plates and incubated at 37°C for 48 h in anaerobic conditions. *L. plantarum* B7 was isolated and spotted into each well of a 96-well plate containing brain heart infusion (BHI) agar. Supplements with 20 mM glucose in 140 mm plates were incubated at 37°C under anaerobic condition for 24–48 h. Plates were then overlaid with 20 mL of TSA (7.5 g agar/L) and overnight culture of *S. typhimurium* at 1×10^7 CFU/mL and incubated at 37°C for 24 h. In the present study, *L. rhamnosus* L34 was used as a positive control and *L.* fermentum L12 was used as a negative control.

Agar spot method

An agar spot method previously described by Spinler et al. [17] was used to determine antimicrobial activity of *L. plantarum* B7 against *S. typhimurium*.

Study in mice

Bacterial preparation

S. typhimurium ATCC 13311 were cultured on Salmonella Shigella agar (SS agar, Oxoid) by incubation at 37°C under aerobic conditions for 24 h. The colonies of *S. typhimurium* ATCC 13311 were harvested and adjusted to a final concentration of 3×10^9 CFU/mL suspended in 0.85% saline.

L. plantarum B7 was previously isolated from a gastric biopsy of a dyspeptic patient and identified by sequence analysis of the amplified 16S rRNA gene product [18]. The *L. plantarum* B7 were maintained at –80°C in MRS broth containing 20% glycerol at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University.

The *L. plantarum* B7 were recovered from 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 h. Colonies of *L. plantarum* B7 were harvested and adjusted to a final concentration of 1×10^8 CFU/mL suspended in 0.85% saline.

Experimental design

Male albino mice, weighing about 20–25 g, were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornpathom, Thailand. The animals were housed in a temperature-controlled room at 25 ± 1°C

under a 12:12 h light–dark cycle. All procedures conducted on animals were approved by the Animal Ethics Committee, Faculty of Medicine, Chulalongkorn University (approval No. 3/57) and conformed with the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council "Guide for the Care and Use of Laboratory Animals" Washington, D.C.: National Academy Press; 1996. Overall, 24 mice were separated without selection into 3 groups as follows:

- Group 1 (Control group, n = 8): mice administered with 0.85% saline 1 mL by oral gavage once a day for 3 days and housed with free access to water and standard food.
- Group 2 (S group, n = 8): mice administered with 3×10^9 CFU *S. typhimurium* 1 mL suspended in 0.85% saline by oral gavage once a day for 3 days and housed with free access to water and standard food.
- Group 3 (S + LP group n = 8): mice administered with 1×10^8 CFU *L. plantarum* B7 suspended 0.85% saline 1 mL by oral gavage. After treatment with *L. plantarum* B7 for 2 h, mice were administered 3×10^9 CFU *S. typhimurium* suspended in 0.85% saline 1 mL by oral gavage for 3 days and housed with free access to water and standard food.

Before administering 3×10^9 CFU *S. typhimurium* or 1×10^8 CFU *L. plantarum* B7, mice were pretreated with streptomycin suspended in drinking water (5 mg/mL) for 3 days as described by Barth et al. [19].

Mouse body weight, activities, and fecal characteristics were recorded daily. Fresh feces were collected to determine *S. typhimurium* infection by stool culture by colony counting and fecal moisture content was measured. All mice were humanely killed by an overdose of thiopental sodium injected intraperitoneally. Blood samples were collected by cardiac puncture to determine tumor necrosis factor-α (TNF-α), interleukin (IL-6), and CXCL1 levels in serum using enzyme-linked immunosorbent assays (ELISAs).

Determination of *S. Typhimurium* in feces: stool culture with colony count

Fresh feces (1 g) were homogenized in 400 µL phosphate-buffered saline at pH 7.4 and prepared with serial dilution 10^{-1}–10^{-7}. A 100 µL suspension was spread on the SS agar plate that was then incubated at 37°C for 24 h. The plates of *S. typhimurium* with colony counts yielding approximately 30–300 colonies were selected. To confirm that the selected colonies were *S. typhimurium*, a triple sugar iron (TSI) slant agar test was used. A single colony of *S. typhimurium* from an SS agar plate was inoculated onto TSI slant agar and incubated at 37°C for 24 h. Appearance of TSI agar positive test result was confirmed by serological testing using *Salmonella* group B antibodies. The number of *S. typhimurium* in each sample was calculated as follows:

