ผลของแลคโตบาซิลลัส แพลนทารัม บี 7 ต่อระดับทีเอ็นเอฟ-แอลฟา อินเตอร์ลิวคิน-1 เบต้า ในซีรัม ลิพิดเปอร์ออกซิเดชัน และการเกิดอะโพโทซิสในเนื้อเยื่อกระเพาะอาหารของหนูแรทที่ติดเชื้อเฮลิโคแบคเตอร์ ไพลอริ

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Effects of *Lactobacillus plantarum* B7 on Serum TNF-α and IL-1β Level, Lipid Peroxidation and Apoptosis of Gastric Mucosa in Rats Infected with *Helicobacter pylori*.

Miss Chompoonut Sunanliganon

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Thesis Title	EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON SERUM TNF-α AND IL-1β LEVEL, LIPID PEROXIDATION AND APOPTOSIS OF GASTRIC MUCOSA IN RATS INFECTED WITH *HELICOBACTER PYLORI*.
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ชมพูนุท สุนันท์ลิกานนท์ : ผลของแลคโตบาซิลลัส แพลนทารัม บี 7 ต่อระดับทีเอ็นเอฟ-แอลฟา อินเตอร์ลิวคิน-1 เบต้า ในซีรัม ลิพิดเปอร์ออกซิเดชัน และการเกิดอะโพโทซิสในเนื้อเยื่อกระเพาะอาหารของหนูแรทที่ติดเชื้อเฮลิโคแบคเตอร์ ไพลอริ. (Effects of *Lactobacillus plantarum* B7 on serum TNF-α and IL-1β level, lipid peroxidation and apoptosis of gastric mucosa in rats infected with *Helicobacter pylori*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.พญ. ดวงพร ทองงาม, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร. สมหญิง ธัมวาสร, 109 หน้า.

การติดเชื้อเฮลิโคแบคเตอร์ ไพลอริเป็นสาเหตุของกระเพาะอาหารอักเสบ และการหลั่งสารที่เกี่ยวข้องกับการอักเสบ แลคโตบาซิลลัส แพลนทารัม บี 7 มีความสามารถในการยับยั้งการหลั่งของทีเอ็นเอฟ-แอลฟาที่เหนี่ยวนำโดยแอลพีเอสของอีโคไลได้มากที่สุดในหลอดทดลอง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการต่อต้านเฮลิโคแบคเตอร์ ไพลอริ ของสารที่หลั่งจากแลคโตบาซิลลัส แพลนทารัม บี 7 ในหลอดทดลอง และผลของการติดเชื้อเฮลิโคแบคเตอร์ ไพลอริ และผลในการต่อต้านการอักเสบของแลคโตบาซิลลัส แพลนทารัม บี 7 ที่ความเข้มข้นต่างๆ ต่อการเปลี่ยนแปลงของระดับทีเอ็นเอฟ-แอลฟา อินเตอร์ลิวคิน-วัน เบต้า ในซีรัม, ผลพยาธิวิทยา, ระดับของเอ็มดีเอ และการเกิดอะโพโทซิสในเนื้อเยื่อกระเพาะอาหารของหนูแรท การทดลองนี้แบ่งออกเป็น 2 ส่วน ส่วนแรก คือการศึกษาการยับยั้งการเจริญเติบโตของเฮลิโคแบคเตอร์ ไพลอริในหลอดทดลอง โดยใช้ supernatant ทั้งที่ไม่ได้ปรับ (pH ดั้งเดิม) และปรับ pH ให้เท่ากับ 7 ของ แลคโตบาซิลลัส แพลนทารัม บี 7 ที่ความเข้มข้น 1, 5 และ 10 เท่า วางลงบน plate ที่เพาะเชื้อเฮลิโคแบคเตอร์ ไพลอริไว้ และทำการแปลผลการยับยั้งการเจริญเติบโตของเฮลิโคแบคเตอร์ ไพลอริ โดยดูจากขนาดของ inhibition zone ส่วนที่ 2 ทำการทดลองโดยใช้หนูแรทเพศผู้สายพันธุ์ Spraque-Dawley แบ่งออกเป็น 4 กลุ่ม คือ กลุ่มควบคุม กลุ่มติดเชื้อ กลุ่มติดเชื้อที่ได้รับแลคโตบาซิลลัส แพลนทารัม บี 7 ความเข้มข้น 10^6 CFUs/mL และ กลุ่มติดเชื้อที่ได้รับแลคโตบาซิลลัส แพลนทารัม บี 7 ความเข้มข้น 10^{10} CFUs/mL หลังติดเชื้อนาน 1 สัปดาห์ หนูในกลุ่มที่ได้รับการรักษาจะถูกป้อนแลคโตบาซิลลัส แพลนทารัม บี 7 ความเข้มข้นดังกล่าวทุกวัน วันละ 1 ครั้งเป็นเวลา 1 สัปดาห์ เมื่อสิ้นสุดการทดลอง ทำการเก็บตัวอย่างเลือดและกระเพาะอาหาร ผลการทดลองในส่วนแรกพบว่าสารที่หลั่งจากแลคโตบาซิลลัส แพลนทารัม บี 7 สามารถยับยั้งการเจริญเติบโตของเฮลิโคแบคเตอร์ ไพลอริได้ตามระดับความเข้มข้นที่เพิ่มขึ้น และใน pH ดั้งเดิมสามารถให้ผลการยับยั้งที่มากกว่า pH ปรับให้เท่ากับ 7 และผลการทดลองในส่วนที่ 2 พบว่าในกลุ่มที่ติดเชื้อพบพยาธิสภาพของกระเพาะอาหารมีการ colonization ของเชื้อและการอักเสบของกระเพาะอาหารอยู่ในระดับน้อยถึงปานกลาง ระดับมาลอนไดอัลดีไฮด์ในกระเพาะอาหาร และการตายของเซลล์กระเพาะอาหารแบบอะโพโทซิสสูงขึ้นอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม แต่ไม่พบการเปลี่ยนแปลงของระดับทีเอ็นเอฟ-แอลฟา และอินเตอร์ลิวคิน-วัน เบต้า ในซีรัมเมื่อเทียบกับกลุ่มควบคุม ส่วนกลุ่มที่ได้รับแลคโตบาซิลลัส แพลนทารัม บี 7 พบพยาธิสภาพของกระเพาะอาหารดีขึ้น ลดระดับของทีเอ็นเอฟ-แอลฟาในซีรัม ลดระดับของมาลอนไดอัลดีไฮด์ในกระเพาะอาหาร และลดการตายของเซลล์กระเพาะอาหารแบบอะโพโทซิส และมีแนวโน้มในการลดระดับอินเตอร์ลิวคิน-วัน เบต้าในซีรัม สรุปผลการทดลองแลคโตบาซิลลัส แพลนทารัม บี 7 สามารถลดการอักเสบของกระเพาะอาหารที่เกิดจากการติดเชื้อเฮลิโคแบคเตอร์ไพลอริ โดยการช่วยให้พยาธิสภาพของกระเพาะอาหารดีขึ้น ลดระดับทีเอ็นเอฟ-แอลฟาในซีรัม ลดภาวะออกซิเดทีฟสเตรส ลดการตายของเซลล์กระเพาะอาหารแบบอะโพโทซิส และอาจจะมีแนวโน้มลดระดับอินเตอร์ลิวคิน-วัน เบต้าในซีรัม และผลที่เกิดเหล่านี้อาจเกี่ยวข้องกับสารที่หลั่งจากแลคโตบาซิลลัส แพลนทารัม บี 7 ในการศึกษาในหลอดทดลอง

สาขาวิชา.....สรีรวิทยา.....ลายมือชื่อนิสิต ชมพูนุท สุนันท์ลิกานนท์

ปีการศึกษา.....2553.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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CHOMPOONUT SUNANLIGANON : EFFECTS OF *LACTOBACILLUS PLANTARUM* B7 ON SERUM TNF-α AND IL-1β LEVEL, LIPID PEROXIDATION AND APOPTOSIS OF GASTRIC MUCOSA IN RATS INFECTED WITH *HELICOBACTER PYLORI*.

ADVISOR : ASSOC.PROF. DUANGPORN THONG-NGAM, M.D.,

CO-ADVISOR : ASSOC.PROF. SOMYING TUMWASORN, Ph.D., 109 pp.

The *H. pylori* infection causes gastric inflammation and the release of inflammatory mediators. *L. plantarum* B7 show the highest *E. coli* LPS-induced TNF-α inhibitory activity *in vitro*. The present study aims to determine anti-*Helicobacter* activity of *L. plantarum* B7 supernatants *in vitro*, and effects of *H. pylori* infection and anti-inflammatory effect of *L. plantarum* B7 doses on serum TNF-α and IL-1β level, histopathology of gastric mucosa, gastric malondialdehyde (MDA) level and apoptosis in gastric epithelial cells in rats. The experiments were divided into two parts. First, *in vitro* inhibition of *H. pylori* growth was examined by using *L. plantarum* B7 supernatants pH 4 and pH 7 at the concentration of 1X, 5X and 10X on plates that were inoculated with *H. pylori*. The inhibitory effect of *H. pylori* was interpreted by size of the inhibition zone. Secondary, male Spraque-Dawley rats were divided into four groups including control, *H. pylori* infected, *H. pylori* infected with *L. plantarum* B7 10^6 CFUs/mL treated, and *H. pylori* infected with *L. plantarum* B7 10^{10} CFUs/mL treated groups. One week after *H. pylori* inoculation, *L. plantarum* B7 10^6 CFUs/mL or 10^{10} CFUs/mL were fed once daily to *L. plantarum* B7 treated groups for one week. Rats were sacrificed and blood and gastric samples were collected at the end of the study. *In vitro* experiment, *L. plantarum* B7 supernatants inhibited *H. pylori* growth in a dose-dependent manner and better at intact pH 4. *In vivo* study, in *H. pylori* infected group, the stomach histopathology revealed mild to moderate *H. pylori* colonization and inflammation. Level of gastric MDA and epithelial cell apoptosis increased significantly when compared with control group. However, there were no significant change of serum TNF-α and IL-1β level compared with control group. *L. plantarum* B7 treatments resulted in improving stomach pathology, decreasing of serum TNF-α level, gastric MDA level and apoptotic epithelial cells, and providing a trend of decreased IL-1β concentration. In conclusion, *L. plantarum* B7 could attenuate *H. pylori*-induced gastric inflammation by the improvement of stomach pathology, and reduction of serum TNF-α level, oxidative stress and gastric epithelial cell apoptosis. Moreover, *L. plantarum* B7 might have a trend to decrease IL-1β concentration. These effects might be involved in the secreted substance of *L. plantarum* B7 from *in vitro* study.

