PROTECTIVE EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON *SALMONELLA* TYPHIMURIUM ASSOCIATED DIARRHEA IN MICE

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ผลของแลคโตบาซิลลัส แพลนทารัม บี 7 ในการป้องกันท้องเสียที่เกิดจากเชื้อซัลโมเนลล่า ไทฟิมูเรียม ในหนูไมซ์

นางสาวศิวพร วงค์เสน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PROTECTIVE EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON *SALMONELLA* TYPHIMURIUM ASSOCIATED DIARRHEA IN MICE
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ศิวพร วงค์เสน : ผลของแลคโตบาซิลลัส แพลนทารัม บี 7 ในการป้องกันท้องเสียที่เกิดจากเชื้อซัลโมเนลล่า ไทฟิมูเรียม ในหนูไมซ์ (PROTECTIVE EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON *SALMONELLA* TYPHIMURIUM ASSOCIATED DIARRHEA IN MICE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. พญ. ดวงพร วีระวัฒกานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. สมหญิง ธัมวาสร, 81 หน้า.

การศึกษานี้จึงวัตถุประสงค์เพื่อศึกษาผลของแลคโตบาซิลลัส แพลนทาลัม บี 7 ในการป้องกันท้องเสียที่เกิดจากเชื้อซัลโมเนลล่า ไทฟิมูเรียม ในหนูไมซ์ โดยการศึกษา *In vitro* เพื่อศึกษาคุณสมบัติของแลคโตบาซิลลัส แพลนทาลัม บี 7 ในการยับยั้งการเจริญของเชื้อซัลโมเนลล่า ไทฟิมูเรียม และการศึกษาในสัตว์ทดลอง โดยทำการแบ่งหนูไมซ์เพศผู้ออกเป็น 3 กลุ่ม โดยหนูทุกกลุ่มจะได้รับสเตร็ปโตมัยซิน (5 มก./มล.) ล่วงหน้าเป็นเวลา 3 วัน ก่อนที่จะได้รับเชื้อแบคทีเรีย หนูกลุ่มควบคุม ได้รับน้ำเกลือความเข้มข้น 0.85% 1 มล. หนูกลุ่ม S ได้รับเชื้อซัลโมเนลล่า ไทฟิมูเรียม ความเข้มข้น $3x10^9$ ซีเอฟยู/มล. และหนูกลุ่ม S + LP ได้รับแลคโตบาซิลัส แพลนทาลัม บี 7 ความเข้มข้น $1x10^9$ ซีเอฟยู/มล. และเชื้อซัลโมเนลล่า ไทฟิมูเรียม ความเข้มข้น $3x10^9$ ซี เอฟยู/มล. ให้กินเป็นเวลา 3 วัน จัดทำบันทึกข้อมูลน้ำหนักตัวของสัตว์ทดลองทุกวัน เมื่อสิ้นสุดการทดลอง ทำการเก็บอุจจาระและตัวอย่างเลือด อุจจาระถูกนำมาตรวจหาปริมาณเชื้อซัลโมเนลล่า ไทฟิมูเรียม และเปอร์เซ็นต์ความชื้น ส่วนตัวอย่างเลือดนำมาวิเคราะห์หาระดับไซโตไคน์ ทีเอ็นเอฟแอลฟา อินเตอร์ลิวคินชนิดที่ 6 และซีเอ็กซ์ซีแอลชนิดที่ 1

ผลการศึกษา *In vitro* พบว่าแลคโตบาซิลลัส แพลนทาลัม บี 7 มีคุณสมบัติในการยับยั้งการเจริญของเชื้อซัลโมเนลล่า ไทฟิมูเรียม และการศึกษาในสัตว์ทดลอง พบว่ากลุ่ม S + LP มีปริมาณเชื้อซัลโมเนลล่า ไทฟิมูเรียมในอุจจาระลดลงอย่างมีนัยสำคัญเทียบกับกลุ่ม S (7.42 ± 0.05 *vs* 8.86 ± 0.02 CFU/g, $p < 0.05$) ผลการศึกษาระดับไซโตไคน์พบว่า กลุ่ม S มีปริมาณทีเอ็นเอฟแอลฟา อินเตอร์ลิวคินชนิดที่ 6 และซีเอ็กซ์ซีแอลชนิดที่ 1 เพิ่มขึ้นอย่างมีนัยสำคัญเทียบกับกลุ่มควบคุม (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, $p < 0.05$) ส่วนกลุ่ม S + LP ระดับไซโตไคน์ทั้งสามตัวมีปริมาณลดลงอย่างมีนัยสำคัญเทียบกับกลุ่ม S (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, $p < 0.05$) ตามลำดับ และลักษณะอุจจาระในกลุ่ม S มีลักษณะก้อนอุจจาระเหลวมีสีอ่อน สำหรับกลุ่ม S + LP อุจจาระมีลักษณะเป็นรูปท่อน สีดำ และเปอร์เซ็นต์ความชื้นในอุจจาระในกลุ่ม S มีเปอร์เซ็นต์ความชื้นในอุจจาระเพิ่มสูงขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับ กลุ่มควบคุม (43.24 ± 2.05% *vs* 14.19 ± 1.57%, $p < 0.05$) ส่วนในกลุ่ม S + LP มีเปอร์เซ็นต์ความชื้นในอุจจาระลดต่ำลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่ม S (24.65 ± 2.08% *vs* 43.24 ± 2.05%, $p < 0.05$) การศึกษานี้แสดงให้เห็นว่าแลคโตบาซิลลัส แพลนทาลัม บี 7 สามารถยับยั้งการเจริญของเชื้อซัลโมเนลล่า ไทฟิมูเรียม ลดปริมาณไซโตไคน์ในกลุ่มชักนำให้เกิดการอักเสบ และทำให้ลักษณะของอุจจาระดีขึ้น แลคโตบาซิลลัส แพลนทาลัม บี 7 จึงสามารถบรรเทาและป้องกันท้องเสียที่เกิดจากเชื้อซัลโมเนลล่า ไทฟิมูเรียม ในหนูไมซ์

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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SIWAPON WONGSEN: PROTECTIVE EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON *SALMONELLA* TYPHIMURIUM ASSOCIATED DIARRHEA IN MICE. ADVISOR: PROF. DUANGPORN WERAWATGANON, M.D., CO-ADVISOR: ASSOC. PROF. SOMYING TUMWASORN, Ph.D., 81 pp.

This study aimed to determine the protective effects of *Lactobacillus plantarum* B7 on *Salmonella* Typhimurium associated diarrhea in mice. *In vitro* investigation of antagonistic activity of *L. platarum* B7 was performed by using agar spot method. *In vivo*, male mice were randomly divided into 3 groups, all groups were 3 days pre-treated with streptomycin (5 mg/mL). Control group (n = 8) was fed with 1 mL of 0.85% saline, S group (n = 8) was fed with $3x10^9$ CFU/mL of *S.* Typhimurium and S + LP group (n = 8) was fed with $1x10^9$ CFU/mL of *L. plantarum* B7and after 2 hours mice were fed $3x10^9$ CFU/mL *S.* Typhimurium. All groups were treated for 3 days. The body weights of mice were measured and recorded daily. At the end of experiment, fresh feces were collected to determine the number of *S.* Typhimurium by stool culture with colony counts. Blood samples were collected to determine TNF-α, IL-6 and CXCL1 levels. The feces were tested for the percentage of fecal moisture content (%FMC) and investigated of characteristics.

In vitro test showed that L. plantarum B7 produced clear zone of inhibitory activity against S. Typhimurium. In vivo test showed that the number of colony of S. Typhimurium in feces significantly decreased in S + LP group vs S group (7.42 ± 0.05 vs 8.86 ± 0.02 CFU/g, $p < 0.05$). The level of TNF-α, IL- 6 and CXCL1 was significantly increased in S group vs 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, $p < 0.05$) and significantly decreased in S + LP group vs S 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, $p < 0.05$), respectively. The fecal characteristics of S group were soft and loose whereas S + LP group had the rod shape and dark color. The %FMC significantly increased in S group vs control group (43.24 ± 2.05% vs 14.19 ± 1.57%, $p < 0.05$) and significantly decreased in S + LP group compared with S group (24.65 ± 2.08% vs 43.24 ± 2.05%, $p < 0.05$). In conclusion, oral administration of L. plantarum B7 can inhibit S. Typhimurium growth, decrease pro-inflammatory cytokine levels, attenuate inflammatory response and improve the moisture content in feces, which can prevent S. Typhimurium associated diarrhea in mice.

Field of Study: Medical Science
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

A	=	Acid
BHI	=	Brain heart infusion
CFU	=	Colony forming unit
CINC-1	=	Cytokine-induced neutrophil chemoattractant type 1
ELISA	=	Enzyme-linked immunosorbent assay
FCM	=	Fecal moisture content
G	=	Gas production
GRAS	=	Generally recognized as safe
H_2S	=	Hydrogen sulfide
IFN-**γ**	=	Interferon-gamma
I**K**B	=	Inhibitor of kappa B
IL-1b	=	Interleukin-1b
IL-6	=	Interleukin-6
K	=	Alkaline
LP	=	*Lactobacillus plantarum* B7
LPS	=	Lipopolysaccharide
M cells	=	Microfold cells or membranous cells
MAPKs	=	Mitogen-associated protein kinase
MRS	=	de Man, Rogosa and Sharpe
N	=	No change of agar color or original agar color
NF-kB	=	Nuclear factor kappa beta
OD	=	Optical density

PBS	=	Phosphate buffer saline
PMN	=	Polymorphonuclear
SCV	=	*Salmonella* containing vacuole
SD	=	Standard deviation
SipA	=	Secreted effectors protein A
SipB	=	Secreted effectors protein B
SPI-1	=	*Salmonella* pathogenicity island-1
SS	=	Salmonella-Shigella
T3SS1	=	Types III secretion system I
T3SS2	=	Types III secretion system II
TLR	=	Toll-like receptors
TNF-α	=	Tumor necrosis factor alpha
TSI	=	Triple sugar iron

CHAPTER I

INTRODUCTION

Background and Rationale

Salmonella Typhimurium infection is one of the major causes of acute gastroenteritis and enterocolitis. It is manifested by changing in the intestine with loose or watery stools, excess water, electrolytes, fat, and other substances in intestinal lumen. It is associated with passaging of three or more loose or liquid stools per day with more than 200 gram of stools per day [1, 2]. Usually, an inflammation damage comes from the brush border of the intestine that leads to leak of water and electrolyte from the lining intestine and decreases the absorption ability of these lost fluids. After 12-72 hours of *S.* Typhimurium infection, the common symptoms occur with diarrhea comprise, nausea, vomiting, abdominal pain, fever and muscle weakness [3]. In most people, the symptoms usually last 4 to 7 days, and can recover without the treatment. However, in some cases diarrhea can develop to be and dangerously induce intestinal inflammation that causes the loss of intestinal lining and lead to a bloody diarrhea which is a systemic infection. In a severe case of *S.* Typhimurium infection, it can spread from the intestine to blood system and lead to death unless the antibiotics treatment is given on time [4]. *S*. Typhimurium infection is one of a worldwide common cause of death each year and the second most common cause of infant death. In 2009, WHO considered diarrhea as the major cause of death in children at the age of 5 years old and the children under 5 years old [5]. There are approximately 94 million worldwide incidences of Salmonella diarrhea which cause around 150,000 deaths [6, 7]. The treatments of Salmonella diarrhea is a causal therapy which mostly uses antibiotics and anti- inflammatory agents. The antibiotic drugs can effectively destroy and reduce the inhibit growth of bacteria. The anti-inflammatory

agents are used to reduce an inflammatory response or decrease the inflammations and also improve the mucosal function of the intestine. Ampicillin, cefotaxime, chloramphenicol and ciprofloxacin are antibiotics commonly used for treatment of Salmonella infection [8]. In term of incredibility, using antibiotic and anti-inflammatory agents can cause the adverse effects such as nausea, vomiting, and stomach cramps or develop to major allergic reactions and disruption of the normal flora species in the gastrointestinal tract. This also includes an interaction with other drugs that can cause tendon and kidney damage. Moreover, it can adjust the host body to a normalflora which has been associated with chronic diseases [9]. It is extremely important to consider that an antibiotic-resistant problem can lead to a treatment with stronger medicines that make the decease more difficult to cure with new or experimental drugs [10].

One of alternative treatments for *Salmonella* diarrhea mostly is a probiotics treatment. Probiotics are naturally live microorganisms. With administering in an adequate amount, they can promote a health benefit on the host [11]. It existence in the human gastrointestinal tract maintains the equilibrium of normal flora bacteria in the intestine. Many strains of probiotics [e.g., *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri, Lactobacillus casei*, *Lactobacillus acidophilus* CL1285, *Escherichia coli* strain Nissle 1917, and certain bifidobacteria and enterococci (*Enterococcus faecium* SF68 and yeast *Saccharomyces boulardii*)] can inhibit the growth, metabolic activity and adhesion in intestinal epithelium cells of pathogenic enteric bacteria (*Salmonella, Shigella, E. coli,* or *Vibrio cholerae*) [12]. The potential mechanisms of probiotic perform functions of prevention and treatment of diarrhea as a modification of intestinal microbial environment and modification of normalflora and probiotic bacteria in order to enhance diarrhea prevention [13]. *Lactobacillus plantarum,* the gram-positive bacteria in the family of *Lactobacillaceae,* is the normal

flora which can be found in the human gastrointestinal tracts and the reproductive systems [14]. In food industry, *L. plantarum* provides a benefit for fermentative food and beverage production such as yogurt, cheese, pickles, beer, wine, and cider etc. In medicine, *L.* plantarum is mostly used as probiotics and biotherapeutics for the prevention and treatment of disease and symptoms including gastrointestinal disease, Salmonella infection and diarrhea [15-17]. Some strains of *L. plantarum* can inhibit the growth of pathogens [18]. It can also prevent the adhesion and invasion of enteropathogen to intestinal epithelial cells [19], promote anti-inflammatory and regulate immunomodulatory activities for reducing inflammatory response [20, 21], enhance the intestinal function to prevent diarrhea [22] and reduce allergenicity from soy flour [23]. Hence, the present study aims to determine the protective effects of *Lactobacillus plantarum* B7 on *Salmonella* Typhimurium by developing diarrhea in mice in order to investigate the mechanisms of inflammatory response and the physical symptoms of *Salmonella* Typhimurium infection in mice.

Research Question

Can *Lactobacillus plantarum* B7 prevent *Salmonella* Typhimurium associated diarrhea, inhibit *S.* Typhimurium growth, reduce the inflammatory response and attenuate the physical symptoms of *S.* Typhimurium infection in mice?

Research Objectives

1. To determine the effects of *L. plantarum* B7 on *S.* Typhimurium-developed diarrhea in mice.
2. To determine the effects of *L. plantarum* B7 on inflammatory response caused by *S.* Typhimurium in mice.

3. To examine whether *L. plantarum* B7 can attenuate the symptoms of *S.* Typhimurium infection in mice.

Hypothesis

L. plantarum B7 can prevent *S*. Typhimurium associated diarrhea by the inhibition of *S*. Typhimurium growth and/or reduction of inflammatory response and attenuation of the physical symptoms in mice.

Expected Benefit and Application

The results of this study will provide an understanding of protective effects of *L. plantarum* B7 on *S.* Typhimurium in developing diarrhea, inhibiting or reducing *S.* Typhimurium infection, decreasing inflammatory response, and attenuating the physical symptoms of *S*. Typhimurium infection in mice. Moreover, this study will be useful for further studies and applications for prevention and treatments of *S.* Typhimurium associated diarrhea.