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

Assay of fecal moisture content

Moisture content of fecal samples was determined by the percentage of water left from fecal drying using a microwave oven. Fresh fecal samples (1 g) were collected, weighed, and recorded as "wet weight of sample." Wet samples were then dried at 101–105°C using a microwave oven. After they were allowed to cool, the samples were then weighed and recorded as "dry weight of sample" [20, 21]. The moisture content of the samples was calculated with the following equation:

$$\begin{aligned} &A = \text{Weight of wet sample (grams)},\\ &B = \text{Weight of dry sample (grams)}\\ &\%\text{Fecal moisture content} = \frac{A-B}{B} \times 100 \end{aligned}$$

Assay of serum cytokine levels

Serum sample preparation

Blood samples were collected via cardiac puncture and allowed to clot for 2 h at room temperature before centrifuging for 20 min at approximately 1000×*g*. The serum was removed and stored at –80°C until determining TNF-α, IL-6, and CXCL1 levels using ELISA kits (R&D Systems).

Statistical analyses

We used PASW Statistics for Windows (version 18.0; SPSS Inc.) for statistical analyses. Continuous data are presented as mean ± standard deviation (SD). Means between groups of animals were compared with a one-way analysis of variance followed by a Tukey post hoc test. Differences were considered statistically significant at $P < 0.05$.

Results

Assay of inhibitory activity of *L. plantarum* B7 against *S. typhimurium* in vitro

The inhibitory effect of *L. plantarum* B7 against *S. typhimurium* is expressed as a clear zone (C) and microcolonies (M) on the lawn of *S. typhimurium* around the spot of *L. plantarum* B7. In the present study, *L. fermentum* L12 was used as a negative control and *L. rhamnosus* L34 was used as a positive control. *L. plantarum* B7 has a clear zone around the spot, which demonstrates inhibitory activity against *S. typhimurium* (**Figure 1**).

Study of mice in vivo

Concentration of S. typhimurium in feces

The mean concentration of *S. typhimurium* in feces from mice in the S + LP group (7.42 ± 0.05 logCFU/g) was significantly less than the concentration in feces from mice in the S group (8.86 ± 0.02 logCFU/g; **Figure 2**)

Serum level of TNF-

Levels of TNF-α in serum of mice from the S group (128.59 ± 12.82 pg/mL) were significantly greater than levels in mice from the control group (53.49 ± 8.90 pg/mL) and mice from the S+LP group (36.15 ± 9.22 pg/mL) (**Figure 3**).

Serum level of IL-6

IL-6 levels in serum of mice from the S group (144.44 ± 8.91 pg/mL) were significantly greater than levels in serum from mice in the control group (66.51 ± 4.04 pg/mL) and mice in the S + LP group (70.36 ± 5.37 pg/mL; **Figure 4**).

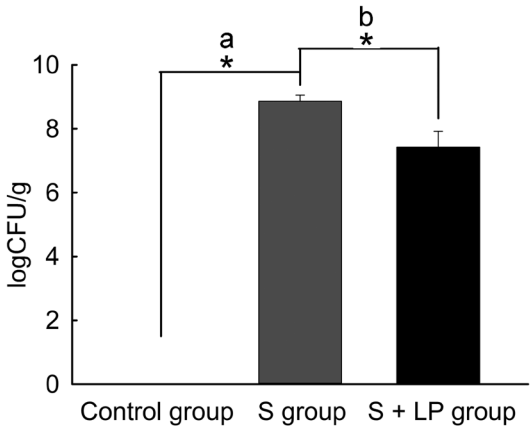

Figure 2. Concentration of *S. typhimurium* in 1 g feces (logCFU/g). Control group (n = 8): mice administered 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice administered *L. plantarum* B7 1×10^8 CFU/mL and *S. typhimurium* 3×10^9 CFU/mL. *$P < 0.05$