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CONTENTS

PAGE

ABSTRACT (THAI)..... iv

ABSTRACT (ENGLISH)..... v

ACKNOWLEDGEMENTS..... vi

CONTENTS..... vii

LIST OF TABLES..... ix

LIST OF FIGURES..... x

LIST OF ABBREVIATIONS..... xii

CHAPTER I BACKGROUND AND RATIONALE 1

CHAPTER II LITERATURE REVIEWS..... 3

The stomach..... 3

Helicobacter pylori (H. pylori)..... 6

TNF-α and IL-1β..... 17

Oxidative stress..... 19

Cell death..... 26

Lactobacillus plantarum..... 32

The model used in this study..... 38

CHAPTER III MATERIALS AND METHODS..... 39

Chemical substances..... 39

In vitro study..... 40

Disk diffusion method..... 45

In vivo study..... 46

Bacteria and animals preparation..... 46

H. pylori inoculation in the rat stomach..... 48

Detection of *H. pylori* infection in gastric tissues..... 48

TNF-α and IL-1β assay (ELISA) 49

Gastric Malondialdehyde (MDA) Determination..... 54

PAGE

BCA Protein Assay……..…… 56
Gastric apoptosis determination…….. 58
Statistical analysis……...… 59
CHAPTER IV RESULTS…….. 60
In vitro study……. 60
Anti-*H. pylori* activity of *L. plantarum* B7 supernatants……. 60
In vivo study…….. 64
H. pylori infection in rat stomach……... 64
Effects of *H. pylori* infection on serum TNF-α level and role of *L. plantarum* B7……..…….. 70
Effects of *H. pylori* infection on serum IL-1β level and role of *L. plantarum* B7…… 72
Effects of *H. pylori* infection on lipid peroxidation in gastric tissue and role of *L. plantarum* B7…… 74
Effects of *H. pylori* infection on apoptosis in gastric tissue and role of *L. plantarum* B7…… 76
CHAPTER V DISCUSSIONS……..… 79
CHAPTER VI CONCLUSIONS…….. 88
REFERENCES…….. 89
BIOGRAPHY…… 109

LIST OF TABLES

TABLE		PAGE
1	*H. pylori* virulence factors and their effects……..……..	9
2	Preparation of TNF-α concentration (pg/mL)……	50
3	Preparation of IL-1β concentration (pg/mL)……..……	51
4	Preparation of MDA concentration (μM)……..	54
5	Preparation of BCA concentration (μg/mL)……..…..	56
6	Means ± SD of inhibition zone diameters (mm) of all *L. plantarum* B7 supernatant concentrations at intact pH 4 and adjusted pH 7……	61
7	Summary of the scores of the bacterial colonization levels and gastric inflammation in all groups…….……..	67
8	Means ± SD of serum TNF-α level (pg/mL) in all groups……..	70
9	Means ± SD of serum IL-1β level (pg/mL) in all groups……	72
10	Means ± SD of gastric MDA level (nmol/mg protein) in all groups…….	74
11	Means ± SD of apoptotic cells (%) in all groups……...	76

LIST OF FIGURES

FIGURE		PAGE
1	Anatomy of stomach……...	4
2	Structure of gastric wall……..	5
3	Characteristic of *H. pylori*……	6
4	*H. pylori* coccoid form……	7
5	Pathogenesis of *H. pylori* infection……..	16
6	The various phases of the lipid peroxidation…….	20
7	The products and pathways relating to lipid peroxidation……...	21
8	Direct and indirect stimulation of neutrophil chemotaxis and activation in *H. pylori* infection……..	24
9	Pathway of neutrophil (PMN) stimulation following initial *H. pylori* infection……	25
10	Morphology of an apoptotic trophoblast cell……..	26
11	Summary of apoptotic signaling pathways…….	28
12	Structure of *L. plantarum*……...	32
13	*H. pylori* colonies on blood agar plate……...	41
14	*L. plantarum* B7 colonies on MRS agar plate…….	41
15	Preparation of *L. plantarum* B7 supernatant……..	42
16	Adjustment of pH and concentration of *L. plantarum* B7 supernatant..	43
17	Preparation of paper disks……	44
18	Disk diffusion method…….	45
19	Diagram of experimental groups…….	47
20	Rapid urease test……	48
21	A standard curve of TNF-α……	52
22	A standard curve of IL-1β…….	53
23	A standard curve of MDA……..	55
24	A standard curve of BCA……...	57

FIGURE		PAGE
25	A bar graph shows the mean ± SD of inhibitory zone diameters (mm) of all *L. plantarum* B7 supernatant concentrations at intact pH 4 and adjusted pH 7……	61
26	Inhibition zone of all concentration of *L. plantarum* B7 supernatant disks at intact pH 4……	62
27	Inhibition zone of all concentration of *L. plantarum* B7 supernatant disks at adjusted pH 7……	63
28	Histopathology of control group (x20)……..……...	65
29	Histopathology of *H. pylori* infected group (x20) and (x40)…….	66
30	Hematoxylin-eosin stained gastric sections (x40)…….	68
31	Hematoxylin-eosin stained gastric sections (x20)…….	69
32	A bar graph shows the mean ± SD of serum TNF-α level (pg/ml) in all groups……	71
33	A bar graph shows the mean ± SD of serum IL-1β level (pg/mL) in all groups……	73
34	A bar graph shows the mean ± SD of gastric MDA levels (nmol/mg protein) in all groups……...	75
35	A bar graph shows the mean ± SD of apoptotic cells (%) in all groups……..……	77
36	Representative gastric sections processed for apoptosis assay by TUNEL reaction (x20)…….	78

LIST OF ABBREVIATIONS

AP-1	=	Activator protein-1
^{o}C	=	Degree Celsius
CagA	=	Cytotoxin-associated gene product A
cag PAI	=	*cag* Pathogenicity Island gene
CAT	=	catalase
CFUs/mL	=	Colony forming units per milliliter
CO_2	=	Carbondioxide
DAB	=	Diaminobenzidine
ELISA	=	Enzyme-linked immunosorbent assay
H&E	=	Hematoxylin and Eosin
H_2O_2	=	Hydrogen peroxide
H. pylori	=	*Helicobacter pylori*
ICAM-1	=	Intercellular adhesion molecule-1
IFN-γ	=	Interferon-gamma
IL-1β	=	Interleukin-1 beta
IL-12	=	Interleukin-12
iNOS	=	Inducible nitric oxide synthase
LPS	=	Lipopolysaccharide
L. plantarum B7	=	*Lactobacillus plantarum* B7
MDA	=	Malondialdehyde
mm	=	Millimeter
NaOH	=	Sodium hydroxide
NAP	=	Neutrophil-activating protein
NF-ĸB	=	Nuclear factor-kappa B
nm	=	Nanometer
nmol/mg protein	=	Nanomole per milligram of protein
NO^{-}	=	Nitric oxide
O_2^{-}	=	Superoxide radical
O.D.	=	Optical density
OH^{-}	=	Hydroxyl radical

pg/mL	=	Picogram per milliliter
PBS	=	Phosphate buffer saline
PUFAs	=	polyunsaturated fatty acids
ROS	=	Reactive oxygen species
SD	=	Standard deviation
SOD	=	Superoxide dismutase
TBARS	=	Thiobarbituric acid reactive substances
TdT	=	Terminal deoxynucleotidyl transferase
TNF-α	=	Tumor necrosis factor-alpha
TUNEL	=	TdT-mediated X-dUTP nick end labeling
VacA	=	Vacuolation cytotoxin A
µg/mL	=	Microgram per milliliter
µL	=	Microliter
µL/cm^2	=	Microliter per square centimeter
µm	=	Micrometer
µM	=	Micromolar

CHAPTER I

BACKGROUND AND RATIONALE

Helicobacter pylori is a gram-negative, spiral shaped bacterium that has the unique ability to colonize the human gastric mucosa, and infects more than half of the world's population. *H. pylori* causes chronic gastritis, plays an etiologic role in the development of peptic ulcer disease, and is considered a risk factor in the development of gastric cancer and gastric lymphoma [1]. In 1994, *H. pylori* was classified into type I carcinogen by World Health Organization (WHO).

H. pylori infection is transferred by fecal-oral transmission and oral-oral transmission [2]. Epidemiology showed a correlation with low socioeconomic status and a high rate of *H. pylori* infection. In developed countries, 40% of the adult population is infected. In developing countries, the rate is as high as 80% [3]. The rate of carriage increases according to age group. Infection is usually acquired during childhood and persists lifelong if not treated specifically. Triple therapy, which combines a proton pump inhibitor or ranitidine bismuth citrate with two antibiotics, is recommended treatment for *H. pylori* infection.

Nowadays, *H. pylori* eradication is suboptimal because current treatment regimens result in adverse side effects, poor compliance, and an increasing prevalence of antibiotic resistance [4]. Therefore, alternative treatments are interesting for *H. pylori* eradication.

Lactobacillus is a gram-positive bacterium that has been detected in diverse environments. It is known as microflora, microorganism which normally inhabits the healthy human body and not disease causing, and used in the production of fermented foods and beverages. *Lactobacillus* is one of probiotic bacteria which probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [5]. The most commonly used organisms in probiotic products belongs to *Lactobacillus* sp., and *Bifidobacterium* sp. [6]. The species *L. plantarum* is commonly found in the human gastrointestinal tract (GI-tract). It is important in the production of a variety of fermented foods such as sauerkraut, Korean kimchi, cheese, sausages and stockfish, and also used as probiotics. Additionally, there is increasing evidence that *L. plantarum* has anti-*Helicobacter* activity [7, 8]

and show the highest *E. coli* LPS-induced TNF-α inhibitory activity *in vitro* [9]. Importantly, *L. plantarum* is acid and bile tolerant, survival passage through the GI-tract, and safe for human and animals [10].

Nowadays, there is rare research in effects of *L. plantarum* on *H. pylori*-induced gastric inflammation. Therefore, this present study will examine the effects of *L. plantarum* B7 on pathophysiology of *H. pylori* infection by monitoring histopathological change in gastric tissue, serum TNF-α and IL-1β level, gastric MDA level, and gastric epithelial cell apoptosis. Moreover, *in vitro* study, an anti-*Helicobacter* activity of *L. plantarum* B7 supernatants was investigated by a disk diffusion method.

CHAPTER II

LITERATURE REVIEWS

The Stomach

The stomach (figure 1) is a muscular J-shaped organ of the digestive tract lining between the esophagus and the duodenum. It is located in the upper left quadrant of the abdomen, just beneath the diaphragm. The concavity of the right, inner curve is called the lesser curvature and the convexity of the left, outer curve is the greater curvature. Its principal function is mechanically and chemically food digestion. The stomach can be divided into several regions on the basis of structure and function. The cardia is a small region just distal to the lower esophageal sphincter that does not secrete acid. The fundus consists of the dome of the stomach that lies above an imaginary horizontal line through the diaphragmatic pinch. The corpus, or body, is the main part of the stomach. Gastric glands in the corpus contain parietal cells, which secrete hydrochloric acid and intrinsic factor, and chief cells, which secrete pepsinogen [11]. The corpus is a reservoir that is a major site of gastric digestion. The pyloric region is subdivided into antrum, pyloric canal, and pyloric sphincter. This region is the distal end of the stomach that contains gastrin - secreting G cells and somatostatin - secreting D cells that sense pH and secrete hormones to regulate acid production in the corpus. All regions of the stomach secrete mucus and bicarbonate.

H. pylori grows predominantly in the antral region of the stomach, along the surface of gastric pit [12]. *H. pylori* – induced gastric inflammation is occurred overall stomach.

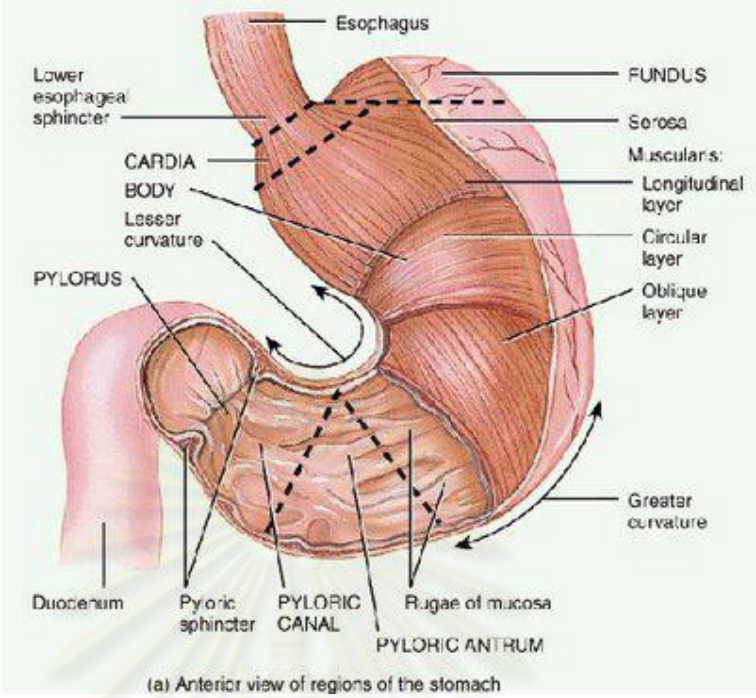


Figure 1 Anatomy of stomach.

The wall of the stomach has four layers (figure 2) consisting of mucosa, submucosa, muscularis propria and serosa together with gastric vessels and nerves [13].