CHAPTER II

LITERATURE REVIEW

Small Intestine and the Major Cell Types

The small intestine is a part of the gastrointestinal tract lining in the stomach. Following by the large intestine that can be divided into three portions: the duodenum, jejunum and ileum. The small intestine performs a function of nutritional digestion. These nutrients are proteins, lipids and carbohydrates. It also absorbs the nutrients into blood system. The process of absorption occurs in the small intestine wall with diffusion. The small intestine's inner wall or mucosa layer is lined with the simple columnar epithelial tissue; the mucosa layer develops into folds called "plica circulars or circular folds" for projection of microscopic finger-like pieces of tissue called intestinal "villi". In the individual epithelial cells, there are finger-like projections called as "microvilli". The major role of the plica circulars, villus and microvillus is to increase the surface area for absorption of the nutrients. In the portion of duodenum and jejunum, there are the largest and most numerous of intestinal villi. However, in the ileum intestinal villi, it is fewer, smaller and mostly found the Peyer's patches. The epithelium of the small intestine consists of enterocytes, M cells, goblet cells, paneth cells, enteroendocrine cells and undifferentiated cells. Enterocytes or absorptive cells perform a function of absorption; they are tall columnar cells with microvilli that specializes for transporting substances including active and passive transport [24, 25]. M cells or microfold cells are intestinal epithelial cells that lack of microvilli on the apical surface. The main function of M cells is to transport antigens or organisms and particles such as the bacteria or pathogens from intestinal lumen across the epithelial barrier into immune cells (macrophages and lymphocytes). Besides, an important role of M cells is to stimulate mucosal immunity [26]. M cells are exploited by several pathogens. They can involve in the pathophysiologic response of bacterial infection or viral

infection that induce inflammation in intestinal epithelial [27]. These processes induce inflammatory response and activate immunity and lead to the increase of pro-inflammatory cytokines and chemoattractant. This release also inflammatory cells as neutrophils infiltrate at inflammatory site [28]. Acute inflammation of the intestine can produce the symptoms of abdominal pain, nausea, vomiting, and acute diarrhea. Besides, the development of a chronic diarrhea can lead to the chronic inflammation that is a cause of inflammatory bowel disease, enterocolitis and gastroenteritis [29].

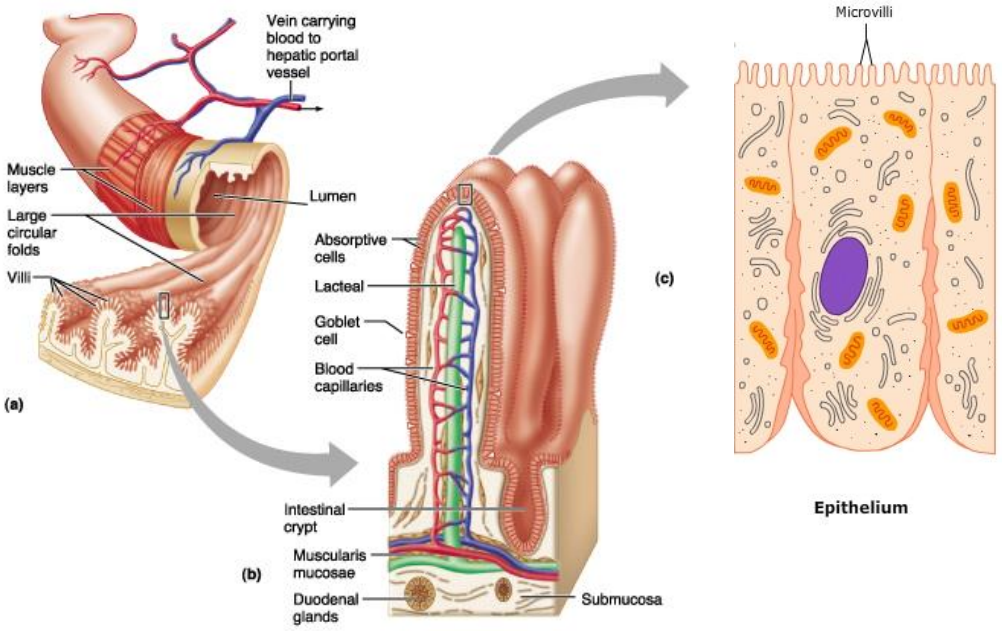

Figure 2-1: The structure of intestinal epithelium cells [30, 31].

General Characteristic of *Salmonella* Typhimurium

Salmonella Typhimurium is gram-negative that belongs in the species of *Salmonella enterica*, non-spore-forming, facultative anaerobic bacteria. It is like other *Enterobacteriaceae* that produce acid on glucose fermentation and reduce nitrates. However, it cannot produce cytochrome oxidase [32]. *S*. Typhimurium belongs in group B of *Salmonella* base on sharing O antigens which show in Table 2-1. The outer membranes of *S.* Typhimurium consist of lipopolysaccharides (LPS) or somatic O that is the basic serovars classification of *Salmonella* bacteria based on the somatic O (lipopolysaccharide) and flagella H antigens described by Kaufmann–White scheme [33]. LPS and O-antigen are important for protecting *S.* Typhimurium from the environment; they also perform functions as virulence factors which determine that *S.* Typhimurium is an important pathogen for the warm-blooded human body, toxicity and alter host immune response and inflammatory reaction [34].

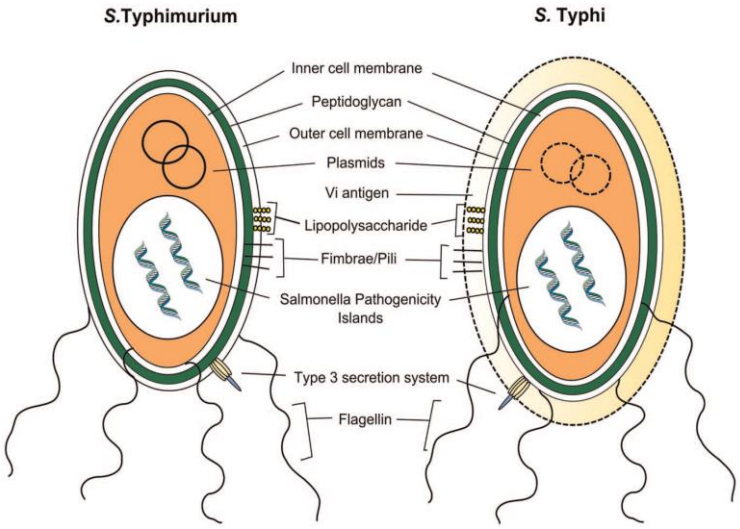

Figure 2-2: The structure and virulence of *Salmonella* Typhimurium [35]

Table 2-1: Some examples of commonly occurring *Salmonella* serotypes and the groups to which they belong [36].

Group	Serotype
A	*S.* Paratyphi A
B	*S.* Paratyphi B
	S. Saintpaul
	S. Agona
	S. Typhimurium
C	*S.* Paratyphi C
	S. Cholerae-suis
	S. Virchow
	S. Thompson
D	*S.* Typhi
	S. Enteriditis
	S. Dublin
	S. Gallinarum

A. *Salmonella* Typhimurium Infection Cycle in Human Host

The infection with *S.* Typhimurium is one of the most common causes of diarrhea. The infections usually occur within 12 to 36 hours after eating or drinking contaminated food and water. *S.* Typhimurium occurs within the first 1–3 hours of infection, while massive neutrophil migration and the secretion of inflammatory cytokines in the intestinal lumen occur within 8–10 hours. Then infection and diarrhea begin approximately 8–72 hours after bacterial colonization [(4)]. The infections of *S.* Typhimurium contribute the homeostasis of intestinal epithelial that leads to excess production of fluids or inhibit absorption of fluids which induce diarrhea and the inflammatory response in human host cells [(37)]. There is a certain percentage of *S.* Typhimurium that survives in the acid environment of the stomach when it enters the small intestine, localizes in the part of terminal ileum and also enters the Peyer's patches. *S.* Typhimurium can spread throughout important organs (lymph node, spleen, liver and bone marrow), localize in gall bladder and increase biofilm release. Then *S.* Typhimurium reseeds to the small intestine via bile release. Finally, *S.* Typhimurium can disperse in the stools and transports to naive hosts for initiating the new infection cycle [(38)].

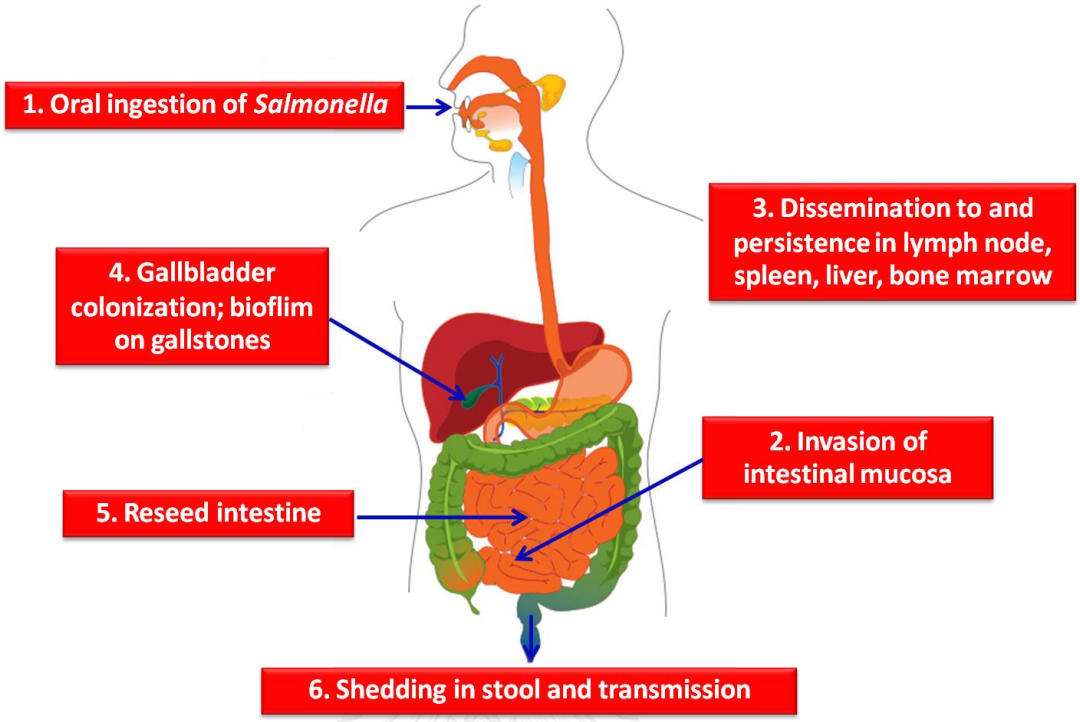

Figure 2-3: The cycle of *S*. Typhimurium in human host (modified from Denise M Monack 2012) [38]

B. Pathophysiology of *Salmonella* Diarrhea

The mechanisms of *S.* Typhimurium infection consist of the 4 important processes. The first process occurs when *S.* Typhimurium appears to adhere with intestinal epithelial cells. Following by the second process, the bacteria invades and crosses intestinal mucosal barrier. The third process is transcytosis, intracellular replication of *S.* Typhimurium, in which it spreads to circulatory systems and other organs. The last process is cytotoxicity which induces inflammatory response and inflammation [39]. In the first process, *S.* Typhimurium appears and adheres to intestinal epithelial cells by binding with Toll-like receptors (TLR) type 2 and type 4 in a host cell membrane [40]. In the second process, "invasion", *S.* Typhimurium can enter via phagocytic cells, "microfold **cells" or "membranous cells (M cells)"** of the intestinal

epithelium by phagocytosis pathway or invade by normal non-phagocytic cells. Then it transports these antigens to lymphoid cells that underlie the epithelium in Peyer's patches [41]. M cells are specific to intestinal epithelial cells which lack of microvilli. In the microvilli, there is a thinner glycocalyx layer without the secretion of mucus or digestive enzymes. M cells play a major role in transepithelial transport via phagocytosis of antigens, microorganisms, intraepithelial macrophages and lymphocytes. Regardless of alter immune response [42], *S.* Typhimurium can invade via non-phagocytic cells by inducing membrane ruffles on the host cell membrane. The important mechanism of non-phagocytic cell invasion is *Salmonella* gene called "***Salmonella* pathogenicity island-1" (SPI-1)**; this *Salmonella* virulence performs a function of encoding type III secretion system (T3SS) or "injectosome" that injects bacterial effector proteins into the host cell membrane and also disrupts actins rearrangements in the host cell membrane. This can induce membrane ruffling and modify phagosome even though the bacteria successfully invades the host cells [43, 44]. In Addition, SPI-1 performs a function that associates with activating innate immune of the host cells and inducing inflammatory response. SPI-1 secretes an effector protein, SipA, in which, it induces polymorphonuclear cell (PMN) recruitment across intestinal epithelial, activates mitogen-associated protein kinase (MAPKs) in the part of nuclear factor kappa beta (NF-kB) signaling, and translocates another effector, SipB, to induce the secretion of chemokines and pro-inflammatory cytokines [45]. SPI-1 that encodes T3SS1 (Types III secretion system I) or T3SS2 (Types III secretion system II) to modify phagosome is called ***Salmonella* containing vacuole (SCV).** It performs a major function in intravascular survival strategies and replication after the entry of the host cells [35]. *Salmonella* containing vacuole (SCV) is the high advantage of *S.* Typhimurium in the part of intracellular replication as it increases the survival rate and disperses to other organs without destroying forms of the immune system of the host cells. In addition, SCV contributes the homeostasis of the host cells; activates NF-kB signaling

pathway; induces the expressions of pro-inflammatory genes; and then increases pro-inflammatory cytokines release (tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), Interleukin-6 (IL-6) and Interleukin -1b (IL-1b)). This also rises chemokines release such as cytokine-induced neutrophil chemoattractant type 1 (CINC-1 or CXCL1). Then it induces the recruitment of inflammatory cells in inflammatory response for an initial process of inflammations, increases cytotoxicity of the host cells that finally leads to pathology in the host cells [46-50].