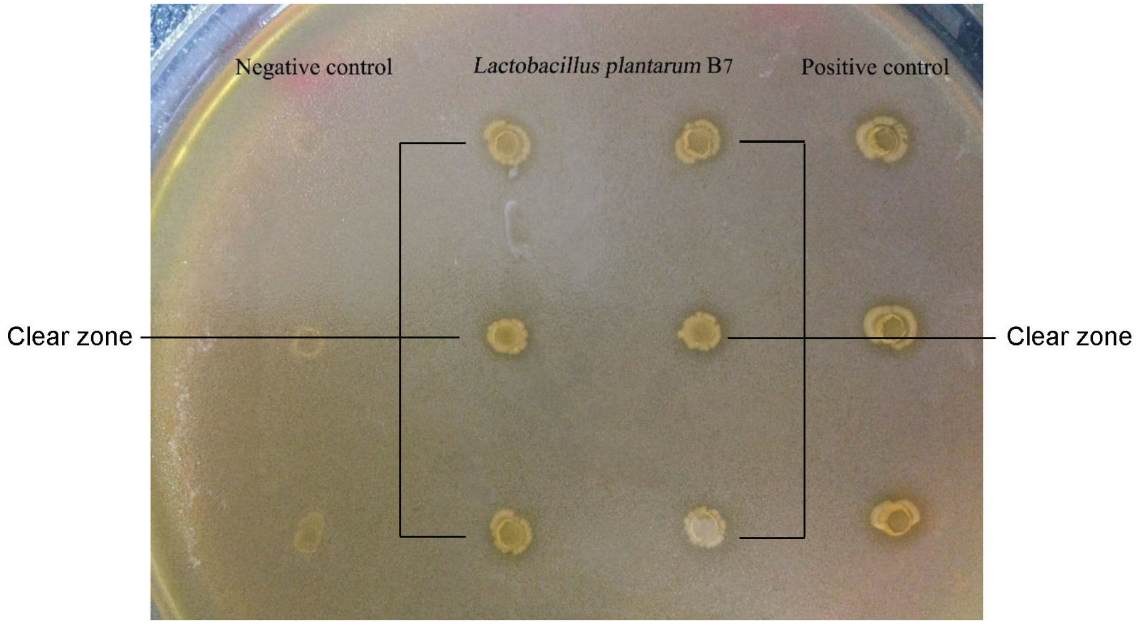

Figure 1. *L. plantarum* B7 spots grown on 20 mM glucose BHI agar under anaerobic conditions at 37°C, 48 h, overlaid with 10^7 *S. Typhimurium* and incubated at 37°C, 24 h, under aerobic conditions. Clear zone (C) and microcolonies (M) around the spot (n = 6).

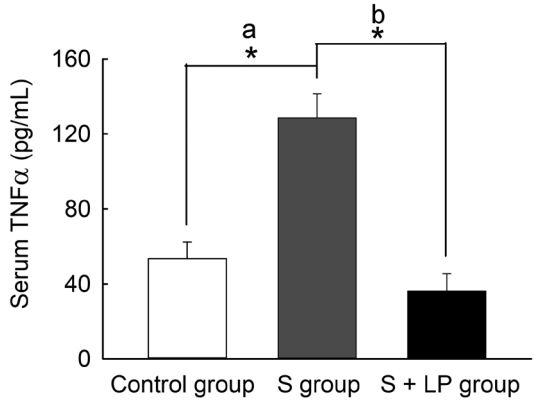

Figure 3. Concentration of serum TNF-α (pg/mL). Control group (n = 8): mice administered 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice administered *L. plantarum* B7 1×10^8 CFU/mL and *S. typhimurium* 3×10^9 CFU/mL. *$P < 0.05$

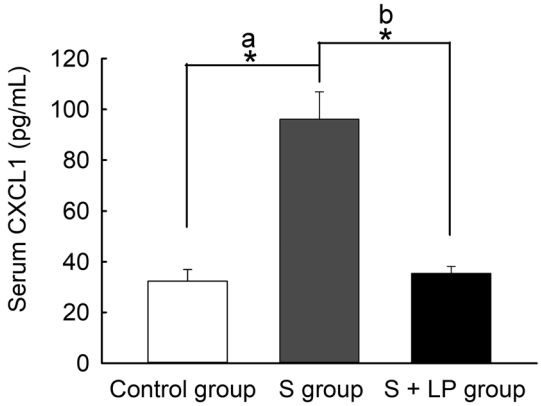

Figure 5. Concentration of serum CXCL1 (pg/mL). Control group (n = 8): mice administered 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice administered *L. plantarum* B7 1×10^8 CFU/mL and *S. typhimurium* 3×10^9 CFU/mL. *$P < 0.05$

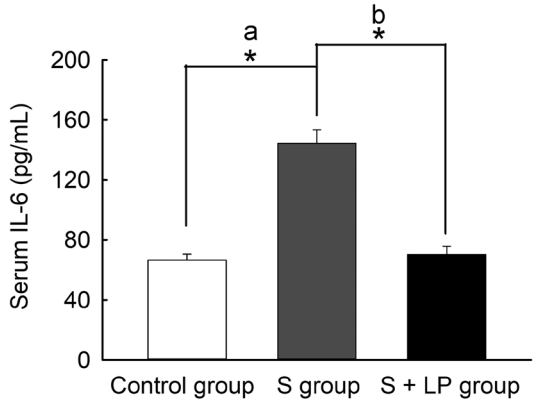

Figure 4. Concentration of serum IL-6 (pg/mL). Control group (n = 8): mice administered with 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice administered *L. plantarum* B7 1×10^8 CFU/mL and *S. typhimurium* 3×10^9 CFU/mL. *$P < 0.05$.