The mucosa, surface of the stomach, consists of an epithelium cover the surface and lines the pits and glands, surrounding connective tissues called the lamina propria, and muscularis mucosae. In the stomach mucosa are folded into numerous folds or rugae. The epithelium contains types of cells with secrete compounds to aid digestion and stomach protection. The lamina propria contains the supporting framework, such as nerve fibers and capillary network, for epithelial cells. The muscularis mucosae, forms the inferior margin of the mucosa and separates of mucosa from submucosa, is thin layer of smooth muscle fibers lying external to the layer of gland [13].

The submucosa, the layer under the mucosa, is consists of the loose connectives tissues which are embedded blood vessels, lymphatic vessels and scattered mononuclear cells including mast cells.

The muscularis propria, the thick muscle coat, is comprised of three layers of smooth muscle: inner oblique layer, middle circular layer and outer longitudinal layer [13]. The nerves and ganglion cells are located between the middle circular and outer longitudinal muscle layers.

The serosa, the outermost layer of the stomach, is a thin covering of loose connective tissues with blood vessels, lymphatic vessels, and nerve fibers [13].

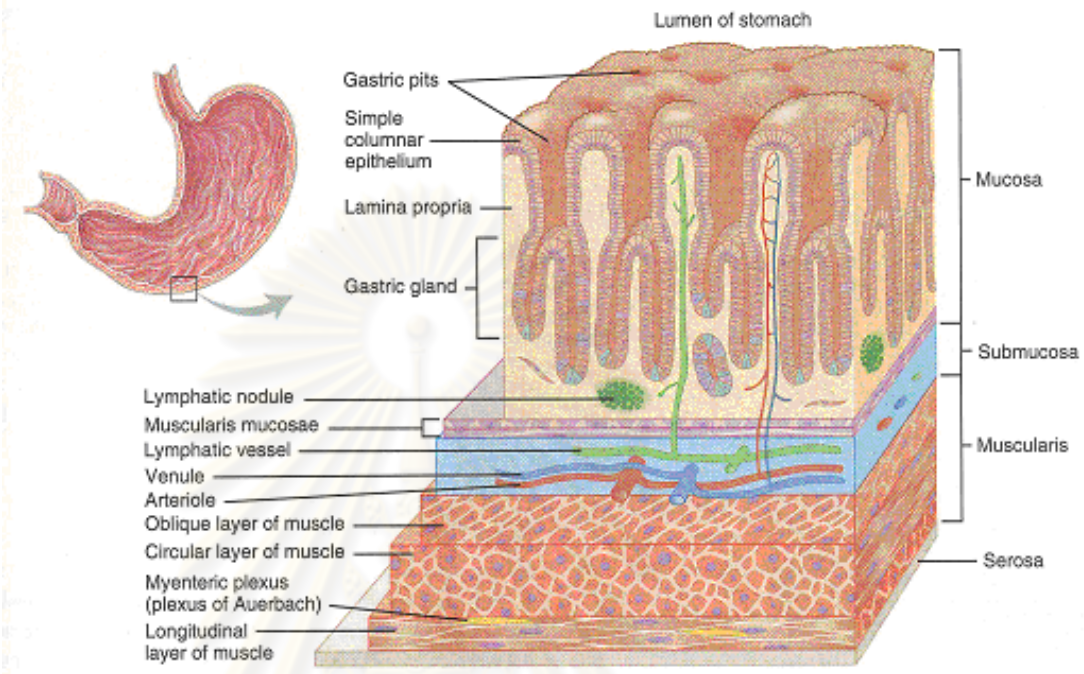


Figure 2 Structure of gastric wall.

***Helicobacter pylori* (*H. pylori*)**

Helicobacter pylori (*H. pylori*) was discovered by two Australian scientists, Barry Marshall and Robin Warren in 1982 but published in 1984 [14]. *H. pylori* bacteria was originally called *Campylobacter pyloridis*, renamed as *Campylobacter pylori* and then later reclassified to *Helicobacter pylori* because its morphological, structural and genetic characteristics has indicated that it should be placed in a new genus [15].

H. pylori is a Gram-negative spiral shaped bacteria measuring approximately 3.5 × 0.5 μm. These bacteria have four to six sheathed flagella at one pole as shown in figure 3. They slowly grow in a moist and microaerophilic condition (5% O_2, 10% CO_2 and 85% N_2) at 37°C [16]. The bacteria are unusual organism with remarkably high level of genetic diversity [17] in that they can survive and multiply in violence-acid environment of the stomach. *H. pylori* is a urease, oxidase and catalase positive organism. Spiral forms of *H. pylori* convert to coccoid forms (figure 4) under conditions of nutrient starvation, antibiotics, oxygen excess, and other stress factors. The coccoid form of *H. pylori* fails to grow on the routine bacteriological media, but nutrient supplementation or animal passage may help to recover. Animal passage of *H. pylori* coccoids was reported to revert in mice [18], but not in gnotobiotic piglets. [19]. These coccoid forms are able to attach to gastric epithelial cells and possess about the same proteins and induce the same cytoskeletal changes as spiral forms suggesting they are viable [20]. The importance of coccoid forms of *H. pylori* is under debate with regard their role to both transmission and treatment failure [21].

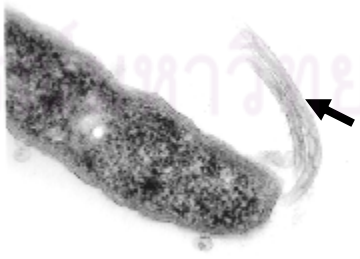


Figure 3 Characteristic of *H. pylori* [16]: *H. pylori* is a gram-negative bacterium which has flagella on the one pole. These flagella help the bacterial movement into the mucus of stomach. Note flagella (arrow).

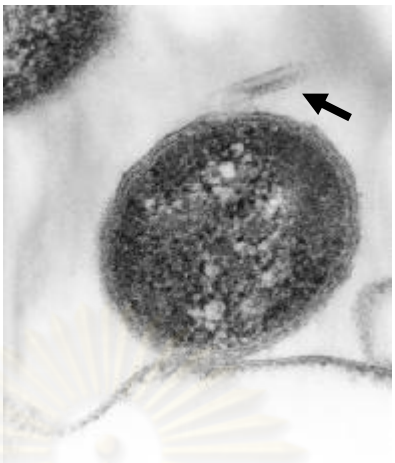


Figure 4 *H. pylori* coccoid form [16]. Note flagella (arrow).

Epidemiology and transmission

H. pylori is a causative agent of a range of human diseases including gastritis, peptic ulcer, gastric adenocarcinoma and gastric lymphoma [1]. In 1994, the International Agency for Cancer Research, an arm of the World Health Organization, classified *H. pylori* as a potential human carcinogen [22]. Approximately half of the world's population is infected with *H. pylori* and the prevalence shows large geographical variations [3].The prevalence of *H. pylori* among adults is typically around more than 80% and under 40% in developing and developed countries, respectively [3]. Moreover, it has been suggested that *H. pylori* infection rates are associated with age, ethnicity, socioeconomic status, sanitary environments and lifestyle. The infection is generally acquired during childhood and usually persists lifelong in the absence of treatment.

In spite of the worldwide spread of *H. pylori* infection, the route of transmission is still unknown. Person-to-person transmission either by the oral-oral, gastro-oral or fecal-oral routes is considered to be the most probable mode [2]. Recent epidemiological study suggests that infected mothers are the main source of *H. pylori* infection in their children [23].

Pathogenesis of *H. pylori* infection

All strains of *H. pylori* persist lifelong and all cause gastric inflammation. However, only 15% of infections result in peptic ulceration and only 0.5%–2% in gastric adenocarcinoma [24]. The disease development depends upon *H. pylori* strain virulence, type and extent of the host immune response to infection and modulating cofactors such as smoking and diet [24].

The main characteristics of bacterial virulence factors are to facilitate colonization of the host, enable the pathogen to avoid host defense mechanisms and cause tissue damage of the host [16]. Interestingly, *H. pylori* virulence factors may damage epithelial cells directly or stimulate them to produce proinflammatory cytokines, thus inducing inflammation [16]. A number of virulence factors of *H. pylori* have been well defined (Table 1).

H. pylori is highly adapted to the stomach environment. By flagella movement, *H. pylori* is able to penetrate the gastric mucus layer thereby escaping the acidic environment in the lumen and approaching the gastric mucosal surface. Additionally, it produces a large amount of urease, representing 5% to 10% of the total protein content of the bacteria [25]. This enzyme is essential for the survival, colonization and pathogenesis of the bacteria. Perhaps the most important role urease plays is to hydrolyze urea into carbon dioxide and ammonia, which aids in buffering the bacteria from the acidic conditions it may encounter in the stomach [25].The colonizing *H. pylori* adhere to gastric epithelium by different bacterial adhesins using hemagglutinins, laminin and Lewis b antigens as receptors [16].

Bacterial properties	**Virulence factors**	**Effects/properties**
Colonizing	Flagella	Active movements through mucin
	Urease	Neutralization of gastric acid
	Adhesins	Anchoring *H. pylori* to the epithelium
Tissue damaging	Proteolytic enzymes	Glucosulfatase degrades mucin
	120-kDa CagA	Related to ulcer and severe gastritis
	VacA	Damage of the epithelium
	Urease	Toxic effect on epithelial cells, disrupting cell tight junctions
	NAP (neutrophil activating protein)	Lead to neutrophil-mediated mucosal injury
	Phospholipase A	Digest phospholipids in cell membranes
Survival	Intracellular surveillance	Prevent killing in phagocytes
	Superoxide dismutase	Prevent phagocytosis and killing
	Catalase	Prevent phagocytosis and killing
	Coccoid forms	Dormant form
	Heat shock proteins	Sheathing antigen
	Urease	Prevent phagocytosis
Other	Lipopolysaccharide	Low biological activity
	Lewis X/Y blood group homology	Autoimmunity

Table 1 *H. pylori* virulence factors and their effects[26].

When *H. pylori* adheres on gastric epithelial cells, it releases several virulence factors, such as VacA, CagA, NAP, LPS, Hsp and OipA, to immune subversion. These *H. pylori* virulence factors are summarized below:

Vacuolating cytotoxin (VacA)

Vacuolating cytotoxin (VacA) is a secreted 87 kDa protein toxin that has direct cell-damaging effects *in vitro*, inducing cytoskeletal changes, apoptosis, and suppression of epithelial cell proliferation and migration, as well as cell vacuolation [27]. VacA forms pores in epithelial cell membranes, allowing egress of anions and small neutral molecules, including urea. [28]. This is important since urea hydrolysis, catalyzed by *H. pylori* urease, protects against gastric acidity [29]. VacA also induces loosening of epithelial tight junctions, potentially allowing nutrients to cross the mucosal barrier to *H. pylori*'s gastric luminal niche [30]. Additionally, recent interest has focused on the immunosuppressive effects of VacA. *In vitro*, VacA can interfere with antigen presentation, which occurs in compartments that are similar to late endosomes [31]. More surprisingly, it can specifically inhibit T cell activation and proliferation [32].

The vacuolating cytotoxin gene A (*vacA*), which encodes VacA, is conserved among all *H. pylori* strains [24]. *vacA* alleles possess one of two types of signal region, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations [33]. Studies of the toxin and its effects on cells have concentrated on *in vitro* effects of the most active (and most disease-associated) s1/m1 form [24].

***cag* PATHOGENICITY ISLAND (*cag* PAI)**

The cytotoxin associated gene A (*cagA*) is the most studied non-conserved gene of *H. pylori. cagA*, which encodes CagA, a 120 to 140 kDa molecular weight highly antigenic protein, is present in approximately 60% of *H pylori* strains [34] and is part of a 40 kilobase pathogenicity island (*cag* PAI) which contains over 30 genes [35].

Genes in the *cag* PAI encode proteins that comprise a type IV secretion system (T4SS) used for translocating bacterial products directly into the host cell cytoplasm [36]. The T4SS can be visualized microscopically as a sheathed, rigid needle linking *H. pylori* to the epithelial cell [37]. The protein CagA is translocated through this needle into host epithelial cells [38], where it is phosphorylated on tyrosine residues by Src family protein tyrosine kinases. Then, the stimulation of cell-signaling pathways and the recruitment of adaptor proteins involved in nucleation of actin polymerization and cytoskeletal rearrangements is occurred [39].