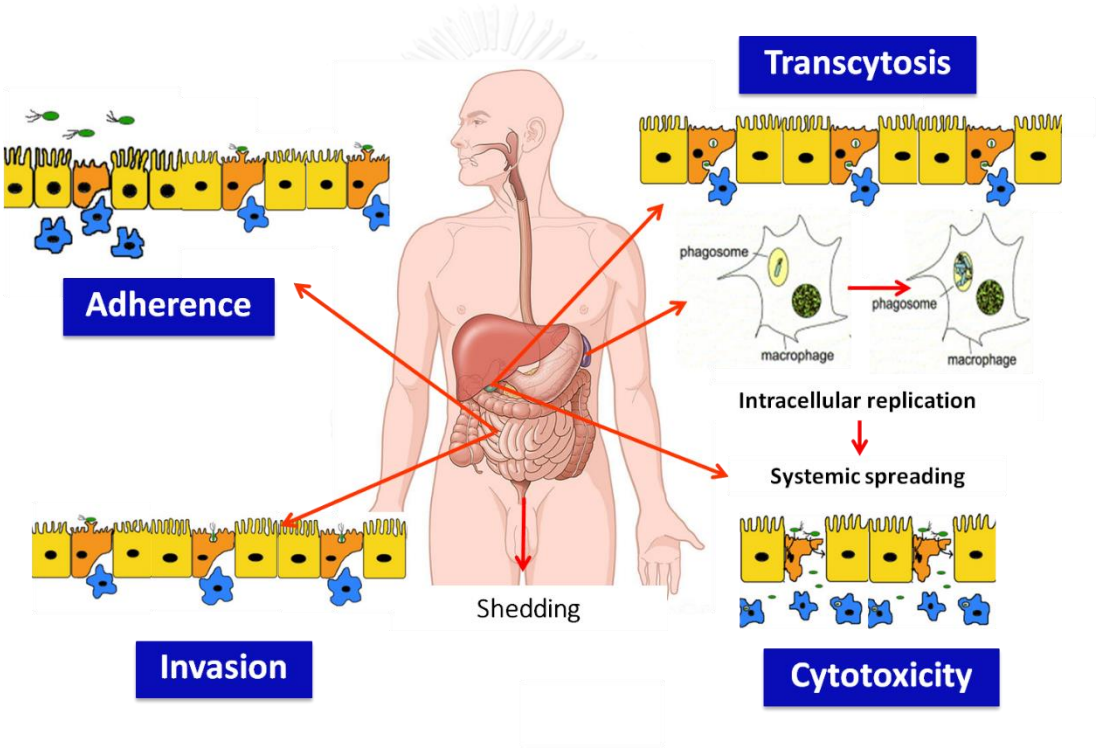

Figure 2-4: The mechanism of *S.* Typhimurium infection (modified from Denise M Monack 2012) [38]

General Characteristic of *Lactobacillus plantarum*

Lactobacillus plantarum is a non-pathogenic gram-positive and non-spore forming rod shape bacteria that belongs in the genus of *Lactobacillus. L. plant*arum. As a facultative heterofermentative bacteria, it can also grow in an anaerobic condition or a low oxygen condition. *L. plant*arum can ferment and convert sugars into lactic acid and/or acetic acid, *in* which, it is commonly known as fermentative lactobacilli or lactic acid bacteria [51]. *L. plantarum* are generally acidophilic that can grow in slight acid media with pH 4.5- 6.4 under an aerobic condition in an anaerobic jar with GasPak or H_2 plus CO_2 generate kits or incubated in jars filled with 90% H_2 or H_2 plus 10% CO_2. The colonies of *L. plantarum* on agar are usually small (2- 3 mm) with round, smooth, convex and white or yellowish features. *L. plantarum* is a normal flora that lives in the human gastrointestinal tract, oral cavity and reproductive system. They can survive under low pH of the stomach and duodenum and grow at 15 ℃ - 40 ℃ [17, 52]. *L. plantarum* can produce lactic acid and antimicrobial substances, for surviving in the gastrointestinal tract, that affect Gram-positive and Gram-negative bacteria. In food industry, some strain of *L. plantarum* provides a benefit in fermentative process of food and beverage production such as yogurt, cheese, pickles, kimchi, beer, wine, and cider etc. [53, 54].

A. *L. plantarum* as Probiotics

The term 'Probiotic' are derived from a prefix '**pro'** that means “for” from Latin and a root-word '**biotic'** or **bios** that means “life” from Greek. In 2001, The World Health Organization (WHO) defined the term 'Probiotics' that "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [55]. Previous studies propose that probiotics have beneficial effects on health of the host and it can be supplemented into a form of powder, tablets and capsules. In

addition, the fermented food can maintain the normal flora bacteria in the gastrointestinal tract and improve a health condition of the human host [56]. For a medical use, some strain of *L. plantarum* such as *L. plantarum* 299v is mostly used as probiotics and biotherapeutics for the prevention and treatment of several diseases or symptoms including gastrointestinal disease and diarrhea [15, 57-63].

Probiotics properties

Table 2-2: The criteria for consider which bacteria as probiotics described by Colum, D., *et al.* 2001 [64] and were summarized in the table

Criteria for characteristic of probiotics bacteria
1. Human origin
2. Generally recognized as safe (GRAS) status
3. Resistance to gastric acidity and bile toxicity
4. Adherence to gut epithelium cells
5. Ability to colonize the gastrointestinal tract
6. Produce antimicrobial substances
7. Ability to modulate and alter the immune system
8. Response to large scale fermentation and commercial production

The probiotics are commonly found in human; it provides various beneficial effects. All probiotics should be recognized as safe (GRAS) microorganisms in general. Probiotics can survive and tolerate the acidic condition of stomach and intestine. It can also grow and colonize in the gastrointestinal tract. Probiotics provide plenty of benefits; it adheres to intestinal epithelium cells and produces antimicrobial substances for destroying the pathogens. It also activates and promotes the immune response. Probiotics should be in food and beverage industry, supplement or application for health production. In addition, several strains of bacteria are used as probiotics; the examples are shown in Table 2-3.

Table 2-3: The bacteria used as probiotics [65].

Lactobacillus spp.	*Bifidobacterium spp.*	Others
L. acidophilus	*B. bifidum animalis*	*Bacillus cereus*
L. rhamnosus	*B. longum*	*Clostridium butyricum*
L. gaserri	*B. breve*	*Escherichia coli*
L. casei	*B. infantis*	*Proprionibacterium*
L. ruteri	*B. lactis*	*Freundendsreichii*
L. bulgaricu	*B. adolescentis*	*Saccharomyces boulardii*
L. plantarum		*Enterococcus feecalis*
L. johnsonii		*Streptococcus thermophiles*
		Lactococcus species
		VSL#3 *(L.bulgaricus,*
		L.plantarum,B.longum,
		B.infantis, B.breve,
		S.salivarius subsp.
		Thermophiles)

B. The Mechanism of Probiotics

Probiotics perform a function of defending the host body from pathogen infection which causes diseases with a diverse mechanism. Previous studies explain that the mechanism of probiotics is against the infection of inhibit pathogens with several mechanisms. The same probiotics have the distinct mechanism that is against various pathogen infections [66].

1. Inhibit Growth of Pathogenic Enteric Bacteria

1.1 Produce Antimicrobial or Inhibitory Substance

Probiotics can produce various antimicrobials or inhibitory substances that have an effect on both of Gram-positive and Gram-negative bacteria. The substances that probiotics can produce are organic acid, hydrogen peroxide, rueterin, diacetyl and bacteriocin [65] which can directly destroy the inhibit growth of pathogens.

1.2 Competition for Nutrition and Prebiotics

Pathogens need the nutrition or prebiotics for growing in the host. Probiotics are live microorganisms which also need the nutrition or prebiotics for growing their activities**.** The high numbers of probiotics in the gastrointestinal tract are able to compete with the pathogens to gain nutrients and prebiotics which are sources of energy for pathogens. This can lead to the lower growth of pathogens in the human body.

2. Block Epithelial Attachment and Improve Barrier and Mucosal Function

2.1 Blocking the Adhesion Area

Probiotics can block and destroy the pathogen infection by displacing pathogens adhered to the gastrointestinal tract. This mechanism benefits the host body by reducing the infection rate and preventing pathogen infection which is the first process of pathogen invasion into the host.

2.2 Produce Mucus Secretion

Probiotics stimulate the host cells by producing the mucus secretion over the intestinal epithelial cells and increasing the thick layer that enhances the protection, inhibition and invasion of pathogens. Moreover, mucus production helps repair and improve epithelial and mucosal barrier function of the host cells.

3. Enhance and stimulate the immunity

Some strains of probiotics stimulate and promote the immune system. Most strains of probiotics are used as an anti-inflammatory. This is because they reduce inflammatory response which causes the inflammatory disease in the gastrointestinal tract [67, 68].

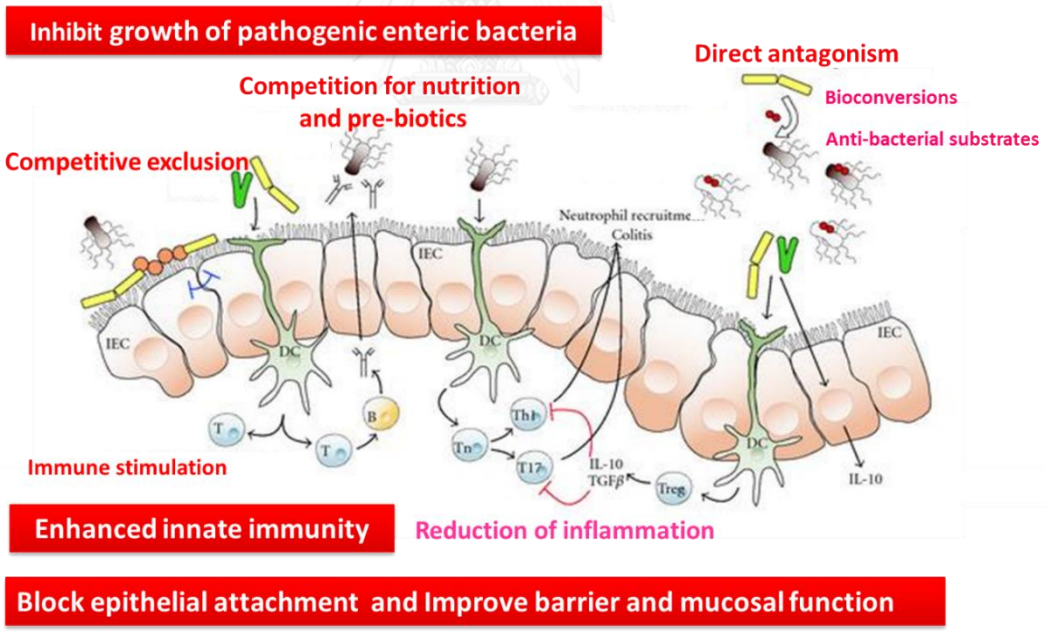

Figure 2-5: The mechanism of action of probiotics (modified from Paul W. O'Toole and Jakki C. Cooney 2008) [66]

C. *L. plantarum* as an Anti–infective Activity of Pathogenic Bacteria

L. plantarum potentially prevents pathogenic bacteria infection (*Salmonella, Shigella*, *Escherichia coli*). It plays a role in the growth of inhibition that reduces the adhesion. It also blocks epithelial attachment or invasion of pathogenic bacteria in intestinal epithelial cells which are the first and second important processes of pathogen infection. In the direct pathway, *L. plantarum* can produce antimicrobial compounds, bactericidal proteins and organic substances including organic acids (lactic acid, acetic acid, butyric acid). With these substances, it can increase the acidity environment in the intestinal lumen leading to the decrease of survival rate, inhibit growth, and adherence to intestinal epithelial cells of pathogenic bacteria. In addition, it produces anti-microbial substances such as organic acid, hydrogen peroxide, rueterin, diacetyl and bacteriocin [65, 69, 70] which can destroy the inhibit growth of pathogens in gastrointestinal tract [71-78]. In the indirect pathway, *L. plantarum* reduces the survival rate and the growth of pathogenic bacteria by competing with the pathogens for nutritious and pre-biotic substances which are the major food of energy synthesis. Both direct and in direct pathways have a potential to prevent and reduce the risk of pathogenic bacteria adherence, invasion and infection.

D. *L. plantarum* Prevent and Improve Intestinal Epithelial and Mucosal Function

Some strains of *L. plantarum* (*L. plantarum* MB452 and *L. plantarum* DSM2648) stimulate the production of mucus by building a thick layer to cover intestinal epithelial cells; enhance the protection layer; inhibit the pathogenic invasion; increase barrier integrity for repairment; and improve epithelial and musical barrier function [22, 79-83]. *L. plantarum* is able to protect intestinal epithelial cells [82, 84] through several different mechanisms such as reducing the adherent areas of pathogens and blocking the invasion of pathogenic bacteria on intestinal epithelial cells.

E. *L. plantarum* Stimulate the Immune Response.

There are specific strains of *L. plantarum that* have a potential to be anti-inflammatory and immunomodulatory agents such as *L. plantarum* L2, *L. plantarum* 299, *L. plantarum* 14917, and *L. plantarum* CS24.2. They can activate innate immune response and enhance innate immunity. They can also stimulate innate immunity to produce and secrete IgA which can destroy the antigen or pathogen, decrease the expression of pro- inflammatory genes and the secretion of pro- inflammatory cytokines (e.g. IFN-**γ**, TNF-**α**). The increase of anti- inflammatory genes induces the secretion of anti- inflammatory cytokines (e.g. IL-4, IL-6, IL-8, and IL-12). In 2010, Bin Wang., *et al.* [85] studies in *vitro* of live *L. plantarum* L2. The result shows that *L. plantarum* can reduce inflammatory response in Caco-2 cells that induce TNF-**α**. Moreover, *L. Plantarum* L2 inhibits the translocation of NF-**K**B to the nucleus and associates with the decrease in I**K**B. The part of down-regulation of I**K**B degradation can inhibit the release of pro-inflammatory cytokines from TNF-**α**-induced cells. In 2012, Raj Kumar., *et al.* [20] investigates the effect of *L. plantarum* Lp91 in colitis mouse model. The result suggests that *L. plantarum* is a strong immunomoduratory efficacy; it induces the expression of anti-inflammatory genes (COX-1, IL-4, IL-6, IL-10 and MUC2) and increases the secretion of anti- inflammatory cytokines. In addition, *L. plantarum* can decrease the expression of pro-inflammatory genes (TNF**α**, COX-2) and reduce pro- inflammatory cytokines release. Many previous studies show that specific strains of *L. plantarum, such as L. plantarum* K8 KCTC10887BP, *L. plantarum* CGMCC No. 1258, *L. plantarum* CS24.2 and *L. plantarm* PTCC 1058, are used as probiotics and biotherapuetics. They are considered as anti-inflammatory and immunomodulatory agents that potentially reduce inflammatory response and decrease inflammation both in *in vitro* and *in vivo* [19, 86-93]

Triple Sugar Iron (TSI) Slant Agar Test

Triple Sugar Iron (TSI) slant agar test is a biochemical test used for identification of *Enterobacteriaceae* by differentiating the fermentation of glucose, lactose and sucrose with or without gas production and hydrogen sulfide (H_2S) production. Triple Sugar Iron (TSI) slant agar is a tube containing the three differential carbohydrate mediums: glucose, lactose and sucrose. It also contains yeast extract, beef extract, peptone, iron (III) citrate, sodium chloride, sodium thiosulphate, phenol red (pH- sensitive dye) and agar.

A. The Principle of TSI Agar Reaction

The sugar fermentation results in the change of a phenol from red to yellow colors. The bacteria ferments only glucose by reducing the concentration of sugar and produces acid products in the slant. During the fermentation, the phenol returns to red. The acid reaction that shows in yellow color of agar, is maintained in the depth of agar, in the butt of the tube. After the bacteria ferments lactose or sucrose, it results in the change of color to be yellow in a slant of the tube. The bacteria with the fermentation containing none of these three sugars results in no changing color of agar. The production of gas and H_2S showing in the butt is noticeable when there is the gas production (CO_2, H_2) due to the sugar fermentation, in which, it appears in forms of bubbles or the fragments of agar. While H_2S production is noticeable from the remains of black precipitates of ferric sulfide; the product results from the reaction of H_2S and ferrous sulfate.

B. TSI Interaction and Results

1. Sugar fermentation

1.1 Glucose fermentation: alkaline slant (red color of slant agar) and acid butt (yellow color of butt agar).

1.2 Lactose and/ or sucrose fermentation: acid slant (yellow slant agar) and acid butt (yellow butt agar).

1.3 Alkaline slant (red slant agar) and alkaline butt (red butt agar): neither glucose, lactose, nor sucrose has been fermented.