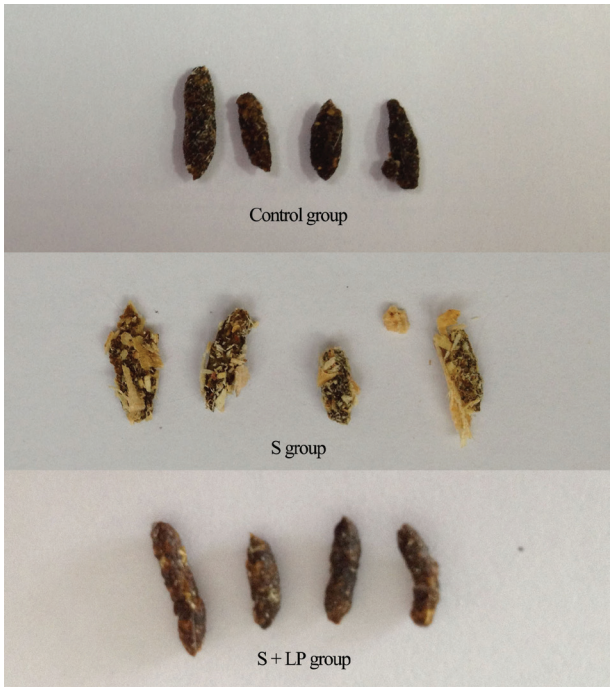

Figure 6. Fecal characteristics of mice in all groups (n = 24). Control group (n = 8): mice administered 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice fed with *L. plantarum* B7 1×10^8CFU/mL and *S. typhimurium* 3×10^9 CFU/mL.

Serum level of CXCL1

CXCL1 levels in serum from mice in the S group (96.09 ± 10.81 pg/mL) were significantly greater than levels in serum from mice in the control group (32.32 ± 4.54 pg/mL) and mice in the S+LP group (35.40 ± 2.77 pg/mL; **Figure 5**).

Fecal characteristics

In the control group, feces were rod shaped, dark colored, and trifling with no sawdust around their surface. In the S group, after being fed with *S. typhimurium* feces were loose, soft, and lighter in color with sawdust covering their surface. Similar in appearance to feces from the control group, feces in the S + LP group were rod shaped, dark colored, and there was little sawdust around the surface (**Figure 6**).

Fecal moisture content

Percentage of mouse fecal moisture content (%FMC) from all groups are presented in **Figure 7**. The %FMC from mice in

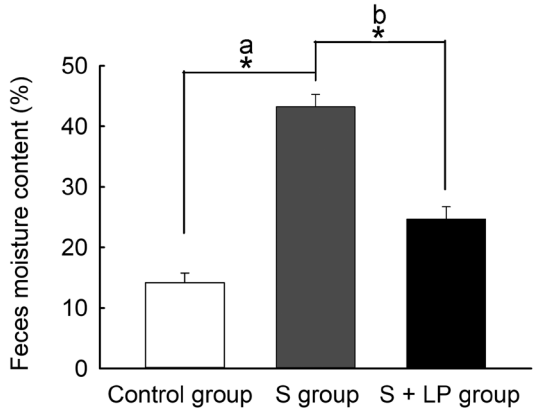

Figure 7. Percentage of feces moisture content (%FMC) in all groups (mean ± SD). Control group (n = 8): mice administered 0.85% saline; S group (n = 8): mice administered *S. typhimurium* 3×10^9 CFU/mL; S + LP group (n = 8): mice administered *L. plantarum* B7 1×10^8 CFU/mL and *S. Typhimurium* 3×10^9 CFU/m*L*. *$P < 0.05$

the S group (43.24 ± 2.05%) was significantly greater than the FMC% from mice in the control group (14.19 ± 1.57%) and mice in the S + LP group (24.65 ± 2.08%).

Discussion

The present study demonstrates that *S. typhimurium* infection is associated with diarrhea in mice and found protective effects of *L. plantarum* B7 treatment.

In the present experiments, all mice underwent pretreatment with streptomycin 5 mg/mL drinking water for 3 days. Pretreatment with streptomycin is considered an effective method when attempting to induce *S. typhimurium* infection, because the treatment results in a decrease in native flora in the GI tract of mice causing higher susceptibility to *S. typhimurium* infection [22]. Mice without streptomycin pretreatment did not develop diarrhea, nor does the pretreatment alone as we found in the present study (results not shown).