The presence of *cag* PAI is associated with a more prominent inflammatory response than is seen with strains lacking this virulence factor. Infection with *H. pylori* CagA positive strains could induce the production of chemokine (IL-8) from gastric epithelial cells to enhance inflammatory response in gastric mucosa [24].Moreover, several studies have shown that infection with CagA positive strains is highly associated with peptic ulcer disease, atrophic gastritis and gastric cancer [24, 40].

Neutrophil-activating protein (NAP)

A water soluble neutrophil activating 150 kDa protein, NAP, expressed by *napA* gene. NAP, a cytosolic protein, is released by bacterial lysis and interacts directly with neutrophils, monocytes, and mast cells, resulting in the activation of their inflammatory function [41]. NAP has been shown to be a chemoattractant for both neutrophils and monocytes during *H. pylori* infection [42]. In addition, it stimulates the production of reactive oxygen intermediate (ROI) of human neutrophils and monocytes.

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) or called endotoxin is the cell envelope of gram-negative bacteria. The LPS of individual *H. pylori* strains are structurally heterogeneous and may have variable degree of endotoxicity [43].

H. pylori LPS has also been implicated in the pathogenesis of *H. pylori* infection [43]. The immunological activity of *H pylori* LPS has been considered to be low [43]. LPS expressed by *H. pylori* is a very weak immunogen compared to that of other gram negative bacteria, but it has been shown to induce some proinflammatory cytokines [44]. Similarly, several studies also showed that secretion of the proinflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin 1 (IL-1) and IL-6 from human mononuclear cells and IL-8 secretion from neutrophils following stimulation with *H. pylori* LPS was significantly lower than that induced by *salmonella* and *Escherichia coli* LPS [45,46]. This low biological activity of *H. pylori* LPS is caused by the phosphorylation pattern of its lipid A component and may prolong *H. pylori* infection and lead to chronic inflammation in the gastric mucosa [43]. Furthermore, *H. pylori* LPS may have an important role in autoimmune responses in the gastric mucosa. The structure of LPS O-specific antigen in different *H. pylori* strains is similar to that of host LewisX or LewisY blood group antigens [47] which are expressed in normal human gastric mucosa [48]. This molecular mimicry may explain about some of the gastric autoantibodies induced by *H. pylori*.

Heat shock proteins (Hsp)

Heat shock proteins (HspA, HspB) are immunodominant antigens in various diseases including *H. pylori* infection. Hsp60, a 60 kDa Hsp, has been shown to induce proinflammatory cytokines by macrophages and gastric epithelial cells [49], which appears to be mediated by Toll-like receptors (TLRs). In addition, it has been shown that Hsp60 is expressed in the follicular dendritic cells of the gastric mucosa in patients with gastric mucosa-associated lymphoid tissue (MALT) lymphoma and that antibodies to human Hsp60 can be detected in MALT lymphoma patients.

Outer inflammatory protein A (OipA)

Outer inflammatory protein A (OipA) is an outer membrane protein of *H. pylori* that contributes to the induction of IL-8 production by epithelial cells [25]. In one study with *H pylori* clinical isolates, those isolates expressing OipA, but not the cag pathogenicity island (cagPAI) proteins were able to induce IL-8 production from gastric epithelial cell lines at 3 times the level of strains that did not express either [50]. When the signaling induced by OipA was compared to cagPAI proteins, the cagPAI proteins was found to induce NF-ΚB activation, while OipA induced phosphorylation of Stat1 [50]. Both of these signaling pathways contribute to induction of IL-8 production, but act in conjunction with one another to fully activate the IL-8 promoter.

Host inflammatory response to *H. pylori*

Despite an apparently vigorous inflammatory response against *H. pylori*, most infected people fail to clear the pathogen spontaneously [51]. The host inflammatory response to *H. pylori* consists of both an innate and an adaptive response (figure 5). The innate response towards bacterial infection is normally an initial non-specific process, which reacts rapidly with several bacterial molecules to signal infectious danger and with the aim of killing the bacteria. On the contrary, the adaptive response is delayed, antigen-specific, leads to the activation of T-, B- and memory cells and is shaped by the innate response [52].

Innate immunity

Recognition of bacterial molecules by the innate immune response is mediated by TLRs (Toll-like receptors) expressed on APCs (antigen-presenting cells) such as monocytes and DCs (dendritic cells). Bacterial contact with monocytes and other APCs contributes to the secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-8. *H. pylori* infection has been shown

to be involved in elevated levels of these cytokines which, in turn, act as local chemoattractants, inducing granulocytic infiltration [53].

Several studies have demonstrated that contact between *H. pylori* and gastric epithelial cells results in a rapid activation of nuclear factor kappa B (NF-κB), regulating a variety of genes whose products are involved in cell growth, inflammation and immune responses, which is followed by increased IL-8 expression [54].

In addition to NF-κB, mitogen-activated protein kinases (MAPKs) have been implicated as mediators of *H. pylori*-induced IL-8 expression. *In vitro* studies utilizing IL-8 reporter constructs have now revealed that *H. pylori*-induced IL-8 gene expression is dependent upon activation of both NF-κB and the early-response transcription factor activator protein 1 (AP-1) (via activation of MAPKs) [55]. Moreover, studies have shown that *H. pylori* strains carrying the cag-PAI induce a far stronger IL-8 response than cag-negative strains, and this response also depends on activation of NF-κB and AP-1 [56]. These results indicate that synergistic interactions between NF-κB and AP-1 within the IL-8 promoter are required for maximal *H. pylori*-induced IL-8 production [55].

Adaptive immunity

Cellular response

Adaptive immune responses to micro-organisms can be predominantly Th-1 (mainly cell mediated) or Th-2 (mainly antibody dependent). *H. pylori* stimulates both responses, though gastric mucosal cytokine profiles suggest that the Th-1 response predominates [57].

The Th1-predominant immune response is associated with elevated levels of the pro-inflammatory cytokines IL-12, IL-18 and TNF-α [58] and characterized by the induction of interferon-γ (IFN-γ) and IFN-γ -related genes [52]. In 2007, Holly, *et al*. [59] have reported that the acquired immune response was detected by 3 weeks after orogastric infection of mice with *H. pylori*, marked by gastric infiltration of T lymphocytes, macrophages, and neutrophils, as well as increased numbers of *H. pylori*-specific T cells, macrophages, and dendritic cells in paragastric lymph nodes. Moreover, they have found the increased expression of TNF-α and IFN-γ (Th1-type inflammatory cytokines) was detected in the stomachs of *H. pylori*-infected mice, but elevated expression of IL-4 (a Th2-type cytokine) was not detected.

This Th1 predominance is unusual for extracellular, toxin-producing bacteria, which commonly are met by B cell activation and high level antibody production (Th2 responses).

Moreover, *H. pylori* induces a strong humoral response, so a Th2-cell response would be expected. However, studies in mice imply that the predominant Th1 response is appropriate to control *H. pylori*. *Helicobacter* colonization density is lower in mice with predominant Th1 responses, whether genetically programmed or manipulated to have Th2 responses by experimental helminth infection [60].

In spite of its obvious propriety, the immune response, and in particular its Th1 component, is a main factor in *H. pylori*–associated pathogenesis [60]. During *Helicobacter* colonization, mice with a predominant Th1 response develop more gastric inflammation than those with a Th2 response [60]. T cell transfer experiments show that these effects are dependent on Th1 cells [60]. Mice genetically manipulated to have a Th-1 response develop inflammation, epithelial apoptosis, and disruption of cell organization in gastric glands even in the absence of *H. pylori* [61]. On the contrary, mice deficient in T cell responses do not show these changes [24]. Interestingly, gastric inflammation and atrophic changes are abrogated in the absence of the key Th1 cytokine IFN-γ [62] and are induced by IFN-γ infusion, even without *Helicobacter* [63].

Humoral response

H. pylori infection induces a vigorous systemic and mucosal humoral response [64]. The humoral response to *H. pylori* leads to the production of antibodies (mainly IgG and IgA) to cell surface and soluble products used for serodiagnosis [64]. Tosi and Czinn [65] reported that binding of IgG to *H. pylori* promoted phagocytosis and killing by polymorphonuclear leucocytes *in vitro*. In addition, studies have shown that sampling of gastric secretions from *H. pylori*-infected individuals also reveals an active mucosal antibody response, primarily of the IgA isotype. Several studies have shown that secretory IgA (sIgA) can interfere with the ability of some enteric pathogens to establish infection [66] and inhibit bacterial adherence [67]. Nevertheless, Clyne, *et al*. [68] have shown that the systemic antibody response and the sIgA response against *H. pylori* do not inhibit the bacteria from adhering to gastric cells *in vitro*. This may explain why chronic infection develops in infected subjects despite a powerful immune response.

Evasion of immune response by *H. pylori*

Despite *H. pylori* provokes the substantial inflammatory response, this bacteria has also evolved the mechanisms for evading and downregulating it.

Evasion of innate response

By avoidance of the immune response, *H. pylori* virulent factors and enzymes provide this function. For example, *H. pylori* prevents nitric oxide (NO), an effective antimicrobial agent, production by host cells by producing the enzyme arginase, encoded by the gene *rocF*. This enzyme competes with NO synthase 2 (NOS2) for the substrate L-arginine and converts it into urea and L-ornithine, rather than NO [69]. Moreover, *H. pylori* produces enzymes involved in ROI scavenging, such as catalase and superoxide dismutase to avoid the negative effects of reactive oxygen intermediates (ROIs) [70]. *H. pylori* is more resistant to phagocytic killing than other gram-negative bacteria. This feature is unique to CagA and VacA positive (Type I) *H. pylori* strains. Following the formation of megasomes as a result of phagosome fusion, the uptake of type I *H. pylori* into macrophages is delayed *in vitro* [71].

H. pylori has evolved its LPS and flagellin to minimize recognition by the innate immune system's toll-like receptors (TLR4 and 5, respectively) [72]. In addition, *H. pylori* LPS has been considered to be low immunological activity [72]. When compared with LPS from *salmonella*, *H. pylori* LPS is 500-fold less lethal toxicity in mice [73] and only weakly activates macrophages [72]. Consequently, it has been suggested that there is selective pressure in *H. pylori* cells to minimize pro-inflammatory activities to permit long-term colonization, enhance inflammation and lead to atrophic gastritis [74].

Evasion of adaptive response

H. pylori has evolved to subvert not only the innate, but also the adaptive immune response. Recent study *in vitro* suggests that VacA may help *H. pylori* persistence by specific immune suppression. VacA has ability to interfere with antigen presentation mediated by MHC class II [75] and downregulate Th1 effects by interacting with calcineurin to block the IL-2 signaling pathway in T-cells [76]. More surprisingly, it can specifically inhibit T-cell activation and proliferation [77].

Accordingly, although there is the host immune response to the bacteria, it is not effective enough to eradicate the infection and may involve in the damage to the gastric epithelium and contribute to tissue injuries.