2. Gas production (CO_2, H_2): bubbles or fragments in the agar butt.

3. Hydrogen sulfide (H_2S) production: black precipitate or black color between the slant and butt, or in the butt.

C. The key of TSI resulting

K= Alkaline

A= Acid

G= Gas production

H_2S= H_2S production

N= No change of agar color or original agar color (rose base or orange)

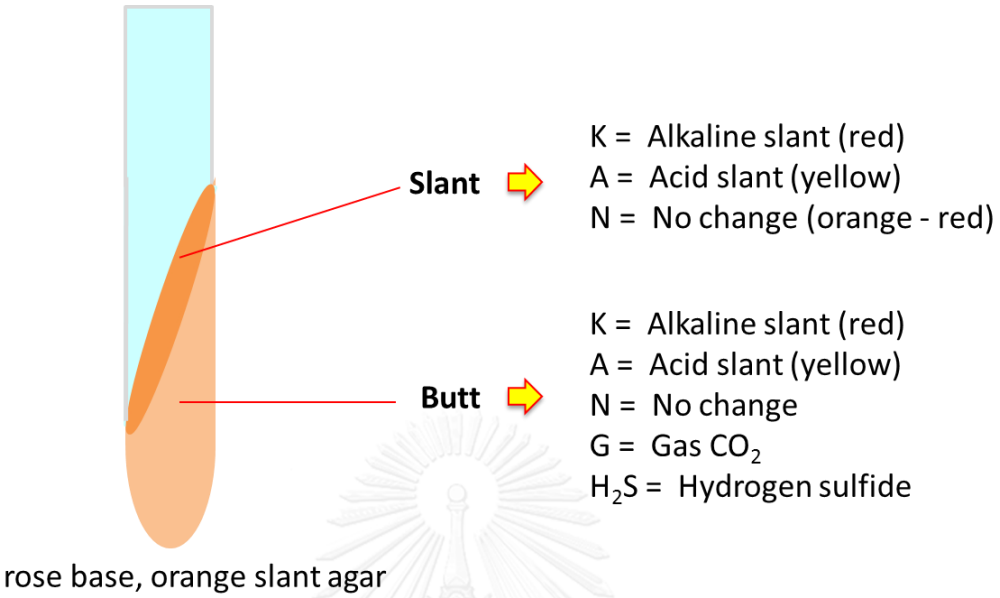

Figure 2-6: TSI slant agar test tube

Table 2-4: TSI slant agar test interaction [94, 95]

Key	Color and appearance	Slant (surface)	Butt
A	Yellow	Lactose and/or Sucrose fermentation and acid production	Glucose fermentation and acid production
G	Yellow with bubbles or fragmentation		Gas production from glucose
K	Red	No sugar fermentation, Fermentation of alkaline production	No sugar fermentation, Fermentation of alkaline production
N	No change or original color	No fermentation of lactose or sucrose	No fermentation of glucose
H_2S	Black		H_2S production
	Not black		No H_2S production

Table 2-5: Typical reactions of TSI slant agar test [96]

Species	Slant (surface)	Butt		H_2S
	Lactose/sucrose	Glucose	Gas	
Salmonella Tphi[2]	-	+	-	+
Salmonella Parathyphi A [2]	-	+	+	-
Salmonella Choleraesuis[2]	-	+	+	-
Salmonella Pullorum[2]	-	+	+	+
Salmonella Paratyphi B[2]	-	+	+	+
Salmonella Typhimurium[2]	-	+	+	+
Salmonella Enteritidis[2]	-	+	+	+
Salmonella Gallinarum[2]	-	+	-	+
Shigella dysenteriae	-	+	-	-
Shigella flexneri	-	+	-	-
Shigella sonnei	-	+	-	-
Shigella boydii	-	+	-	-

Proteus vulgaris	+	+	[+]	+
Proteus marabilis	-	+	+	+
Proteus morganii	-	+	+	-
Proteus rettgeri	-	+	-	-
Serratia marcescens	-	+	-	-
Enterobacter hafniae	-	+	+	-
Enterobacter areogenes	+	+	+	-
Enterobacter cloacea	+	+	+	-
Escherichia coli(1)	+	+	+	-
Citrobacter freundii	+	+	+	+
Klebsiella pneumonia	+	+	+	-
Alcaligenes faecalis	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Yersinnia enterocolitica	-	-	-	-

CHAPTER III
MATERIALS AND METHODS

Chemical Substances

Absolute Alcohol (Merck Germany)

Assay Diluent RD1-14 (R&D Systems, Minneapolis, MN, USA)

Assay Diluent RD1-18 (R&D Systems, Minneapolis, MN, USA)

Assay Diluent RD1-63 (R&D Systems, Minneapolis, MN, USA)

Brain Heart Infusion Agar (BHI) (BD Bacto, Heidelberg, Germany)

Calibrator Diluent RD5-3 (R&D Systems, Minneapolis, MN, USA)

Calibrator Diluent RD5T (R&D Systems, Minneapolis, MN, USA)

Calibrator Diluent RD6-12(R&D Systems, Minneapolis, MN, USA)

De Man, Rogosa, Sharpe agar (MRS agar) (Oxoid, Basingstoke, UK)

De Man, Rogosa, Sharpe broth (MRS agar) (Oxoid, Basingstoke, UK)

Distilled water

Ethyl alcohol

Formalin solution

Glucose

NaCl (Merck, Germany)

Phosphate buffer saline (PBS)

Mouse CXCL1 Conjugate (R&D Systems, Minneapolis, MN, USA)

Mouse CXCL1 Standard (R&D Systems, Minneapolis, MN, USA)

Mouse IL-6 Conjugate (R&D Systems, Minneapolis, MN, USA)

Mouse IL-6 Standard (R&D Systems, Minneapolis, MN, USA)

Mouse TNF-α Conjugate (R&D Systems, Minneapolis, MN, USA)

Mouse TNF-α Standard (R&D Systems, Minneapolis, MN, USA)

Salmonella O Group Antisera (S&A reagents lab, Bangkok, Thailand)

Salmonella Shigella agar (SS agar) (Oxoid, Basingstoke,UK)

Stop solution for mouse CXCL1 (R&D Systems, Minneapolis, MN, USA)

Stop solution for mouse IL-6 (R&D Systems, Minneapolis, MN, USA)

Stop solution for mouse TNF-α (R&D Systems, Minneapolis, MN, USA)

Streptomycin (General Drugs House, Bangkok, Thailand)

Substrate solution for mouse IL-6 (R&D Systems, Minneapolis, MN, USA)

Substrate solution for mouse CXCL1 (R&D Systems, Minneapolis, MN, USA)

Substrate solution for mouse TNF-α (R&D Systems, Minneapolis, MN, USA)

Tryptone Soya Agar (TSA) (BD Bacto, Heidelberg, Germany)

Wash buffer (R&D Systems, Minneapolis, MN, USA)

0.85% saline

EQUIPMENT

Anaerobic Chamber (Envimed, England)

Autoclave (Hirayama, Japan)

Autopipetts (Gilson, France)

Centrifuge

Frogger (DAN-KAR CCRP, USA)

Hot air oven

Incubator

Spectrophotometer

Vortex mixer

***In Vitro* Study**

A. Antagonistic Activity Assay of *L. plantarum* B7 Against *S*. Typhimurium Using Agar Spot Method.

According to the agar spot method previously described by Spinler *et al.* [97], this method was applied in order to determine antimicrobial activities of *L. plantarum* B7 against *S.* Typhimurium.

B. Bacterial Preparation

1. *S.* Typhimurium ATCC 13311 was grown on the Tryptic soya agar at 37 ^{o}C under aerobic conditions for 24 h and adjusted at the final concentration of 1x10^{7} CFU/mL.
2. The frozen culture of *L. plantarum* B7 was pre-cultivated on MRS agar at 37 ^{o}C for 48 h in an anaerobic condition. Then, a single colony was isolated and subcultured on de Man, Rogosa and. Sharpe (MRS) broth two times in 96 well plates and incubated at 37 ^{o}C, 48 h in an anaerobic condition.
3. *L. plantarum* B7 was transferred from 96 well plate by using a frogger and spotted onto Brain heart infusion (BHI) agar supplemented with 20 mM glucose in 140 mm plates. These plates were incubated at 37 ^{o}C under anaerobic conditions for 48 h.
4. The plates were overlaid with 20 mL of Tryptic soft agar (TSA) (7.5 g agar/L) containing overnight culture of 1x 10^{7} CFU/mL *S.* Typhimurium.
5. After that they were incubated at 37 ^{o}C 24 h. In this study, *L. rhamnosus* L34 was used as a positive control and *L.* fermentum L12 was used as a negative control.

***In Vivo* Study**

A. Bacterial Preparation

1. *S.* Typhimurium ATCC 13311 was cultured on Salmonella-Shigella agar (SS agar) (Oxoid, Basingstoke, United Kingdom) and incubated at 37 ^{o}C under aerobic conditions for 24 h. The colonies of *S.* Typhimurium ATCC 13311 were harvested and adjusted at the final concentration of 3x 10^{9} CFU/mL in 0.85 % saline solution.

2. *L. plantarum* B7 previously isolated from Thai dyspeptic patients at King Chulalongkorn Memorial Hospital and stored in MRS broth (Oxoid, Basingstoke, United Kingdom) with 20% glycerol at -80 ^{o}C. This strain was recovered from a frozen stock and cultivated anaerobically twice on MRS agar in an anaerobic jar (10% CO_2, 10% H_2 and 80% N_2) at 37 ^{o}C for 48 h.

3. The colonies of *L. plantarum* B7 were harvested and adjusted to the final concentration of 1x 10^{9} CFU/mL in 0.85 % saline solution.

B. Animal Preparation

Male albino mice, weighing about 20-25 grams, were purchased from the National Laboratory Animal Center located on Salaya Campus of Mahidol University in Nakornpathom, Thailand. The animals were housed in a controlled temperature room at 25 ± 1ºC with 12:12 hour light-dark cycle. All mice were received proper care in accordance with the Ethics Committee for Animal Care and Use of Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

C. Experimental Protocol

1. Albino male mice were randomly divided into the following 3 groups:

Group 1 (Control group, *n*=8): Mice were fed with 0.85% saline 1 mL by oral gavage feeding once a day for 3 days and housed with a free access to water and standard food.

Group 2 (*Salmonella* group or S group, *n*=8): Mice were fed with $3x10^9$ CFU *S.* Typhimurium 1 mL and suspended 0.85% saline by oral gavage feeding once a day for 3 days and housed with a free access to water and standard food.

Group 3 (*Salmonella* + *Lactobacillus* or S + LP group *n*=8): Mice were fed with $1x10^9$ CFU *L. plantarum* B7 and suspended 0.85% saline 1 mL by oral gavage feeding. After the mice were treated with *L. plantarum* B7 2 hours, they were fed with $3x10^9$ CFU *S.* Typhimurium and 1 mL of suspended 0.85% saline by oral gavage feeding for 3 days. The mice were also housed with a free access to water and standard food.

2. All groups of mice were pre-treated with streptomycin suspended in drinking water (5 mg/mL) for 3 days as previously described by Manja Barthel *et al* [98] before treatment with 1 mL of $3x10^9$ CFU *S.* Typhimurium or 1 mL of $1x10^9$ CFU *L. plantarum* B7 in 0.85 % saline solution by oral gavage feeding.

3. Body weights of the mice were measured and recorded daily. This included the investigation and recording of their physical symptoms (mice activities and fecal characteristics).

4. After the treatment of $3x10^9$ CFU *S.* Typhimurium (1 mL) or $1x10^9$ CFU *L. plantarum* B7 (1 mL) for 3 days, the fresh feces were collected to determine *S.* Typhimurium infection by culture and colony count, investigate the fecal characteristics and measure the moisture content in feces.

5. Mice were sacrificed with an overdose of intraperitoneal thiopental sodium injection. Blood samples were collected by cardiac puncture to determine TNF-**α** level, IL-6 level and CXCL1 level in serum using enzyme-linked immunosorbent assay (ELISA) kit.

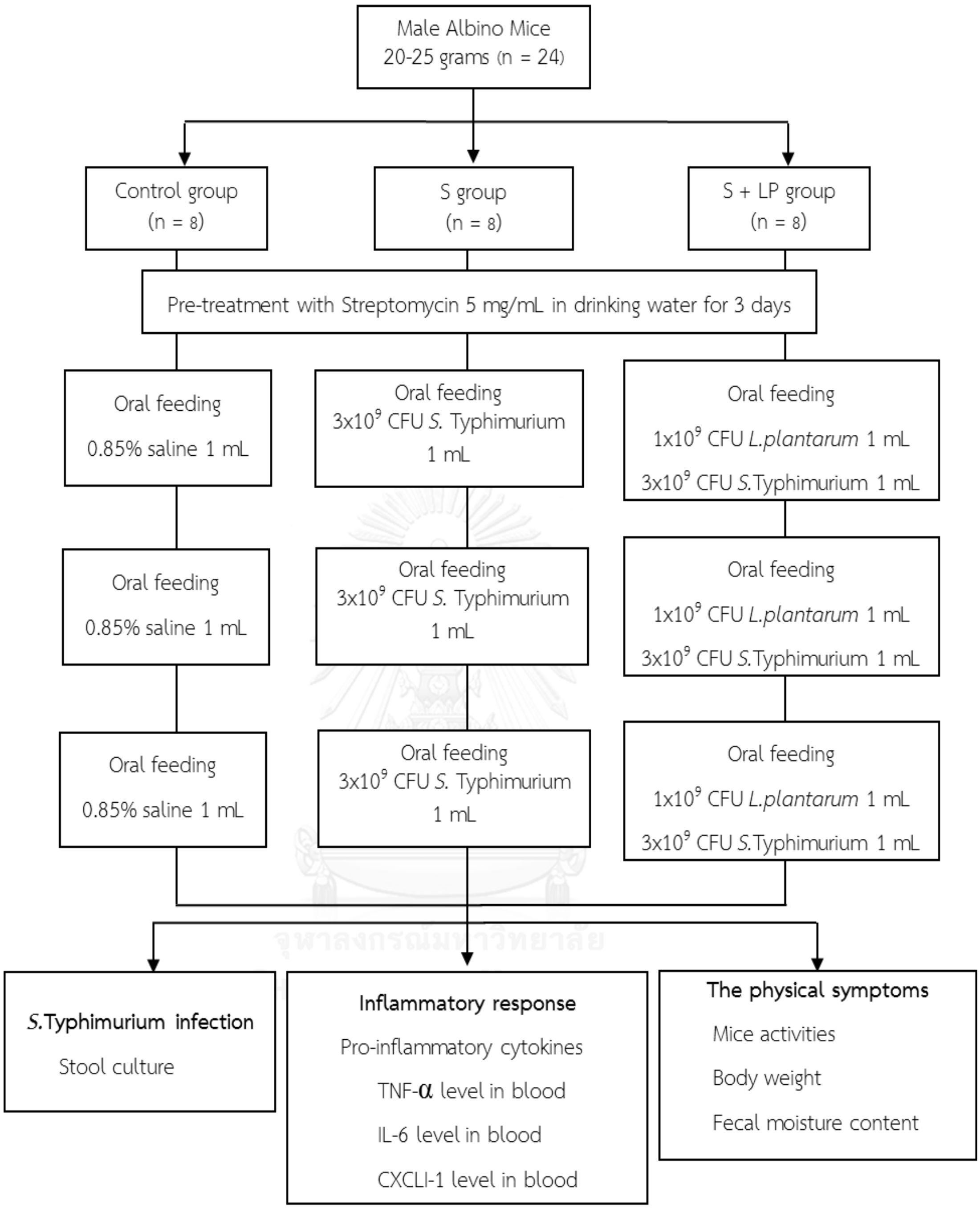

Figure 3-7: Schematic diagram of experimental protocol

Data Collection

A. Determination of *S.* Typhimurium in Feces: Stool Culture with Colony Count

1. Fresh feces (approximately 1 gram) were weighed and homogenized in 400 µL of phosphate buffer saline (PBS) pH 7.4.and prepared the serial diluted 10^{-1}-10^{-7} suspension in PBS. A 100 µL of each suspension was plated on SS agar via a spreader technique and the plates were incubated at 37 ^{o}C for 24 hours. The plates with *S.* Typhimurium colonies of 30-300 were selected for counting and calculated according to the equation below.

2. Triple Sugar Iron (TSI) slant agar test was applied here in order to confirm the selected colonies of *S.* Typhimurium. The single colony of *S.* Typhimurium from SS agar plate was inoculated onto TSI slant agar and incubated at 37 ^{o}C for 24 hours. The result of TSI agar test was determined and the culture was tested with *Salmonella* group B antibodies.