The body weight of each mouse was measured every day and no significant differences were found between the treatment and control groups (results not shown). However, the body weight of mice in the control group tended to increase. By contrast, after mice were infected with *S. typhimurium* in both S and S + LP groups, body weight tended to decrease. However, the differences were not significant. Nevertheless, mice in the S group showed reduced activity and had ruffled fur. It is possible that the infection with *S. typhimurium* could have caused the reduced activity and appetite.

In the study in vitro, we found that *L. plantarum* B7 inhibits growth of *S. typhimurium* as seen by the clear zone around the spot. *L. plantarum* B7 is a probiotic with antagonist activity properties against pathogenic bacteria. Our findings are consistent with previous studies [23–25] showing that *L. plantarum* B7 produces effects against pathogen infections by various mechanisms. *L. plantarum* B7 can reduce or inhibit pathogen growth by producing antimicrobial or inhibitory substances such as organic acids, hydrogen peroxide, 3-hydroxypropionaldehyde, diacetyl, and bacteriocin [26], which have adverse effects on other bacteria and pathogens. These substances can kill, reduce, or inhibit the growth of pathogens directly [27]. Moreover, organic acid production from *L. plantarum* B7 can modulate and change the pH environment, thus increasing acidity that can either decrease or inhibit survival rates and growth of pathogens.

In vivo, we found antipathogenic bacterial properties of *L. plantarum* B7 in our mouse model of *S. typhimurium*-associated gastroenteritis and diarrhoea. We found a decrease in *S. typhimurium* concentration in feces as was found by stool culture and colony counts. Aside from antimicrobial substances that inhibit the growth of *S. typhimurium*, *L. plantarum* B7 may reduce and inhibit the infection by these pathogenic bacteria by competitive exclusion. Our present findings are consistent with those of Shahlaa et al. who showed that administration of *L. plantarum* 10^9 CFU/mL after infection of mice with 0.5×10^5 CFU/mL of *S. typhimurium* can attenuate necrosis, degenerative changes, and inflammatory cell infiltration. Treatment or even pretreatment with *L. plantarum* can improve the histopathological outcome [28]. Hiroki et al. also demonstrated that mice administered with heat-killed *L. plantarum* b240 for 3 weeks were protected from infection with *S. typhimurium*. *L. plantarum* b240 was considered to inhibit the binding and invasion of *S. typhimurium* into epithelial cells and decreased the translocation of *S. typhimurium* into other organs (Payer's patches, mesenteric lymph nodes, spleen, and liver) [29]. Chompoonut et al. suggested that *L. plantarum* B7 plays a preventive role against pathogenic bacterial infection by demonstrating it inhibited growth of *Helicobacter pylori* in vitro. Moreover, they found *L. plantarum* B7 attenuated the histopathology of gastric inflammation induced by *H. pylori* [30]. *L. plantarum* B7 are a type of so called "friendly bacteria" for human hosts and can live and survive in the human GI tract. Offering many supposed benefits for human hosts, probiotics can improve and modulate the commensal bacterial balance in the GI tract and protect humans from pathogenic infections [31].

We found proinflammatory cytokines (TNF-α, IL-6, and CXCL1) were significantly decreased in the S + LP group. This is consistent with previous findings that *L. plantarum* reduces levels of proinflammatory cytokines [32–35]. Panpetch et al. found that *L. plantarum* B7 can reduce TNF-α in vitro [36]. Dick et al. found that *L. plantarum* 423 and *E. mundtii* ST4SA

may alleviate physical symptoms of infection with *S. typhimurium*. *L. plantarum* 423 alone was more effective than *E. mundtii* ST4SA or a combination of both bacterial types [37].

Conclusions

L. plantarum B7 is effective against infection by *S. typhimurium* by inhibiting its growth, decreasing serum levels of inflammatory cytokines (TNF-α, IL-6, and CXCL1), and improving fecal moisture content and characteristics, thus alleviating diarrhea in mice infected with *S. typhimurium*. Further research is required to clarify other protective mechanisms of *L. plantarum* B7. These findings may be applicable to further studies of the prevention and treatment of *Salmonella*-associated infections.

Author contributions. SW and DW contributed substantially to the conception and design of the present study. ST acquired the data and SW analyzed and interpreted the data. DW contributed substantially to drafting the manuscript, and SW and ST critically contributed to its revision. All authors approved the final version submitted, and take the responsibility for the statements made in the published article.

Acknowledgments. This study was supported by the 90th Anniversary Fund of Chulalongkorn University (Ratchada phiseksomphot Endowment Fund) and The Grant of Ratchadaphiseksomphot, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Conflict of interest statement. The authors have each completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

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