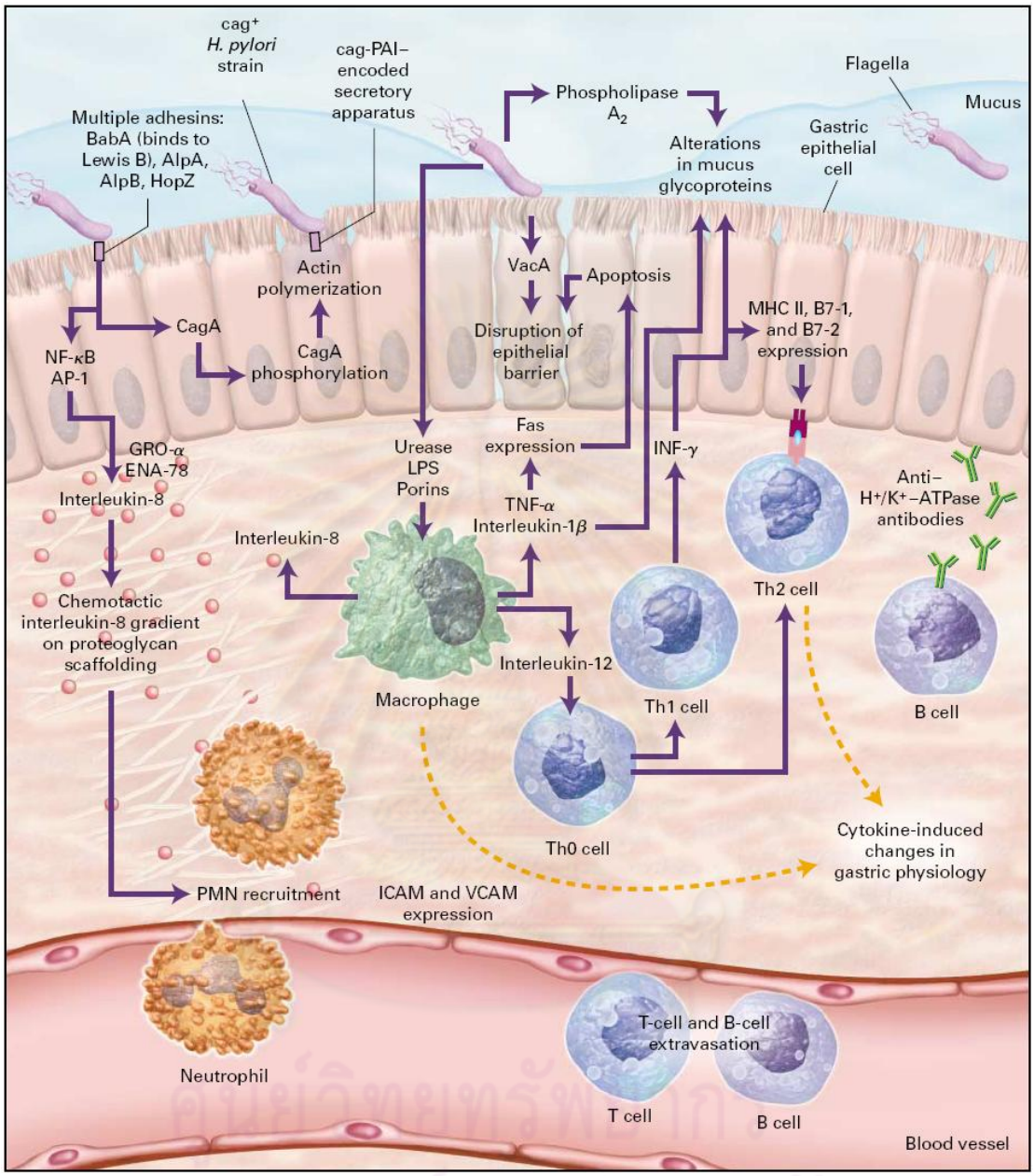


Figure 5 Pathogenesis of *H. pylori* infection [78]: The host response to *H. pylori* participates in the induction of gastric epithelial damage and therefore has an integral role in *H. pylori* pathogenesis. *H. pylori* resides in the gastric lumen and colonizes the gastric epithelium using urease. This microorganism adheres on epithelial cells by bacterial adhesion protein. Then, virulent factors are delivered into host cells. Particularly, CagA associates with many pathological conditions. For instance, injection of CagA results in the activation of NF-ΚB that cause production of IL-8 and other inflammatory mediators inducing gastric inflammation.

TNF-α and IL-1β

Tumour necrosis factor-α (TNF-α)

TNF-α, comprised of 235 amino acids, is a cytokine with pleiotropic functions produced mainly by activated macrophages in response to tissue injury or infection. TNF-α has been shown to activate neutrophils and promote T and B cell proliferation. Moreover, it also up-regulate cell adhesion molecules expression on endothelial cell, induce a variety of cells to produce a host of additional cytokines [79], elicit expression of chemokine and IL-1 and IL-6 that provide a chemotactic gradient allowing the directed migration of leukocyte into a site of inflammation. TNF-α is a major mediator of inflammation as well as apoptosis and immunity, and it has been associated with the pathogenesis of a wide spectrum of human diseases, including diabetes, cancer, sepsis, rheumatoid arthritis and inflammatory bowel diseases [80]. TNF-α is beneficial in activating the innate immune response. However, the inappropriate production of TNF-α contributes to inflammation, tissue damage and organ injury. Additionally, TNF-α is endogenous pyrogen, if it was produce in numerously, can cause a number of metabolic change in a variety of cell types and lead to the hepatic acute-phase response, and other systemic effects namely fever, anorexia, thrombocytosis and anemia [81]. Several microbial agents induce TNF-α secretion and a potent stimulator is the lipopolysaccharide (LPS) of gram negative bacteria [82].

Interleukin-1 (IL-1)

The interleukin 1 (IL-1) superfamily consists of IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RA). IL-1α and IL-1β, the proinflammatory cytokines, are associated with immune defense against infection. In contrast, the IL-1RA is a molecule that competes for receptor binding with IL-1α and IL-1β, blocking their role in immune activation. Both IL-1α and IL-1β are produced by activated macrophages, monocytes and dendritic cells [83]. They form an important part of the inflammatory response of the body against infection. These cytokines increase the expression of adhesion factors on endothelial cell to enable transmigration of leucocytes to the site of infection. Moreover, IL-1, called an endogenous pyrogen, re-set the hypothalamus thermoregulatory center, resulting in an increased body temperature that helps immune system to fight the pathogen [84].

Interleukin-1 beta (IL-1β)

IL-1β is a member of the interleukin 1 cytokine family. This cytokine is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by

caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is associated with a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [85].

IL-1 receptors

IL-1α and IL-1β exert their effects by binding to specific receptors. Two distinct receptor types have been isolated that bind both forms of IL-1.

First, IL-1RI, an 80 kDa membrane bound receptor protein, has been cloned from mouse and human cells [86]. It is found on T cells, fibroblasts, keratinocytes, endothelial cells, synovial lining cells, chondrocytes, and hepatocytes [85], and belongs to the Ig super family.

IL-1RII, a second type of IL-1 receptor, is found on B cells, neutrophils, and bone marrow cells [85]. This receptor has an apparent molecular weight of about 68 kDa and is also a member of the Ig super family.

***H. pylori* and TNF-α and IL-1β**

H. pylori infection is characterized by enhanced production of proinflammatory mediators such as TNF-α, IL-1β, IL-2, IL-6 and IL-8 and infiltration of lamina propria with inflammatory cells.

H. pylori LPS and released surface proteins stimulate lamina propria mononuclear cells and macrophage to produce proinflammatory cytokines such as TNF-α, IL-1β, and generation of reactive oxygen species(ROS) [87]. TNF-α and IL-1β are potent inducers of IL-8 expression in many cell types. Furthermore, *H. pylori* is capable to interacting with epithelial cell surface to produce IL-8. The release of these inflammatory mediators results in expression of CD11b/CD18 on leukocytes and ICAM-1 on endothelial cells, migration of leukocyte into a site of inflammation, and finally generation of ROS.

Several studies have demonstrated an increase in various inflammatory cells including monocytes, macrophage and PMNs activation when they are exposed to *H. pylori* extracts *in vitro* [88, 89]. Recently, study has shown an increase in the leukocyte adhesion and emigration in mesenteric venules following exposed to *H. pylori* extracts [90]. In 2006, Amanda and co-workers [91] have reported the significant expression of TNF-α, IL-8, IL-12, Nox1 and iNOS in biopsies from gastritis patients caused by *H. pylori* infection. Moreover, in gastric cancer group, the significant increase of TNF-α, IL-1β, IL-8, IL-10, IL-12, Nox1 and iNOS expression were shown.

Oxidative stress

Oxidative stress has been defined as a disturbance of the balance between antioxidants and prooxidants (free radicals and other reactive oxygen and nitrogen species) with increased levels of prooxidants resulting in potential cell damage [92]. This imbalance can be an effect of low dietary intake of antioxidants, depletion of endogenous antioxidants and/or increased formation of free radicals and other reactive species.

Free radicals and reactive oxygen species

Free radicals are atoms or molecules that possess one unpaired electron on their outer orbital. They are very unstable and quite reactive and are produced in both normal metabolic and pathological processes [92]. Common examples of free radicals are superoxide anion (O_2^-), hydroxyl radical (OH^-), transition metals such as iron and copper, nitric oxide (NO^-), and peroxynitrite [93].

The term reactive oxygen species (ROS) is a collective one that includes not only oxygen-centered radicals such as O_2^- and OH^- but also some nonradical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen, and hypochlorous acid (HOCI) [93]. ROS are generated from endogenous and exogenous sources such as diet, cigarette smoke, air pollution and radiation. Excess of ROS are thought to mediate a large portion of the tissue damage produced upon inflammation, ischemia and reperfusion and may also be associated with the pathogenesis of many diseases including inflammatory and neurodegenerative disease, heart disease and carcinogenesis [94].

ROS reactions tend to proceed as chain reactions and not terminated until the ROS are deactivated by a chain reaction breaking antioxidant [95]. However, these species may perturb the cell's natural antioxidant defense systems, resulting in damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates and lipids [96].

Reactive oxygen species and lipid peroxidation

Phospholipids are essential components of the membranes. Accordingly, damage to the phospholipids will compromise the viability of the cells. The peroxidation of lipids is a hallmark of oxidative damage. The polyunsaturated fatty acids (PUFAs) present in the membranes phospholipids are particularly susceptible to attack by hydroxyl radical (OH^-), peroxyl radicals (ROO^-) and alkoxyl radicals (RO^-) [97]. Their susceptibility increasing as the number of double bonds increases. OH^-, a very reactive species, can result in the peroxidation of many PUFA

molecules because the reactions involved in this process are part of a cyclic chain reaction. In addition to damaging cells by destroying membranes, lipid peroxidation can bring about the formation of reactive products that themselves can react with and damage proteins and DNA [97].

Lipid peroxidation and assessment

ROS can react with double bonds of PUFAs resulting in lipid peroxidation as shown in figure 6. Conjugated dienic hydroperoxides, the initial products of lipid peroxidation, are unstable compounds that tend to degrade rapidly to a variety of products such as short chain alkanes and aldehydes as shown in figure 7. All these products of degradation and decomposition are used in assessing oxidative stress, including hydroperoxides (LOOH), commonly expressed as conjugated dienes (CD), as well as the widely used end products malondialdehyde (MDA), F2-isoprostanes (F2I), exhalation of the alkanes pentane and ethane gases, and cytotoxic aldehydes [92] (figure 7).

Equation 1 - initiation phase of lipid peroxidation

$$LH + OH^{\bullet} \rightarrow L^{\bullet} + H_2O$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

Equation 2 - Propagation/Chain Reaction phase, oxygen independent stage

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$$

Figure 6 The various phases of the lipid peroxidation [92]. The initiation phase of lipid peroxidation starts with the abstraction of a hydrogen atom from the target fatty acid (LH) to form a fatty acid radical ($L^{\cdot}$). $L^{\cdot}$, product of this initiation reaction, rapidly rearranges to form a conjugated diene structure. The extremely rapid addition of oxygen to $L^{\cdot}$ forms a fatty acid peroxyl radical ($LOO^{\cdot}$). This is capable of reacting with other PUFAs, beginning a new chain of oxidation, thus forming a lipid hydroperoxide (LOOH) on the original PUFA and generating a new fatty acid radical ($L^{\cdot}$). The propagation phase of lipid peroxidation is the initiation of a new chain by a lipid peroxyl radical ($LOO^{\cdot}$) and the breakdown of lipid hydroperoxides (LOOH) to more radical intermediates [92, 97].