   The number of *S.* Typhimurium in each sample was calculated by the following equation:

$$\text{Number of bacteria (CFU/mL)} = \frac{\text{No. of colonies on plate X reciprocal of dilution of sample}}{\text{Volume of sample}}$$

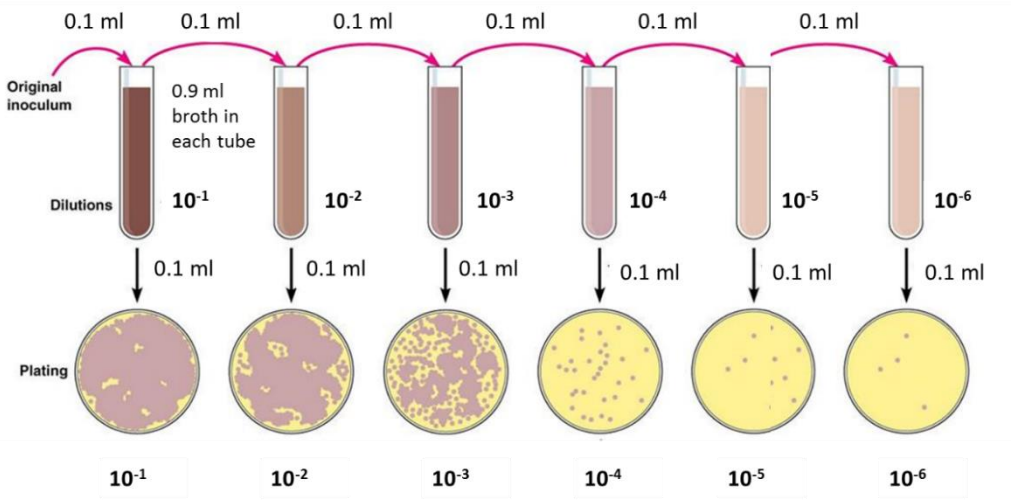

Figure 3-8: Serial dilution for *S.* Typhimurium samples plating [99]

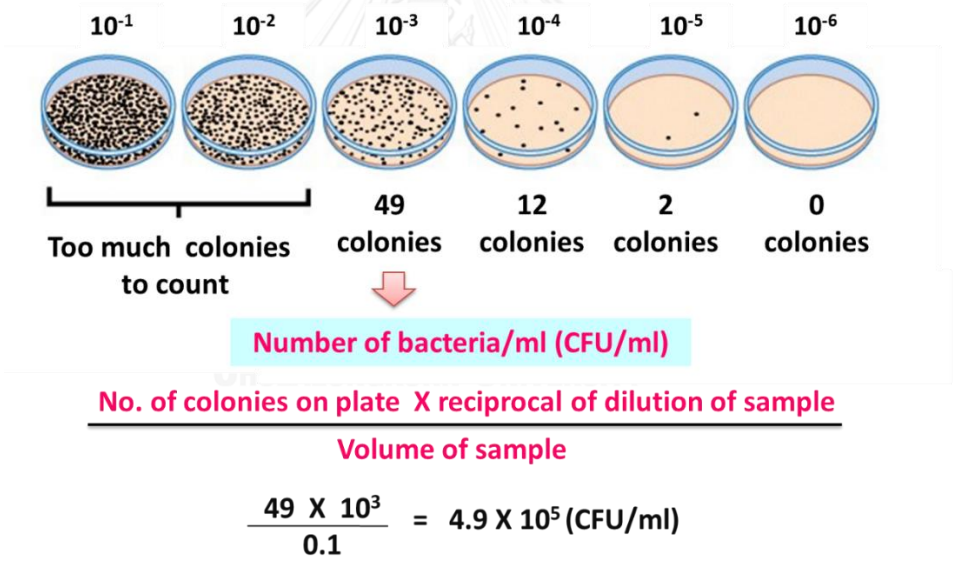

Figure 3-9: Calculation of the number of *S.* Typhimurium in samples [100]

B. Assay of Fecal Moisture Content

In order to determine the percentage of water in fecal sample, fresh feces (approximately 1 gram) were collected, weighed and recorded as “wet weight of sample”. The sample was then dried at temperature 101°C to 105°C by using the microwave oven. After allowing the sample to cool, the sample was weighed and recorded as the “dry weight of sample” [101, 102]. Sample drying was repeated until the dry weight was not changed.

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

$$\text{\% Fecal moisture content} = \frac{A - B}{B} \times 100$$

A = Weight of wet sample (grams)

B = Weight of dry sample (grams)

C. Assay of Serum Cytokine Levels

1. Sample Preparation

Blood samples were collected via cardiac puncture and left for 2 hours at room temperature before centrifugation 20 minutes at approximately 1000 × *g*. The serum was removed and stored at -80 °C for the determination of TNF-α, IL-6 and CXCL1 level by enzyme-linked immunosorbent assay (ELISA) using R&D Systems.

2. Assay of Serum Tumor Necrosis Factor Alpha (TNF-α)

1. The level of serum TNF-α was measured by enzyme-linked immunosorbent assay (ELISA) using a colorimetric commercial kit from R&D Systems (Minneapolis, MN, USA). This procedure used the quantitative sandwich ELISA by pre-coated a monoclonal antibody specific for mouse TNF-α on microplate.

2. After adding the standard samples into the wells, any presented mouse TNF-α was bound by immobilized antibody. They were washed for removing the unbound materials, then added an enzyme-linked polyclonal antibody specifically for mouse TNF-α to binds with the captured TNF-α.

3. Then they were washed again to remove the unbound antibody-enzyme reagent and added substrate solution into a microplate. The enzyme reaction occurred in blue color of the product and the reaction stopped after adding stop solution which appeared in yellow product. The intensity of the color was measured in a suitable proportion for the amount of mouse TNF-α bounded in the initial step. Then the sample values were read off the standard curve.

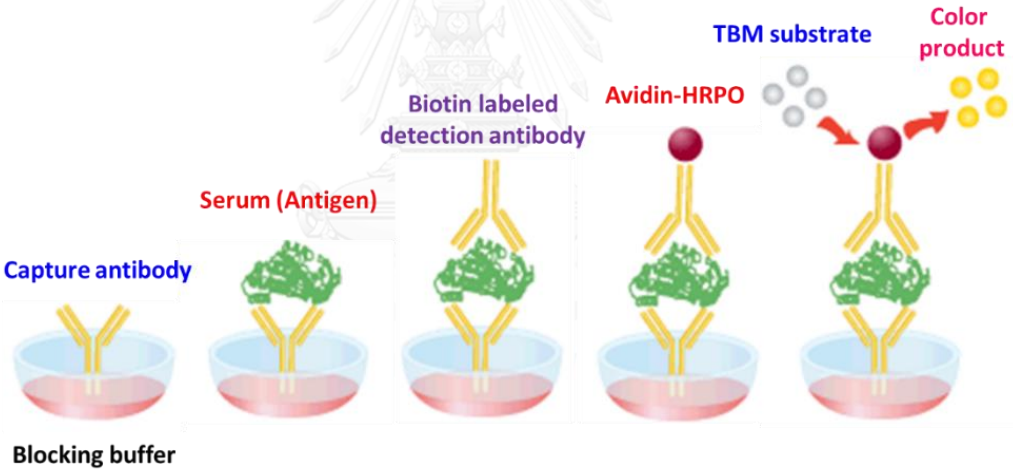

Figure 3-10: Enzyme-linked immunosorbent assay (ELISA) technique [103]

2.1 Assay Protocol

1. TNF-α standard was prepared as a stock solution at the concentration of 7000 pg/mL and then set a dilution series of standards with known concentrations.
2. Assay diluent RD1-63 and the standards or samples were mixed together in each well and incubated for 2 hours at room temperature.
3. After 2 hours, the solution in each well was wash away repeatedly. Mouse TNF-α conjugate was added into the wells and were subsequently incubated at room temperature for 2 hours.
4. After being washed again, and added a substrate solution into wells which were previously incubated for 30 minutes at room temperature and protected from light.
5. Finally, added stop solution, the reaction was stopped and the optical density of each well was measured within 30 minutes at 450 nm with wavelength correction set at 540 or 570 nm.

2.2 Calculations

The duplicate readings for each standard and sample were average zero standard optical density (O.D). The standard curve was created by reducing the data. The mean absorbance for each standard was plotted on the y-axis against the concentration on the x-axis and plotted a best fit curve. The best fit line can be determined by regression analysis and the contents of TNF-**α** in the samples were expressed in pg/mL.

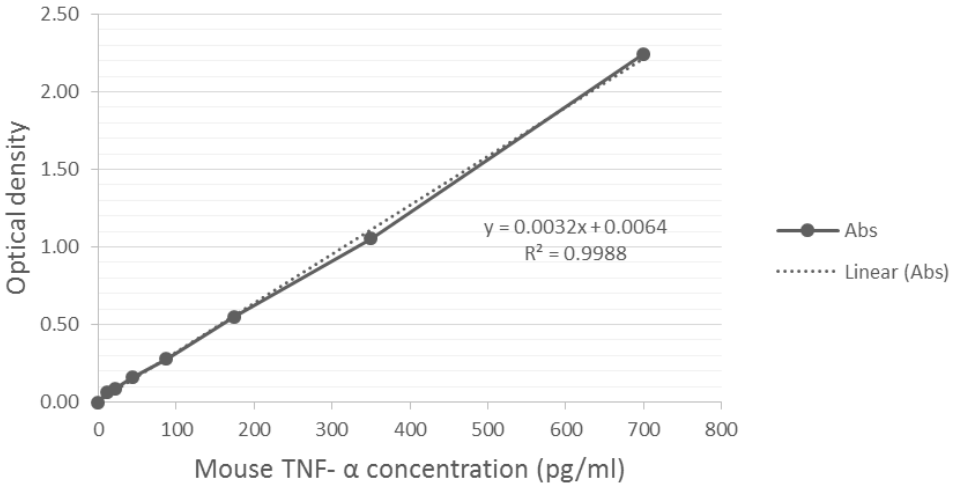

Figure 3-11: Example of TNF-**α** standard curve

3. Assay of serum Interleukin 6 (IL-6)

1. The level of serum IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) by using a colorimetric commercial kit from R&D Systems (Minneapolis, MN, USA). This procedure was applied the quantitative sandwich ELISA by pre-coated a monoclonal antibody which specific for mouse IL-6 on the microplate.
2. The strands samples were added into the wells and any mouse IL-6 presented was bound by the immobilized antibody.
3. They were washed for removing the unbound substances and added an enzyme-linked polyclonal antibody specific for mouse IL-6 into the wells.
4. Then they were wash again and added substrates solution into the wells. The enzyme reaction was occurred with blue product and the reaction was stopped when added the stop solution occurred with turn to yellow product.
5. The intensity of the color measurement was in proportion to the amount of mouse IL-6 bound in the initial step. The sample values were then read off the standard curve.

3.1 Assay Protocol

1. IL-6 standard was prepared as a stock solution at the concentration of 500 pg/ml and then set a dilution series of standards with known concentrations.
2. Assay diluent RD1-14, the standards and samples were mixed together in each well and were incubated for 2 hours at room temperature.
3. Then the wells was washed away repeatedly, following by added mouse IL-6 conjugate and the wells were subsequently incubated at room temperature for 2 hours.

4. After being washed again, substrate solution was added into wells incubated for 30 minutes at room temperature protected from light.
5. Finally, stop solution was added to stop the reaction of wells, and measured the optical density of each well within 30 minutes at 450 nm with wavelength correction set at 540 or 570 nm.

3.2 Calculations

The duplicate readings for each standard and sample were average zero standard optical density (O.D).The standard curve was created by reducing the data used computer software capable of generating a four parameter logistic (4-PL) curve-fit. The standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. The best fit line can be determined by regression analysis and the contents of mouse IL-6 in the samples were expressed in pg/mL.

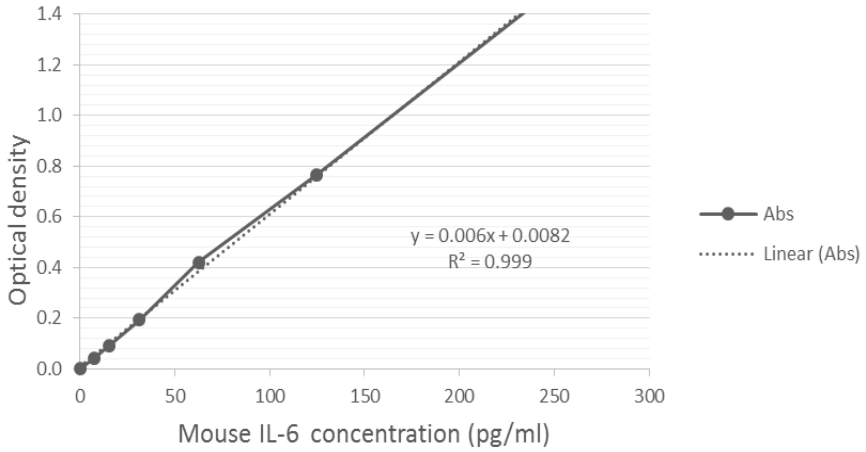

Figure 3-12: Example of IL-6 standard curve

4. Assay of Serum Cytokine-Induced Neutrophil Chemoattractant 1 (CINC-1/CXCL1)

1. The level of serum CINC-1 or CXCL1 was measured by enzyme-linked immunosorbent assay (ELISA) using a colorimetric commercial kit from R&D Systems (Minneapolis, MN, USA).This procedure was applied by adding the quantitative sandwich enzyme immunoassay technique by pre-coated polyclonal antibody which is specific to mouse CXCL1 on a microplate.
2. Standards and samples were added into the wells and any mouse CXCL1 presented was bound by the immobilized antibody.
3. After that they were washed to remove unbound substances and added an enzyme-linked polyclonal antibody specifically for mouse CXCL1 into the wells.
4. They were washed again for removing unbound antibody-enzyme reagent. After adding a substrate solution, the enzyme reaction was appearance with a blue product and turns to yellow product when added stop solution into the wells. The intensity of the color measured was in proportion to the amount of mouse CXCL1 bounded in the initial step. The sample values were read off the standard curve.

4.1 Assay Protocol

1. CXCL1 standard was prepared as a stock solution at the concentration of 1000 pg/ml and then set a dilution series of standards with known concentrations.
2. Assay diluent RD1-18, the standards and samples were mixed together in each well and were incubated for 2 hours at room temperature.

3. Then the wells were washed away repeatedly, following added mouse CXCL1 conjugate and the wells were subsequently incubated at room temperature for 2 hours.
4. After being washed again, substrate solution was added into wells incubated for 30 minutes at room temperature protected from light.
5. Finally, stop solution was added to stop the reaction of wells, and measured the optical density of each well within 30 minutes at 450 nm with wavelength correction set at 540 or 570 nm.

4.2 Calculations

The duplicate readings for each standard and sample were average zero standard optical density (O.D).The standard curve was created by reducing the data used computer software capable of generating a four parameter logistic (4-PL) curve-fit. The standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. The best fit line can be determined by regression analysis and the contents of mouse IL-6 in the samples were expressed in pg/mL.

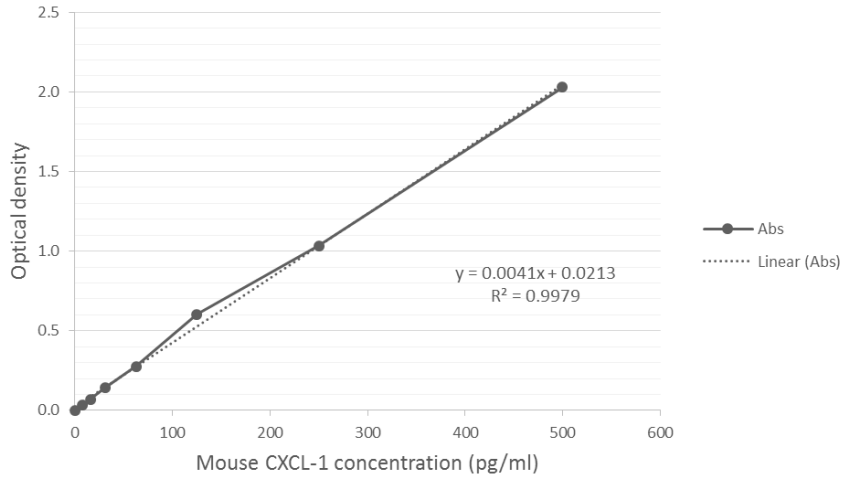

Figure 3-13: Example of CXCL1 standard curve

Data Analysis

Statistical analysis is performed by the Statistics Package for the Social Sciences (SPSS) software version 18.0 for windows. Continuous data is presented as mean ± standard deviation (SD). Mean comparison among groups of animals is carried out with one way analysis of variance (one-way ANOVA) following by Tukey post hoc test. Differences are considered statistically significant at $p < 0.05$.

CHAPTER IV
RESULTS

In Vitro Study

A. Antagonistic Activity of *L. plantarum* B7 against *S.* Typhimurium

The character of inhibitory activity was shown as a clear zone (C) around the colony. In this study, *L. fermentum* L12 and *L. rhamnosus* L34 was used as a negative control and positive control, respectively. The results in Figure 4-14showed that *L. plantarum* B7 has a clear zone around the spot. , which demonstrated that it has an inhibitory activity against *S.* Typhimurium.

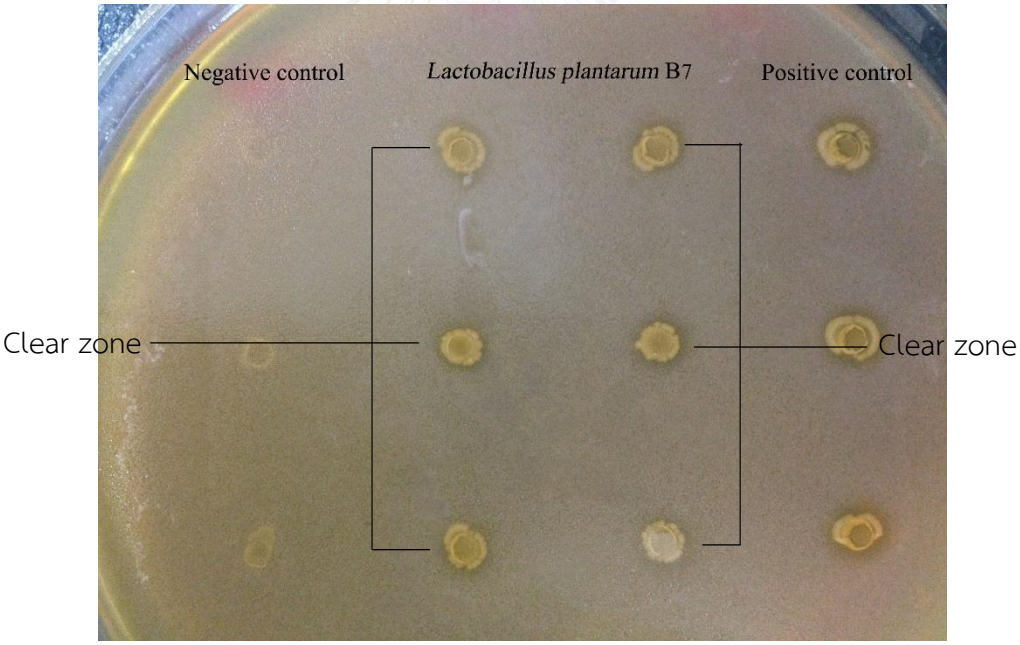

Figure 4-14: *Lactocillus platarum* B7 spots grown on 20 mM glucose BHI agar in anaerobic condition at 37 ^{o}C , 48 h overlaid with 10^{7} *S.* Typhimurium and incubated at 37 ^{o}C, 24 h in aerobic condition (n=6).

In Vivo Study

A. The Number of *S*. Typhimurium in Feces

In comparison of the number of *S*. Typhimurium in feces among the groups of mice, the number of *S.* Typhimurium in 1 gram of feces in S group was significantly increased when compared with control group (8.86 ± 0.02 *vs* 0.00 ± 0.00, $p < 0.05$), while the number of *S.* Typhimurium was significantly decreased in S + LP group as compared to *Salmonella* group (7.4.2 ± 0.05 *vs* 8.86 ± 0.02, $p < 0.05$) as shown in Figure 4-15.

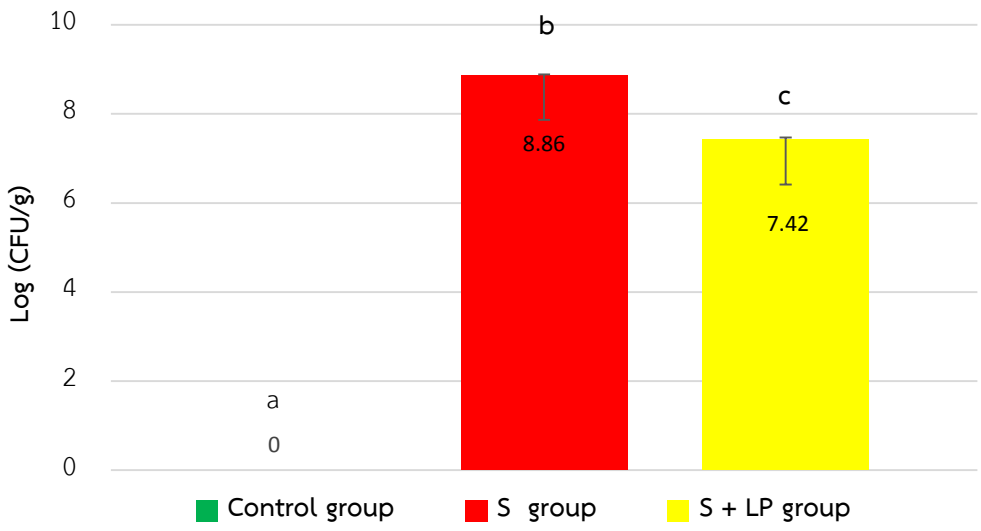

Figure 4-15: The number of S. Typhimurium in feces (CFU/g) (mean ± SD)

[ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

B. Serum TNF-α level

As shown in Figure 4-16, the level of TNF-α serum in S group was significantly increased when compared with control group (128.59 ± 12.82 *vs* 53.49 ± 8.90, $p < 0.05$). After administration of *L. plantarum* B7 in S + LP group ,however, TNF-α level was significantly decreased as compared to S group (36.15 ± 9.22 *vs* 128.59 ± 12.82, $p < 0.05$)

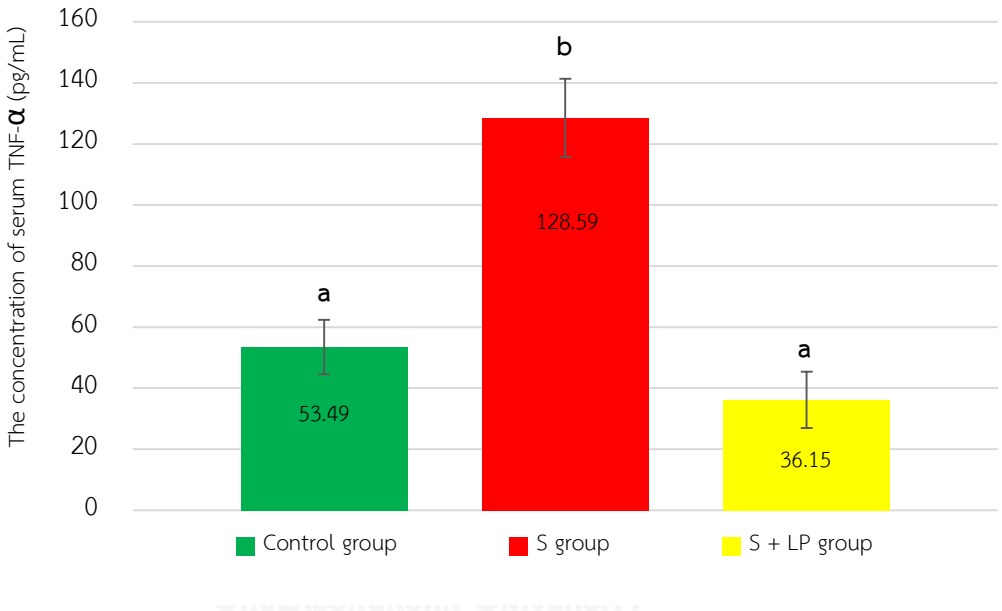

Figure 4-16: The concentration of serum TNF- α (pg/mL) in all groups (mean ± SD)
[ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

C. Serum IL-6 level

The level of serum IL-6 in all groups was represented in Figure 4-17. The level of serum IL-6 in S group was significantly increased when compared with control group (144.44 ± 8.91 *vs* 66.51 ± 4.04, $p < 0.05$). However, the level of serum IL-6 in S + LP group was significantly decreased as compared to S group (70.36 ± 5.37 *vs* 144.44 ± 8.91, $p < 0.05$)

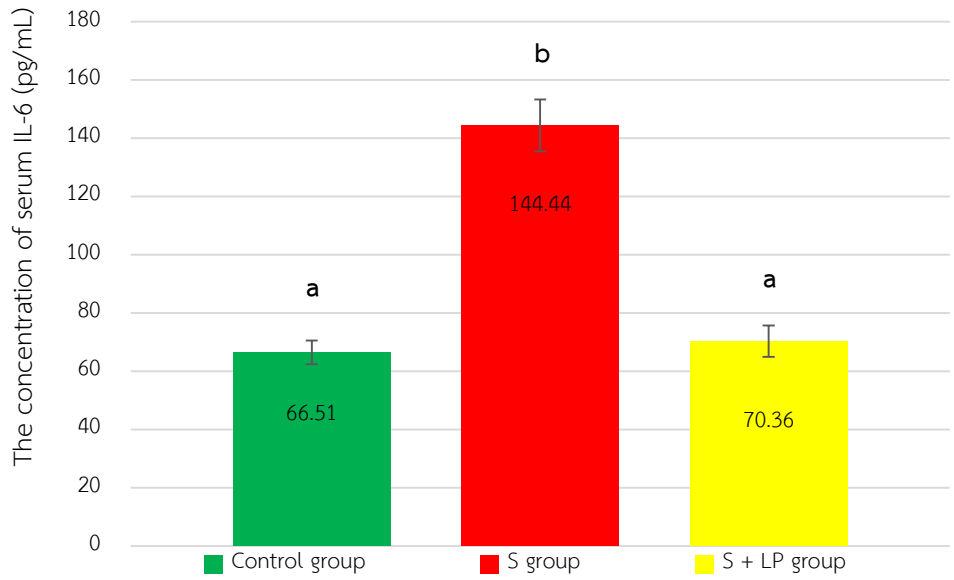

Figure 4-17: The concentration of serum IL-6 (pg/mL) in all groups (mean ± SD)

[ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

D. Serum CXCL1 level

Serum CXCL1 Levels were presented in Figure 4-18, serum CXCL1 levels significantly was increased in S group when compared with control group (96.09 ± 10.81 *vs* 32.32 ± 4.54, $p < 0.05$). In contrast, administration of *L. plamtarum* B7 in S + LP group was significantly decreased the level of serum CXCL1 when compared with S group (35.40 ± 2.77 *vs* 96.09 ± 10.81, $p < 0.05$).

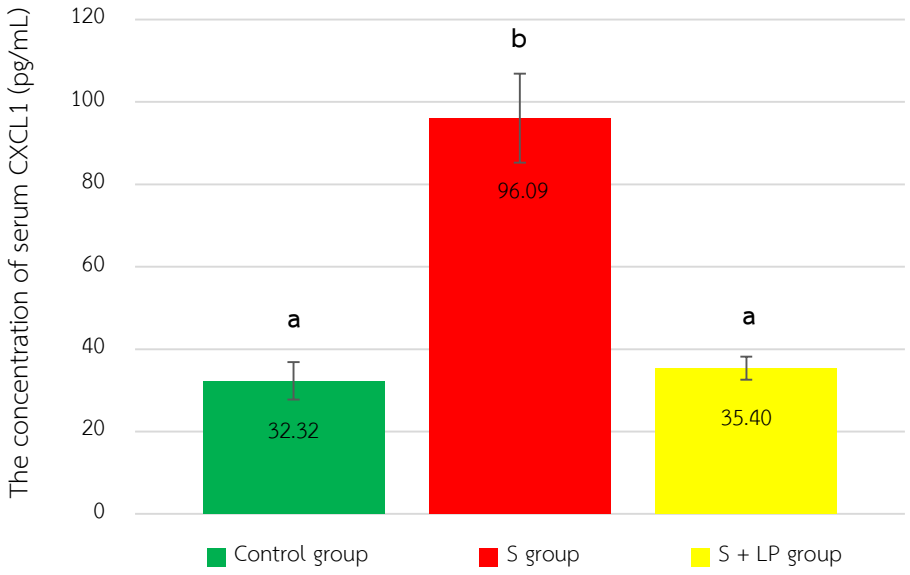

Figure 4-18: The concentration of serum CXCL1 (pg/mL) in all groups (mean ± SD) [ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

E. Change of Body Weight

As shown in Figure 4-19, there was no significant difference ($p < 0.05$) of the body weight among groups. In control group, the body weight trend was higher. S group and S + LP group, the body weight trend was lower after it was fed with *L. plantarum* B7 and *S.* Typhimurium.

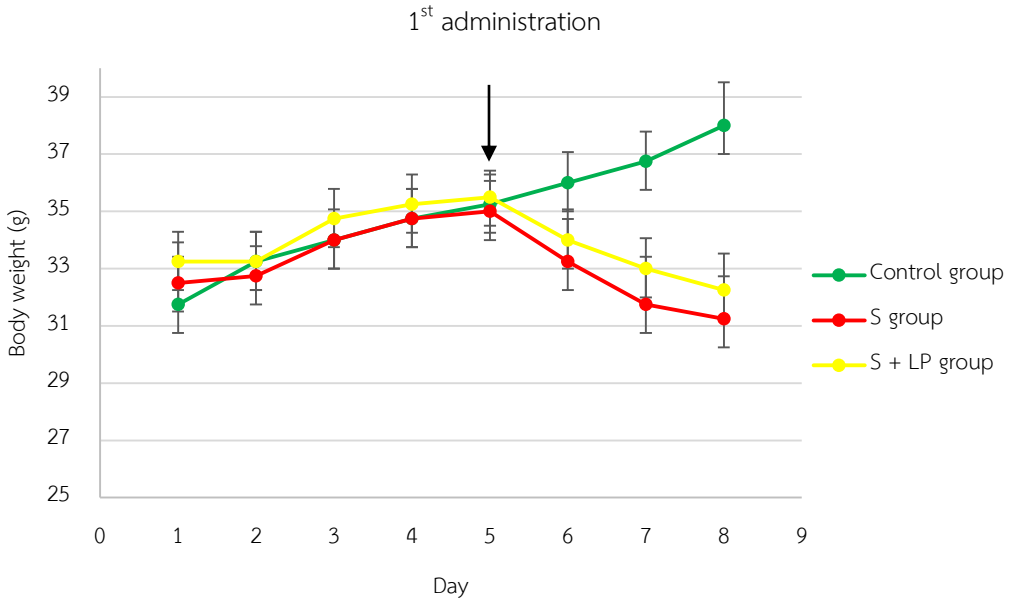

Figure 4-19: Changes of body weight in all groups, n=32 (mean ± SD)

[ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

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F. Fecal Characteristics after Being Fed with *S*. Typhimurium and *S*. Typhimurium + *L. plantarum* B7

Figure 4-20 showed the characteristics of the feces of mice in all groups. In control group, the feces were rod shape, dark color and trifling or no saw dust around the surface. In S group, after being fed with *S.* Typhimurium, the feces were lose, soft, the dark color reducing with the saw dust covered the surface. The feces of S + LP group had the rod shape, dark color and have a little of saw dust around the surface.