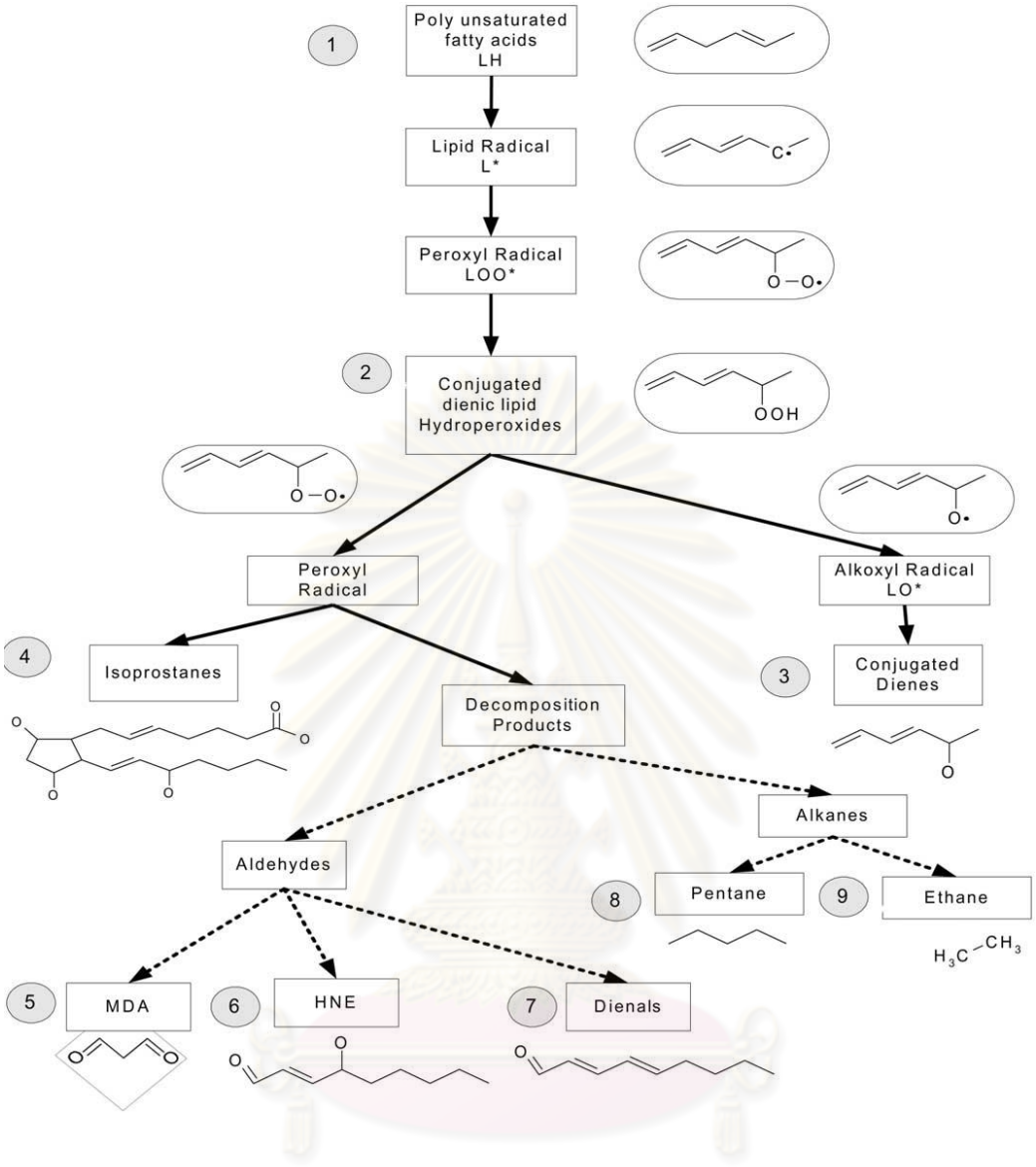


Figure 7 The products and pathways relating to lipid peroxidation. (1): polyunsaturated fatty acids, (2): lipid hydroperoxides, (3): conjugated dienes, (4): isoprostances, (5): MDA, (6): HNE, (7): dienals, (8,9): alkanes [92].

One of the major secondary oxidation products of peroxidized PUFAs is malondialdehyde (MDA) [98]. MDA is a biomarker of oxidative damage to lipids and most frequently determined by the thiobarbituric acid reactive substances (TBARS) assay. After its reaction with thiobarbituric acid (TBA) at 100 ˚C in acidic media, absorbance of pink chromogen is measured spectrophotometrically at 532 nm.

***H. pylori* and oxidative tissue damage**

H. pylori infection induces inflammatory responses, leading to oxidative stress and tissue damage. *H. pylori* stimulates neutrophil chemotaxis and activation both directly by bacterial factors and indirectly via the induction of interleukin-8 (IL-8) [99,100] and other cytokines involved in the inflammatory cascade as shown in figure 8. In addition, water soluble extracts of *H. pylori* up-regulate neutrophil adhesion to endothelial cells which occurs via CD11b/CD18 dependent interactions with intercellular adhesion molecule-1 (ICAM-1) [101] (figure 9). Hatz and co-workers [102] recently showed increased expression of ICAM-1 and VCAM-1 in *H. pylori* positive gastric mucosa which will facilitate neutrophil endothelial adhesion and extravasation. The gastric epithelium, the primary microbial-host interface, is now recognized as a major source of IL-8 [103]. *In vitro* study, *H. pylori* type I strains ($CagA^+$ and $VacA^+$) and cytokines such as TNF-α and IL-1β increased IL-8 expression in gastric epithelial cell lines [104]. Moreover, once attracted to the site of infection, neutrophils are themselves a source of inflammatory cytokines, including IL-8, TNF-α and IL-1β, thereby further amplifying the cellular response to infection [105]. Because of gastric epithelial IL-8 production and neutrophil activation induced by *H. pylori*, the great amounts of toxic ROS are generated. Consequently, gastric epithelial cells and neutrophils represent a main source of ROS, produced during *H. pylori* infection. In addition to IL-8 secretion, the inflammatory response to *H. pylori* infection involves in infiltration of the gastric mucosa with inflammatory cells and induction of other cytokines including TNF-α, IL-1β, IL-6, IL-12 and IFN-γ, that is also oxidative. The H_2O_2, generated by TNF-α and other cytokines, is another source of ROS that contributes to oxidative stress [106]. Since H_2O_2, in the presence of $Fe2^+$ (or Cu^+), can catalyzes the generation of the highly reactive hydroxyl radical, by Fenton reaction [107], an increase in H_2O_2 concentration induced by cytokines makes also TNF-α expression a good oxidative stress marker. Additionally, *H. pylori* also induces inducible nitric oxide synthase (iNOS) expression. In resent study, increased iNOS activity has also been observed in the gastric mucosa of patients with gastritis [108], duodenal ulcer [109] and gastric cancer [110] caused by *H. pylori* infection. iNOS is induced by a variety of stimuli, including cytokines, bacterial lipopolysaccharide and products from the bacterial wall [111], and its expression leads to nitric oxide (NO^-) production. NO^- is a reactive and unstable free radical gas that can cross cell membranes easily by diffusion without depending on any release or uptake mechanisms. The high levels of NO^- produced by iNOS, mainly in macrophages

and neutrophils, mediates cytotoxicity as the first line of host-defense against invading microorganisms [112]. Other possible mechanisms of $NO^{\cdot}$-mediated cytotoxicity have been proposed. Namely, the generation of peroxynitrite by a reaction between $NO^{\cdot}$ and superoxide anion ($O_2^{\cdot-}$) may play a significant role in the cytotoxic process [113]. Moreover, $NO^{\cdot}$ may inactivate several antioxidant enzymes, including catalase, glutathione peroxidase, and superoxide dismutases [114]. Thus, as mentioned before, iNOS expression induced by *H. pylori* contributes to oxidative stress. Another oxidative enzyme induced by *H. pylori* in gastric disease is NADH oxidase 1 (Nox1) [115]. It constitutively produces both $O_2^{\cdot-}$ and H_2O_2. Increased expression of Nox1 mRNA moderately increases $O_2^{\cdot-}$ generation, which contributes to a reduction in aconitase activity [116]. Zhang and colleagues [117] found increased production of ROS in the gastric mucosa is correlated to *H. pylori* density. However, *H. pylori* can resist to antimicrobial action of ROS. For example, *H. pylori* produces enzymes involved in ROS scavenging, such as superoxide dismutase (SOD) and catalase (CAT) [70]. Therefore, the inflammatory response induced during *H. pylori* infection does not appear to confer protective immunity, but may lead to the excess production of ROS, oxidative burst caused by phagocytic cells, and gastric tissue damages.

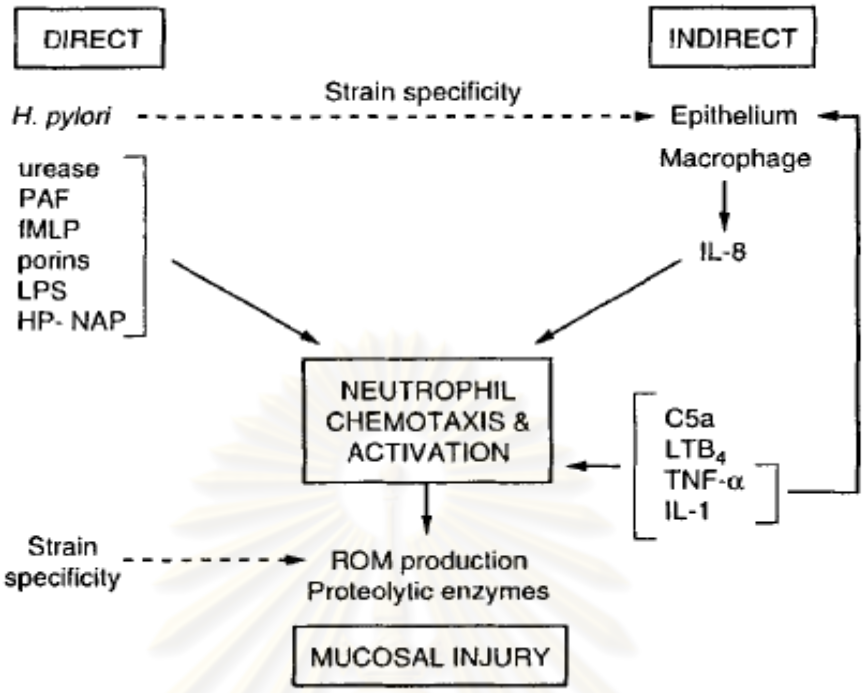


Figure 8 Direct and indirect stimulation of neutrophil chemotaxis and activation in *H. pylori* infection. PAF:platelet activating factor, *f*MLP: N-formyl-methlonyl-leucyl-phenylalanine, LPS: lipopolysaccharide, HPNAP: *H. pylori* neutrophil activating protein, IL: interleukin, C5a: complement 5a, LTB_4: leukotriene B_4, TNF-α: tumour necrosis factor-α, ROM: reactive oxygen metabolite [118].

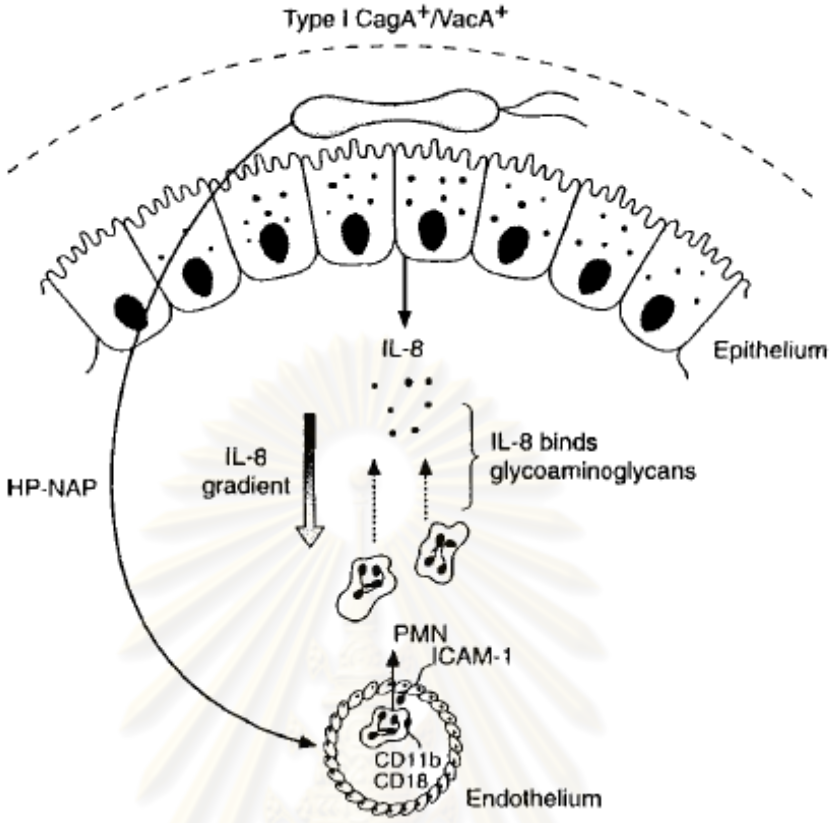


Figure 9 Pathway of neutrophil (PMN) stimulation following initial *H. pylori* infection. CagA: cytotoxin-associated protein, VacA: vacuolating toxin, IL: interleukin, HPNAP: *H. pylori* neutrophil activating protein, ICAM-1: intercellular adhesion molecule-1 [118].

Cell death

Cell death can follow two distinct pathways, apoptosis or necrosis.

Necrosis

Necrosis appears to be the consequently of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents, and is a relatively passive process involved in rapid cellular ATP depletion. Necrosis is morphologically characterized by a dramatic increase in cell volume and rupture of the plasma membrane, with spilling of the cellular contents into the intercellular milieu [119]. This release of the dying cells' contents into the extracellular space can cause further tissue damage by affecting neighboring cells or by attracting proinflammatory cells to the lesion [120].

Apoptosis

Apoptosis, a form of cell death, is divided into two pathways. First is the time dependent pathway, or senescent apoptosis at the end of a cell's natural lifespan. Second, an altruistic or self-defensive form of apoptotic cell death occurs in several pathological situations and response to severe DNA damage. It is thought that this altruistic apoptosis serves to abolish mutated cells before they can proliferate to form a potentially neoplastic clone, and before the end of their expected lifespan [121]. Apoptosis constitutes a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells [122]. Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation [123,124] and formation of apoptotic bodies as shown in figure 10. Importantly, the morphological changes accompanying apoptosis lead to the death of a cell without inflammatory sequelae, unlike necrosis.

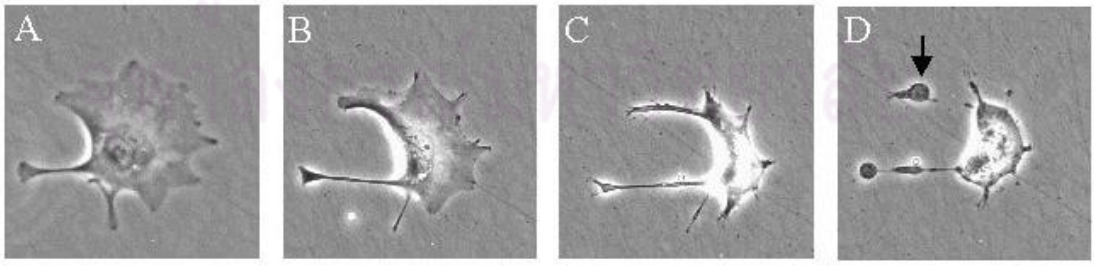


Figure 10 Morphology of an apoptotic trophoblast cell as captured by time-lapse microscopy (images were taken from [125]). Apoptotic cells display distinctive morphology during the apoptotic process. (A): The cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. (B): The breakdown of chromatin in the nucleus often contributes

to nuclear condensation and in many cases the nuclei of apoptotic cells take on a “horse-shoe” like appearance. (C): Cells continue to shrink, packaging themselves into a form that permits for their removal by macrophages. To avoid many of the problems involved in necrotic cell death, these phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion. In order to facilitate their phagocytosis by macrophages, apoptotic cells often ungergo plasma membrane changes that motivate the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. (D): The end stages of apoptosis are often characterised by the appearance of membrane blebs. Moreover, the small vesicles called apoptotic bodies are also sometimes observed (D, arrow) [125].

Regulation of apoptosis

There are two major pathways of apoptosis initiation. First, the extrinsic pathway is mediated by death receptors on the cell surface. The second one, called the intrinsic pathway, activates the mitochondria. Interestingly, both pathways induce apoptosis by caspases activation. Virtually, several protease families are implicated in apoptosis, the most prominent being caspases [126]. Caspases are cysteine-containing, aspartic acid-specific proteases which exist in the soluble cytoplasm, mitochondrial intermembrane space and nuclear matrix of actually all cells [127] as inactive proforms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis [125].

In the extracellular pathway, TNF-R1, CD95 (Fas), TRAIL-R1 and TRAIL-R2 are death receptors that belong to the tumour necrosis factor (TNF) receptor superfamily and can signal for apoptosis. These death receptors are activated by their ligands such as TNF, CD95 ligand (Fas ligand) and the TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), respectively [128,129]. Apoptosis induced by ligation of cell surface receptors represents a pathway almost exclusively controlled by caspases. Following ligand binding, a conformational change in the intracellular domains of the receptors reveals the presence of a death domain which allows the recruitment of various apoptotic proteins, namely TRADD and FADD, to the receptor [125,130]. This protein assembly is called the DISC (death-inducing signalling complex) which then activates an apical caspase, pro-caspase 8. Finally, activated caspase-8 at the DISC is capable of initiating apoptosis via the caspase cascade as summarized in figure 11.

The intracellular death pathway, triggering apoptosis without involving cell surface receptors, is activated by a direct effect of mitochondrial stressors like oxygen radicals,

chemotherapeutics and some bacterial toxins [125]. These apoptotic signals lead to the activation of the pro-apoptotic members of the bcl-2 family of proteins. The stimulated pro-apoptotic bcl-2 proteins, such as Bad, Bax and Bid, result in the formation of the Permeability Transition pore (PT pore) in the mitochondrial membrane. Following the formation of the PT pore, cytochrome C is released from mitochondria into cytosol, where it binds to a protein called Apaf-1 (apoptotic protease activating factor 1) [125]. Apaf-1 contains binding sites for cytochrome C and dATP, and oligomerizes with other Apaf molecules. This complex, christened the apoptosome, recruits and binds pro-caspase 9 by using the CARD (caspase recruitment domain) of Apaf-1 [131]. Then, mature caspase 9 is released from the multiprotein complex and induces apoptosis by activation of the more distal caspases 3 and 7, and this can be seen in figure 11.

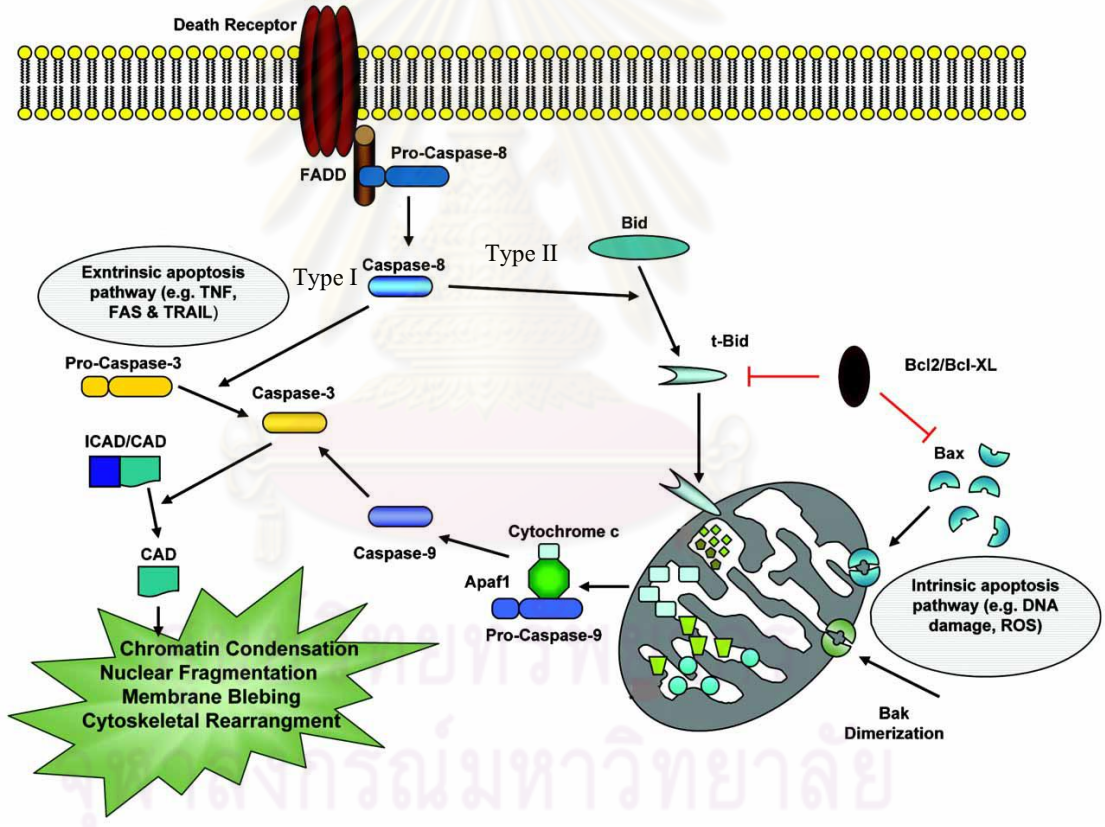


Figure 11 Summary of apoptotic signaling pathways as seen through activation of death receptor (extrinsic) or mitochondrial (intrinsic) pathway. Regarding to the death receptor pathway, in type I cells, extrinsic signals bind to their receptors and trigger intracellular signaling. This results in activation of caspase 8 and induction of apoptosis via the caspase cascade. Moreover, in type II cells, caspase 8 activation by extrinsic stimuli such as CD95/Fas ligand involves in mitochondria-dependent signaling and induces apoptosis by stimulation of the pro-apoptotic bcl-2 family

member Bid. In mitochondrial pathway, apoptotic signals, such as cell stress and free radical damage, activate pro-apoptotic bcl-2 proteins, namely Bad, Bax and Bid, leading to the formation of the PT pore in the mitochondrial membrane. Then, cytochrome C is released into the cytosol and binds Apaf-1 to form a multiprotein complex called apoptosome. Formation of the apoptosome leads to activation of caspase 9 and the induction of apoptosis [125]. Additionally, other bcl-2 family members like bcl-2 and bcl-XL are anti-apoptotic and can prevent apoptosis induction by stabilization of the mitochondrial membrane [132].

***H. pylori* and gastric epithelial cell apoptosis**

H. pylori induces gastric epithelial cell apoptosis both *in vitro* [133, 134] and *in vivo* [121]. Several studies have shown that the *H. pylori* colonized stomach contains more apoptotic epithelial cells than normal [135]. Moreover, the increased numbers of apoptotic epithelial cells decrease to normal after eradication of *H. pylori* [135]. *H. pylori* induces apoptosis both directly by the production of specific virulence factors, such as VacA and urease, and indirectly via the host inflammatory response. Cover, *et al*. [136] have recently shown that VacA induces apoptosis of gastric epithelial cells. By co-culturing gastric AGS cells with either VacA producing or isogenic *vacA*-null mutant *H. pylori* strains, they demonstrated that only VacA producing bacteria can contribute to an elevated number of apoptotic cells. Moreover, a recent study reported that *H. pylori*-induced apoptosis is accelerated by the urease of *H. pylori* [137]. In addition to VacA and urease, a study in rats showed that inoculation of *H. pylori* lipopolysaccharide to the gastric epithelium leads to a marked increase in apoptosis, with numerous apoptotic cells present not only in the superficial epithelium but also deeper in the gastric glands [138].