Figure 4-20: The feces characteristics of mice.

Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

G. Fecal Moisture Content

The percentage of fecal moisture content (% FMC) in all groups is represented in Figure 4-21, S group significant increases of %FMC when compared with control group (43.24 ± 2.05% *vs* 14.19 ± 1.57%, $p < 0.05$), S + LP group significantly decrease of %FMC when compared with S group (24.65 ± 2.08% *vs* 43.24 ± 2.05%, $p < 0.05$).

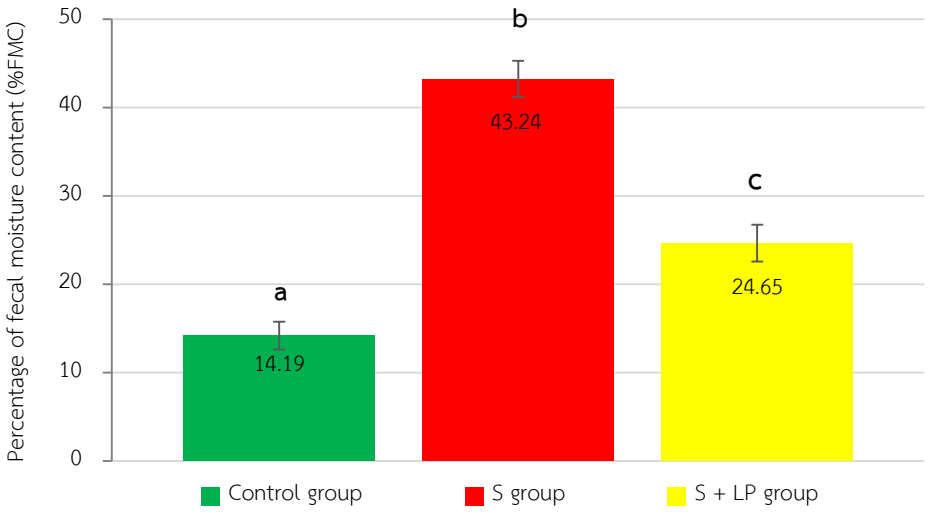

Figure 4-21: Percentage of fecal moisture content in all groups (mean ± SD)

[ab] Different superscript letters indicate significant differences ($p < 0.05$). Control group (n=8): mice fed with 0.85% saline; S group (n=8): mice fed with S. Typhimurium $3x10^9$ CFU/mL; S + LP group (n=8): mice fed with *L. plantrum* B7 $1x10^9$ CFU/m and *S.* Typhimurium $3x10^9$ CFU/mL.

CHAPTER V
DISCUSSION AND CONCLUSIONS

Regarding to this present study, it is well recognized that *S.* Typhimurium is one of the most common causes of acute gastroenteritis and worldwide diarrhea. In addition, using antibiotics or anti-inflammatory agents for medication even cause adverse effects. The model of *S.* Typhimurium diarrhea has remained rarely understandable. There is still insufficient research in terms of pathophysiological and epidemiological studies. This study demonstrated protective effects of *L. plantarum* B7 on *S.* Typhimurium developed diarrhea. The findings demonstrated how *L. plantarum* B7 became probiotic with anti-infection of enteropathogenic bacteria, inhibit growth of bacteria, anti-inflammatory activities and attenuated inflammatory responding to *S.* Typhimurium infected mice.

Pre-treatment with Streptomycin

The mouse model of *S*. Typhimurium infection with pre-treatment of streptomycin was developed by Bohnhoff and co-worker [104]. The pre-treatment with streptomycin led to a transient clearance and increased a susceptibility in the normal flora. In this experiment, all groups of mice were pre-treated with 5 mg/mL streptomycin in drinking water. This was a great advantage for induction of infected *S.* Typhimurium. By doing this, it affected a decrease of the normal flora in GI tract as well as highly increasein susceptibility to *S.* Typhimurium infection. It can also induce diarrhea in mice as well. After an oral infection of *S.* Typhimurium, the animals were ill. The reduced activities had the soften stool and high moisture content.

The Mouse Model of *S.* Typhimurium Associated Diarrhea

The *Salmonella* diarrhea in mice is rarely understandable. Many previous studies usually inoculated *S.* Typhimurium in genetically susceptible mice (C57BL/6 and *BALB/c* mice). That resulted in histopathological and pro-inflammatory cytokines changes. H. Woo and colleague in 2008 [105] used BALB/c.D2^{Nramp1} mice inoculated with *S.* Typhimurium-induced diarrhea. The animal lost about 30% of their body weight change and got a change of histopathological. In 2010, Hiroki and his colleagues [73] inoculated *C57BL/6* mice with *S.* Typhimurium and investigated the effect of heat-killed *L. plantarum* on *S.* Typhimurium infection. These studies showed that C57BL/6 mice had susceptibility for *S.* Typhimurium infection. In addition, the study of Shahlaa M. Salih and Zahraa K. Zedan [77] showed that the oral infection with 0.5 x 10^5 CFU/mL of *S.* Typhimurium can be effective to an intestinal histopathological change. The histopathological section showed a necrosis, degenerative changes, inflammatory cells infiltration and damages of microvilli which is a cause of diarrhea.

In the preliminary study for developing mouse model of *Salmonella* infection in this study, C57BL/6, *BALB/c* and albino mice were oral infected with 10^3-10^5 CFU/mL of *S.* Typhimurium, but it was not able to induce diarrhea in these mice. The animals could do normal activities without showing symptoms of illness and changing of histopathological of intestine. However, when the high dose of *S.* Typhimurium (3 x 10^9 CFU/mL) was inoculated in miceit has an effective result for albino mice. The oral infection with 3 x 10^9 CFU/mL of *S.* Typhimurium for 3 days was able to induce diarrhea in a group of albino mice. The experiment resulted in the decrease of mice's activities; they had a symptom of an illness, spiky hair and soft stools. Thus, this experiment became a model for an acute infection of *S.* Typhimurium associating with diarrhea in mice.

The Changes of Body Weight

During the experimental process, the body weight of each mice was measured daily and the result showed no significant difference of body weight in S groups and S + LP group after they were administrated with the bacterial. In the control group, the body weight of mice tended to increase. On the other hand, in S group and S + LP group, the body weight of mice tended to decrease after they were infected with *S.* Typhimurium. The experiment of mice in the S group resulted in the decrease of mice's activities, sickness and spiky hair. It was possible that the symptoms were resulted from pathogen infection and trauma under the handling or technical feeding. The feeding process might cause an illness in the animals and reduce their intakes of food and water.

Effects of *L. plantarum* B7 Against *S.* Typhimurium

In vitro study, *L. plantarum* B7 grown on MRS broth, was spotted on the 20 mM glucose BHI agar. Then it was incubated in anaerobic condition at 37 °C; overlaid for 48 h with 10^7 *S.* Typhimurium; and incubated at 37 °C for 24 h in aerobic condition, (n=6). The result showed that *L. plantarum* B7 could inhibit the growth of *S.* Typhimurium as a clear zone was seen around the spot of *L. plantarum B7.* *In vitro* study demonstrated that *L. plantarum* B7 was probiotic which had an inhibitory effect against *S.* Typhimurium which was in agreement with the previous studies [65, 72, 106-109]. Moreover, *L. plantarum* B7 was probiotic which had effects on defending against pathogen infection with several mechanisms. Previous studies explained that *L. plantarum* B7 could reduce or inhibit growth of pathogen in virtue of producing antimicrobial substances or inhibitory substances such as organic acid, hydrogen peroxide, rueterin, diacetyl and bacteriocin [65]. These had adverse effects on the pathogen by showing an ability to kill, reduce or inhibit growth of pathogen directly.

Moreover, *L. plantarum* B7 could modulate and change the pH environment in the human gastrointestinal tract; it increased an acidic condition leading to the decline or inhibition of pathogen's survival rate and growth. In addition, *L. plantarum* could produce bacteriocin which inhibited the growth of Gram positive and Gram negative bacteria [78, 108, 109].

L. plantarum B7 as an Anti-infection of _S._ Typhimurium and Pathogenic Bacteria

In vivo study presented the properties of *L. plantarum* B7 as an anti-pathogenic bacteria in the mouse model. In this experiment, mice were orally administrated with 1 x 10^9 CFU *L. plantarum* B7 in 1 mL of 0.85 % saline. After 2 hours, the mice was orally infected with 3 x 10^9 CFU/mL *S.* Typhimurium in 0.85 % saline for 2 days. The results presented a significant decrease of *S*. Typhimurium colonies in feces which were determined by stool culturing and colony counting. Moreover, the decrease of *S.* Typhimurium could improve the diarrhea symptom or illness in the mice. Corresponding to the study of Shahlaa M. Salih and colleagues [77], it has also shown that the administration of *L. plantarum* 10^9 CFU/mL (0.1 mL) infecting with of 0.5 × 10^5 CFU/mL of *S*. Typhimurium (0.1 mL), can attenuate necrosis, degenerative changes and inflammatory cells infiltration of histological sections when it is compared to the group of *S*. Typhimurium infection. Being treated or pre-treated with *L. plantarum* can improve the histopathological effect of *S*. Typhimurium in mice. In addition to the study of Hiroki Ishikawa *et al.*[73] the manifested mice are orally administered with heat-killed *L. plantarum* b240 for 3 weeks; the result shows that *L. plantarum* b240 can protect against *S.* Typhimurium infection in mice. Moreover, the mechanism study shows that, *L. plantarum* b240 can inhibit the binding and invasion of *S*. Typhimurium into epithelial cells. It can also decrease the translocation of *S*. Typhimurium into other organs (payer's patch, mesenteric lymph node, spleen and liver). Furthermore, the

previously study reveals that *L. plantarum* is against pathogenic bacteria infection. In 2012, for example, Chompoonut S. *et al.*[18] demonstrates in vitro *L. plantarum* B7 that it can inhibit the growth of *H. pylori.* In vivo study, the mice were fed with 10^6 and 10^{10} CFU/mL of *L. plantarum* B7 after they were infected with *H. pylori* for one week.

The outcome of the study, presents that *L. plantarum* B7 can attenuate histopathology of *H. pylori* as it induces gastric inflammation with a dose dependent. The previously studies also show that *L. plantarum* has several mechanisms which are against the pathogenic bacteria infection. In a direct mechanism, *L. plantarum* can produce and secrete anti-microbial substances. *L. plantarum* can colonize and adhere to intestinal epithelial cells. These processes can decrease and block the binding sites of pathogen leading to the decline and prevention of pathogen infection. In addition, *L. plantarum* B7 can reduce a survival rate and disrupt the growth of pathogen with its effective capability of pre-biotics and nutrition. *L. plantarum* B7 is a friendly bacterial organism for human host. They can live and survive in human GI tract by providing benefits for human host; they can improve and modulate the commensal bacterial balance in GI tract and protect pathogen infection [14, 22, 62, 71]. In addition, *L. plantarum* can inhibit growth and prevent Gram positive and Gram negative bacterial infection (*in vitro* and *in vivo*) [78, 107, 108].It is also beneficial to food and medical industry.

L. plantarum B7 Alleviate Pro-inflammatory Cytokines

In this study, after the mice were fed with *L. plantarum* B7, the pro-inflammatory cytokines (TNF-α, IL-6 and CXCL1) significantly decreased. It is likewise another study that has also presented *L. plantarum* as an anti-inflammatory which can reduce pro-inflammatory cytokine [91, 110, 111].Furthermore, *in vitro* study of Wimonrat P. and the colleagues in 2008 [91], finds that *L. plantarum* B7 can reduce TNF-α 74.52%

in LPS-induced gastric epithelial cells. According to the study of Leon M. T. Dicks and Kim ten Doeschate [112], the rat were orally fed with 2 x 10^8 CFU *S.* Typhimurium as well as treated with 1 x 10^8 CFU *L. plantarum* 423 and *E. mundtii* ST4SA. These studies suggests that both *L. plantarum* 423 and *E. mundtii* ST4SA can alleviate the physical symptoms of *S.* Typhimurium infection. Additionally, these studies suggest that only being orally administered with *L. plantarum* 423 can make it more effective than *E. mundtii* ST4SA or combination of *L. plantarum* 423 and *E. mundtii* ST4SA. Corresponding to this study, S + LP group expresses the reduction of *S.* Typhimurium colonies. When it is compared to the S group, the alleviation of pro-inflammatory cytokine level in serum can reduce the fecal moisture contents and improve fecal characteristics which attenuate the physical symptom of *S.* Typhimurium infection. The protective effects of *L. plantarum* B7 on *S.* Typhimurium associated diarrhea in mice were proposed as described in Figure 5-22.

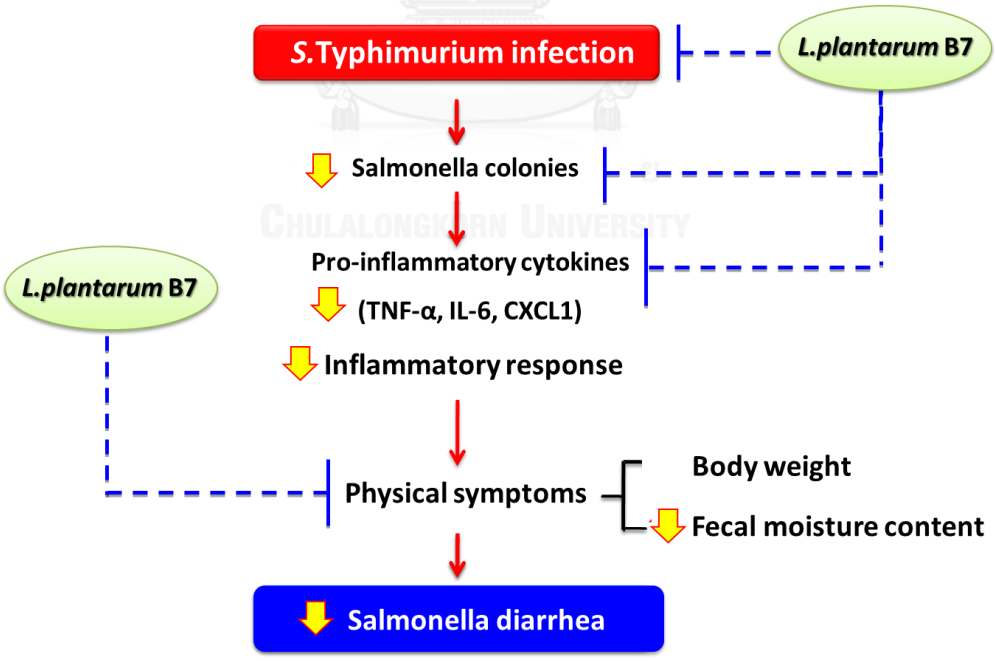

Figure 5-22: Summary of effect of *L. plantarum* B7 on *S.* Typhimurium associate diarrhea

Conclusion

The protective effects of *L. plantarum* B7 on inhibiting the growth and reducing *S.* Typhimurium infection; decreasing inflammatory response; and attenuating physical symptom of *S.* Typhimurium infection in mice, are examined in this study. The findings can be summarized as follows:

1. *In vitro* study, *L. plantarum* B7 can inhibit the growth of *S*. Typhimurium.
2. Oral administering with 3 x 10^9 CFU/mL of *S.* Typhimurium in mice for 3 days resulted in an elevation of pro- inflammatory cytokines (TNF-**α**, IL-6, CXCL1), an increase of the fecal moisture content percentage (%FMC) and changes of the fecal characteristics in mice (soft and loose fecal).
3. Oral administering with 1 x 10^9 CFU/mL of *L. plantarum* B7 resulted in the decrease of *S.* Typhimurium colonization, the alleviation of pro-inflammatory cytokines (TNF-**α**, IL-6, CXCL1) production and the decrease of fecal moisture content.

Overall, *L. plantarum* B7 is a potentially effective probiotic which can protect against *S.* Typhimurium infection. It can inhibit and reduce the growth of *S.* Typhimurium, decrease inflammatory cytokines (TNF-**α**, IL-6, CXCL1) production, reduce the fecal moisture content and improve the fecal characteristics which demonstrate the attenuation of the physical symptoms of *S.* Typhimurium associated diarrhea in mice. However, further research is required to clarify more precise mechanisms of protective effects of *L. plantarum* B7 on *S.* Typhimurium associated diarrhea in mice.

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APPENDIX

The number of *S.* Typhimurium in feces

Descriptives

StoolLog

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control group	8	.00000	.000000	.000000	.00000	.00000	.000	.000
S group	8	8.86463	.020000	.007071	8.84790	8.88135	8.834	8.891
S+LP group	8	7.41663	.054949	.019427	7.37069	7.46256	7.326	7.482
Total	24	5.42708	3.966433	.809645	3.75221	7.10196	.000	8.891

Multiple Comparisons

Dependent Variable: StoolLog

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control group	S group	-8.864625*	.016880	.000	-8.90717	-8.82208
	S+LP group	-7.416625*	.016880	.000	-7.45917	-7.37408
S group	Control group	8.864625*	.016880	.000	8.82208	8.90717
	S+LP group	1.448000*	.016880	.000	1.40545	1.49055
S+LP group	Control group	7.416625*	.016880	.000	7.37408	7.45917
	S group	-1.448000*	.016880	.000	-1.49055	-1.40545

*. The mean difference is significant at the .05 level.

StoolLog

Tukey HSD[a]

Group	N	Subset for alpha = .05		
		1	2	3
Control group	8	.00000		
S+LP group	8		7.41663	
S group	8			8.86463
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

Serum TNF-α

Descriptives

TNFalpha

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control group	8	53.48875	8.895728	3.145115	46.05174	60.92576	40.188	66.125
S group	8	128.58600	12.824279	4.534067	117.86463	139.30737	103.000	141.906
S+LP group	8	36.14513	9.218964	3.259396	28.43788	43.85237	25.500	50.500
Total	24	72.73996	42.184207	8.610815	54.92713	90.55279	25.500	141.906

ANOVA

TNFalpha

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38628.570	2	19314.285	176.340	.000
Within Groups	2300.098	21	109.528		
Total	40928.667	23			

Multiple Comparisons

Dependent Variable: TNFalpha

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control group	S group	-75.097250*	5.232792	.000	-88.28687	-61.90763
	S+LP group	17.343625*	5.232792	.009	4.15400	30.53325
S group	Control group	75.097250*	5.232792	.000	61.90763	88.28687
	S+LP group	92.440875*	5.232792	.000	79.25125	105.63050
S+LP group	Control group	-17.343625*	5.232792	.009	-30.53325	-4.15400
	S group	-92.440875*	5.232792	.000	-105.63050	-79.25125

*. The mean difference is significant at the .05 level.

TNFalpha

Tukey HSD[a]

Group	N	Subset for alpha = .05		
		1	2	3
S+LP group	8	36.14513		
Control group	8		53.48875	
S group	8			128.58600
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

Serum IL-6

Descriptives

IL6

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control group	8	66.5063	4.03595	1.42692	63.1321	69.8804	59.88	71.88
S group	8	144.4363	8.90884	3.14975	136.9883	151.8842	132.63	155.38
S+LP group	8	70.3594	5.36867	1.89811	65.8711	74.8477	59.86	76.80
Total	24	93.7673	37.14768	7.58274	78.0812	109.4534	59.86	155.38

ANOVA

IL6

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30867.506	2	15433.753	371.961	.000
Within Groups	871.352	21	41.493		
Total	31738.859	23			

Multiple Comparisons

Dependent Variable: IL6

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control group	S group	-77.93000*	3.22075	.000	-86.0481	-69.8119
	S+LP group	-3.85313	3.22075	.468	-11.9713	4.2650
S group	Control group	77.93000*	3.22075	.000	69.8119	86.0481
	S+LP group	74.07688*	3.22075	.000	65.9587	82.1950
S+LP group	Control group	3.85313	3.22075	.468	-4.2650	11.9713
	S group	-74.07688*	3.22075	.000	-82.1950	-65.9587

*. The mean difference is significant at the .05 level.

IL6

Tukey HSD[a]

Group	N	Subset for alpha = .05	
		1	2
Control group	8	66.5063	
S+LP group	8	70.3594	
S group	8		144.4363
Sig.		.468	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

Serum CXCL1

Descriptives

CXCL1

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control group	8	32.31700	4.536163	1.603776	28.52467	36.10933	25.756	39.073
S group	8	96.09450	10.811452	3.822425	87.05590	105.13310	77.610	109.561
S+LP group	8	35.39913	2.774436	.980911	33.07964	37.71861	30.780	39.439
Total	24	54.60354	30.724672	6.271647	41.62965	67.57743	25.756	109.561

ANOVA

CXCL1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20695.993	2	10347.997	213.858	.000
Within Groups	1016.132	21	48.387		
Total	21712.126	23			

Multiple Comparisons

Dependent Variable: CXCL1

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control group	S group	-63.777500*	3.478047	.000	-72.54416	-55.01084
	S+LP group	-3.082125	3.478047	.655	-11.84879	5.68454
S group	Control group	63.777500*	3.478047	.000	55.01084	72.54416
	S+LP group	60.695375*	3.478047	.000	51.92871	69.46204
S+LP group	Control group	3.082125	3.478047	.655	-5.68454	11.84879
	S group	-60.695375*	3.478047	.000	-69.46204	-51.92871

*. The mean difference is significant at the .05 level.

CXCL1

Tukey HSD[a]

Group	N	Subset for alpha = .05	
		1	2
Control group	8	32.31700	
S+LP group	8	35.39913	
S group	8		96.09450
Sig.		.655	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

Fecal moisture content

Descriptives

FMC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control group	8	14.19250	1.570630	.555302	12.87942	15.50558	11.950	17.060
S group	8	43.24000	2.046377	.723503	41.52919	44.95081	39.380	45.850
S+LP group	8	24.65250	2.084478	.736974	22.90983	26.39517	21.690	27.790
Total	24	27.36167	12.406347	2.532435	22.12293	32.60041	11.950	45.850

ANOVA

FMC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3463.104	2	1731.552	472.259	.000
Within Groups	76.997	21	3.667		
Total	3540.101	23			

Multiple Comparisons

Dependent Variable: FMC

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control group	S group	-29.047500*	.957409	.000	-31.46072	-26.63428
	S+LP group	-10.460000*	.957409	.000	-12.87322	-8.04678
S group	Control group	29.047500*	.957409	.000	26.63428	31.46072
	S+LP group	18.587500*	.957409	.000	16.17428	21.00072
S+LP group	Control group	10.460000*	.957409	.000	8.04678	12.87322
	S group	-18.587500*	.957409	.000	-21.00072	-16.17428

*. The mean difference is significant at the .05 level.

FMC

Tukey HSD[a]

Group	N	Subset for alpha = .05		
		1	2	3
Control group	8	14.19250		
S+LP group	8		24.65250	
S group	8			43.24000
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.000.

Body weigh

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
BW_1	Control group	8	31.7500	1.66905	.59010	30.3546	33.1454	30.00	34.00
	S group	8	32.5000	1.41421	.50000	31.3177	33.6823	30.00	34.00
	S+LP group	8	33.2500	1.03510	.36596	32.3846	34.1154	32.00	34.00
	Total	24	32.5000	1.47442	.30096	31.8774	33.1226	30.00	34.00
BW_2	Control group	8	33.2500	1.03510	.36596	32.3846	34.1154	32.00	34.00
	S group	8	32.7500	1.03510	.36596	31.8846	33.6154	32.00	34.00
	S+LP group	8	33.2500	1.03510	.36596	32.3846	34.1154	32.00	34.00
	Total	24	33.0833	1.01795	.20779	32.6535	33.5132	32.00	34.00
BW_3	Control group	8	34.0000	1.06904	.37796	33.1063	34.8937	32.00	36.00
	S group	8	34.0000	.00000	.00000	34.0000	34.0000	34.00	34.00
	S+LP group	8	34.7500	1.03510	.36596	33.8846	35.6154	34.00	36.00
	Total	24	34.2500	.89685	.18307	33.8713	34.6287	32.00	36.00
BW_4	Control group	8	34.7500	1.03510	.36596	33.8846	35.6154	34.00	36.00
	S group	8	34.7500	1.03510	.36596	33.8846	35.6154	34.00	36.00
	S+LP group	8	35.2500	1.03510	.36596	34.3846	36.1154	34.00	36.00
	Total	24	34.9167	1.01795	.20779	34.4868	35.3465	34.00	36.00
BW_5	Control group	8	35.2500	1.03510	.36596	34.3846	36.1154	34.00	36.00
	S group	8	35.0000	1.06904	.37796	34.1063	35.8937	34.00	36.00
	S+LP group	8	35.5000	.92582	.32733	34.7260	36.2740	34.00	36.00
	Total	24	35.2500	.98907	.20189	34.8324	35.6676	34.00	36.00
BW_6	Control group	8	36.0000	1.06904	.37796	35.1063	36.8937	34.00	38.00
	S group	8	33.2500	1.48805	.52610	32.0060	34.4940	32.00	36.00
	S+LP group	8	34.0000	1.06904	.37796	33.1063	34.8937	32.00	36.00
	Total	24	34.4167	1.66594	.34006	33.7132	35.1201	32.00	38.00
BW_7	Control group	8	36.7500	1.03510	.36596	35.8846	37.6154	36.00	38.00
	S group	8	31.7500	1.66905	.59010	30.3546	33.1454	30.00	34.00
	S+LP group	8	33.0000	1.06904	.37796	32.1063	33.8937	32.00	34.00
	Total	24	33.8333	2.49637	.50957	32.7792	34.8875	30.00	38.00
BW_8	Control group	8	38.0000	1.51186	.53452	36.7361	39.2639	36.00	40.00
	S group	8	31.2500	1.48805	.52610	30.0060	32.4940	30.00	34.00
	S+LP group	8	32.2500	1.28174	.45316	31.1784	33.3216	30.00	34.00
	Total	24	33.8333	3.33188	.68012	32.4264	35.2403	30.00	40.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BW_1	Between Groups	9.000	2	4.500	2.305	.124
	Within Groups	41.000	21	1.952		
	Total	50.000	23			
BW_2	Between Groups	1.333	2	.667	.622	.546
	Within Groups	22.500	21	1.071		
	Total	23.833	23			
BW_3	Between Groups	3.000	2	1.500	2.032	.156
	Within Groups	15.500	21	.738		
	Total	18.500	23			
BW_4	Between Groups	1.333	2	.667	.622	.546
	Within Groups	22.500	21	1.071		
	Total	23.833	23			
BW_5	Between Groups	1.000	2	.500	.488	.620
	Within Groups	21.500	21	1.024		
	Total	22.500	23			
BW_6	Between Groups	32.333	2	16.167	10.778	.001
	Within Groups	31.500	21	1.500		
	Total	63.833	23			
BW_7	Between Groups	108.333	2	54.167	32.500	.000
	Within Groups	35.000	21	1.667		
	Total	143.333	23			
BW_8	Between Groups	212.333	2	106.167	51.849	.000
	Within Groups	43.000	21	2.048		
	Total	255.333	23			

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
BW_1	Control group	S group	-.75000	.69864	.540	-2.5110	1.0110
		S+LP group	-1.50000	.69864	.104	-3.2610	.2610
	S group	Control group	.75000	.69864	.540	-1.0110	2.5110
		S+LP group	-.75000	.69864	.540	-2.5110	1.0110
	S+LP group	Control group	1.50000	.69864	.104	-.2610	3.2610
		S group	.75000	.69864	.540	-1.0110	2.5110
BW_2	Control group	S group	.50000	.51755	.606	-.8045	1.8045
		S+LP group	.00000	.51755	1.000	-1.3045	1.3045
	S group	Control group	-.50000	.51755	.606	-1.8045	.8045
		S+LP group	-.50000	.51755	.606	-1.8045	.8045
	S+LP group	Control group	.00000	.51755	1.000	-1.3045	1.3045
		S group	.50000	.51755	.606	-.8045	1.8045
BW_3	Control group	S group	.00000	.42956	1.000	-1.0827	1.0827
		S+LP group	-.75000	.42956	.212	-1.8327	.3327
	S group	Control group	.00000	.42956	1.000	-1.0827	1.0827
		S+LP group	-.75000	.42956	.212	-1.8327	.3327
	S+LP group	Control group	.75000	.42956	.212	-.3327	1.8327
		S group	.75000	.42956	.212	-.3327	1.8327
BW_4	Control group	S group	.00000	.51755	1.000	-1.3045	1.3045
		S+LP group	-.50000	.51755	.606	-1.8045	.8045
	S group	Control group	.00000	.51755	1.000	-1.3045	1.3045
		S+LP group	-.50000	.51755	.606	-1.8045	.8045
	S+LP group	Control group	.50000	.51755	.606	-.8045	1.8045
		S group	.50000	.51755	.606	-.8045	1.8045
BW_5	Control group	S group	.25000	.50592	.875	-1.0252	1.5252
		S+LP group	-.25000	.50592	.875	-1.5252	1.0252
	S group	Control group	-.25000	.50592	.875	-1.5252	1.0252
		S+LP group	-.50000	.50592	.592	-1.7752	.7752
	S+LP group	Control group	.25000	.50592	.875	-1.0252	1.5252
		S group	.50000	.50592	.592	-.7752	1.7752
BW_6	Control group	S group	2.75000*	.61237	.001	1.2065	4.2935
		S+LP group	2.00000*	.61237	.010	.4565	3.5435
	S group	Control group	-2.75000*	.61237	.001	-4.2935	-1.2065
		S+LP group	-.75000	.61237	.452	-2.2935	.7935
	S+LP group	Control group	-2.00000*	.61237	.010	-3.5435	-.4565
		S group	.75000	.61237	.452	-.7935	2.2935
BW_7	Control group	S group	5.00000*	.64550	.000	3.3730	6.6270
		S+LP group	3.75000*	.64550	.000	2.1230	5.3770
	S group	Control group	-5.00000*	.64550	.000	-6.6270	-3.3730
		S+LP group	-1.25000	.64550	.153	-2.8770	.3770
	S+LP group	Control group	-3.75000*	.64550	.000	-5.3770	-2.1230
		S group	1.25000	.64550	.153	-.3770	2.8770
BW_8	Control group	S group	6.75000*	.71548	.000	4.9466	8.5534
		S+LP group	5.75000*	.71548	.000	3.9466	7.5534
	S group	Control group	-6.75000*	.71548	.000	-8.5534	-4.9466
		S+LP group	-1.00000	.71548	.360	-2.8034	.8034
	S+LP group	Control group	-5.75000*	.71548	.000	-7.5534	-3.9466
		S group	1.00000	.71548	.360	-.8034	2.8034

*. The mean difference is significant at the .05 level.

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