In 1998, Wilson, *et al*. [139] showed the increased proinflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12 and IFN-γ in *H. pylori* infected gastric mucosa. The inflammatory mediators IFN-γ and TNF-α augment gastric epithelial cell apoptosis induced by *H. pylori* [140]. A postulated mechanism for their interaction is through the upregulation of the Fas receptor on the gastric epithelial cell by IFN-γ [140] and the interaction of Fas with closely related TNF-α receptors [141]. Studies have shown that expression of Fas receptors is increased in epithelial and lamina propria cells in *H. pylori*–associated gastritis, or gastric epithelial cell lines incubated with *H. pylori* alone or in combination with IFN-γ or TNF-α [142, 143]. The ligand for the Fas receptor is commonly expressed by activated T lymphocytes and natural killer cells. *In vivo* study,

Wang, *et al*. [143] reported that FasL mRNA expression is higher in gastric T lymphocyte cells from *H. pylori* infected patients compared to uninfected subjects. As mentioned before, these suggest that increased Fas receptor expression would be forecasted to increase the sensibility of gastric epithelial cells to T cell killing and the local T cells induce apoptosis in gastric epithelial cells by means of interactions through Fas and FasL [121, 143]. Additionally, study has observed that the expression of Fas ligand mRNA is also increased in gastric epithelial cells during *H. pylori* infection. This implies that *H. pylori*–induced apoptosis not only involves killing of epithelial cells by FasL-expressing T lymphocytes, but also occurs by fratricide and/or suicide mediated by a FasL-Fas interaction among epithelial cells [142].

H. pylori-induced host inflammatory responses lead to ROS production, oxidative stress and apoptosis, respectively. Several studies have shown that increased inducible nitric oxide synthase (iNOS) activity is also examined in the gastric mucosa of patients with gastritis [108], duodenal ulcer [109] and gastric cancer [110] caused by *H. pylori* infection. iNOS expression contributes to high level of NO^- production. NO^-, a reactive and unstable free radical gas, has been reported to induce apoptosis by increasing ceramide generation through caspase-3 activation, induction of mitochondrial permeability transition, and activation of the Fas system [144]. Moreover, study has shown that the H_2O_2, generated by TNF-α and other cytokines in response to *H. pylori* infection, induces apoptosis by activation of caspase 3 both through either up-regulation of the Fas/FasL system or stimulation of mitochondrial pathways. Once H_2O_2 acts upon mitochondria, it causes a disruption of mitochondrial membrane potential and the release of cytochrome C to the cytosol [145]. Then, cytochrome C binds to Apaf-1 and becomes an essential component of the apoptosome. Assembly of the apoptosome complex initiates the caspase cascade by first activating caspase 9. In addition, transcription factors can be modulated by H_2O_2. Study has reported that NF-ΚB and AP-1, the ubiquitous transcription factors, are activated by H_2O_2. Following the activation, these transcription factors might drive transcription of pro-apoptotic genes or perhaps cause expression of inhibitors of survival related proteins [146]. Finally, *In vitro* studies have shown that incubation with *H. pylori* leads to up-regulation of pro-apoptotic proteins (Bak and Bax) and downregulation of anti-apoptotic proteins (Bcl-2 and Bcl-XL) [147].

Detection of apoptosis by TUNEL assay

Terminal deoxynucleotidyl nucleotide nick-end labelling (TUNEL) is an *in situ* histochemical method that identifies cells containing fragmented DNA, a hallmark of apoptosis. Several studies have used the TUNEL assay as a surrogate marker to detect apoptotic cells in gastric biopsy samples [121,135].

Genus *Lactobacillus*

Scientific classification of *Lactobacillus*

Kingdom	:	Bacteria
Division	:	Firmicutes
Class	:	Bacilli
Order	:	Lactobacillales
Family	:	Lactobacillaceae
Genus	:	*Lactobacillus*
Species	:	*L. plantarum*, *L. salivarius*, *L .acidophilus*, *L. rhamnosus*, etc.

Lactobacillus

Lactobacillus is a gram-positive facultative anaerobic bacterium which some strain microaerophilic to anaerobic. It is a member of the lactic acid bacteria group, defined as the large group of beneficial bacteria that produce lactic acid as an end product of the fermentation process. Genus *Lactobacillus* consists of more than 50 different species [148].

Lactobacillus has been detected in diverse environments. It is a microflora, microorganism which normally inhabits the healthy human body and not disease causing, in the oral cavity, gastrointestinal tract and vagina [149, 150].

Additionally, it is also used in the production of foods that prepared by mean of lactic acid fermentation such as dairy products, fermented vegetables and fermented meats. *Lactobacillus* is found in plant materials such as foodstuffs, silages and agriculture products [151]. Some species of *Lactobacillus* is used industrially for the production of milk, dairy products and cereal products including beer, wine and cider.

Interestingly, *Lactobacillus* is known as probiotic bacteria which probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [5]. Probiotics are non-pathogenic microbial organisms which can survive passage through the gastrointestinal tract and are believed to have potential beneficial health effects. The desirable properties of probiotic bacteria include having generally regarded as safe status, acid,

and bile stability, adherence to intestinal cells, persistence for some time in the gut, antagonism against pathogenic bacteria and modulation of the immune response [152]. Bacteria of human origin were originally required for safety reasons and because probiotic efficacy appeared to be host-specific. In addition to *Lactobacillus* sp., the most commonly used organism in probiotic products is *Bifidobacterium* sp. [6]. Other organisms have also been used including *Bacillus* sp., and yeast such as *Saccharomyces boulardii*.

Lactobacillus plantarum

Lactobacillus plantarum (*L. plantarum*), a very flexible and versatile species, is a non-pathogenic gram-positive bacterium naturally existing in human saliva and gastrointestinal tract. As a member of the lactic acid bacteria, it is commonly used in food fermentation. Examples of food containing *L. plantarum* include pickles, brined olives, Korean kimchi, sauerkraut, sourdough, yogurt, cheese, sausages and stockfish. The wide use of *L. plantarum* in food makes it suitable for the development of probiotics. Being used as a probiotic, its biotherapeutic applications have been increasingly recognized [153].

Structure

L. plantarum is rod-shaped as shown in figure 12. Its genome is the largest among all lactic acid bacteria and has been fully sequenced.

Metabolism

L. plantarum is a facultative anaerobic bacterium meaning it can grow both in the presence and absence of oxygen. In the presence of oxygen, it can convert oxygen into hydrogen peroxide in a manganese-dependent way, which gives itself a high hydrogen peroxide tolerance. In contrast, when oxygen is absent, it is capable to undergo fermentation and turn sugars into lactic acid or alcohol (heterofermentative). The lactic acid produced is a combination of D- and L-isomers. Additionally, *L. plantarum* has the ability to liquefy gelatin [153].

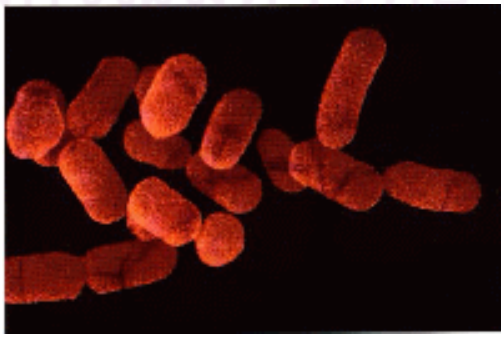


Figure 12 Structure of *L. plantarum* (http://www.bacferm.com.au/silac/micro/micro.html).

L. plantarum as probiotics

According to the definition of the World Health Organization (WHO) [5], probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Recommended properties for a probiotic microbe include survival of the gut, persistence in the host, and proven safety for human consumption [154]. The claimed health effects include the reduction of gastrointestinal infection and inflammatory disease risk, or modulatory effects on the immune system [10].

Survival in the human GI-tract

L. plantarum can survive low pH of the stomach and duodenum, resisting the effects of bile acids in the upper small intestine when ingested, and temporarily colonizing the gastrointestinal tract by binding to the intestinal and colonic mucosa [153].

In 2001, Haller, *et al*. [155] reported that different strains of *L. plantarum* are found to show a high tolerance to the consecutive exposure to hydrochloric acid (pH 2) and bile salts. This was observed both for strains isolated from intestinal samples and for those isolated from fermented foods. 0.003–10% of the *L. plantarum* cells survived those conditions compared to no survival and very limited survival (0–0.001%) for *L. sakei* and *L. paracasei*, respectively. In a study performed in Sweden, investigators have shown that *L. plantarum* 299v can be recovered from jejunum and rectum biopsies in 11 of the 13 participants, even 11 days after cessation of a 10-day treatment period [156]. Bin, *et al.* [157] also reported that after intake of *L. plantarum* L2 for 28 days, a significant increase in live *L. plantarum* is found in the rats' feces, small intestine and colon. The bacterial levels remained high even after the *L. plantarum* L2 administration had been stopped for two weeks. Moreover, recent study showed that following *L. plantarum* 299v consumption, *L. plantarum* 299v can be recovered in faecal samples in the majority of subjects from the probiotic-treated group, indicating that the probiotic strain survives passage through the gastrointestinal tract [158].

Safety of *L. plantarum*

In safety aspects, *L. plantarum* has a long history of natural occurrence and safe use in a variety of food products including its well known use in large numbers in sauerkraut and olive preparations [10]. Recently, study demonstrated that *L. plantarum* NCIMB 8826, isolated from the human saliva, do not induce macroscopic or histological inflammation or abnormal translocation through the intestinal barrier in mice [159]. Another investigation showed that after

an intravenous injection of Sprague-Dawly rats with 10^8 CFU of *L. plantarum* 299v, this organism can not be recovered from the heart and blood, when the rats are sacrificed 96 h after injection. So even if the intestinal barrier was crossed, no infection took place, showing the apparent safety of the organism [160]. Finally, a recent study showed that *L. plantarum* is not found in bacteremia cases [161].

Anti-*H. pylori* activity and immunomodulatory effects of *L. plantarum*

Several studies have been shown that *L. plantarum* 299V induce mucin gene expression to interfere with the actions of *H. pylori* [7, 8].In 2006, investigation showed *L. plantarum* is able to inhibit *H. pylori* growth *in vitro* [162]. Bin and colleagues [157] have been reported that among the tested strains, *L. plantarum* L2 has the most ability to adhere to Caco-2 and IEC-6 cell lines. Furthermore, *L. plantarum* L2 was also found to induce a considerable level of IL-10 from PBMCs, but low levels of pro-inflammatory cytokines including TNF-α, IFN-γ and IL-12. Resent study demonstrated that *L. plantarum* is capable to inhibit epithelial barrier dysfunction and reduce IL-8 secretion induced by TNF-α [163]. Interestingly, in 2008, Panpetch and co-workers [9] reported that *L. plantarum* B7 show the highest *E. coli* LPS-induced TNF-α inhibitory activity *in vitro*.

For these mentioned reason, *L. plantarum* may be an ideal candidate for the development of probiotics.

Possible mechanisms of probiotic action

The mechanisms of probiotic action on *H. pylori* infection are unclear, but there are a number of proposed possibilities from in vitro studies of host epithelial or immune cell responses to probiotic strains as described following.

Normally, probiotics such as lactic acid bacteria and bifidobacteria are able to produce organic acids, hydrogen peroxide and carbon dioxide to inhibit potential pathogens. Furthermore, many probiotics have been found to produce more defined antimicrobial substances [164]. Coconnier, *et al*. [165] found that the anti-*Helicobacter* substances in the *L. acidophilus LB* strain were different from lactic acid. Bernet-Camard, *et al*. [166] reported *L. johnsonii* La1, shown to be beneficial in several clinical and experimental studies in treatment of *H. pylori* infection, is found to release non-bacteriocin antimicrobial substances. Also, some *Bifidobacterium* strains have been found to release heat-stable proteinaceous antimicrobial compounds against *H. pylori in vitro* [167].

Lactic acid bacteria that produce antimicrobial substances have considerable advantage in the competition with other micro-organisms, including pathogens and other harmful bacteria. There are two main types of substances produced by lactic acid bacteria that implicated in the inhibition of *H. pylori*: short chain fatty acids (SCFAs) and bacteriocins.

Firstly, SCFAs such as formic, acetic, propionic, butyric and lactic acids are produced during the metabolism of carbohydrates by probiotics and have an important role in decreasing pH. Midolo and colleagues [168] reported the growth inhibition of *H. pylori* by acetic and lactic acid in a dose dependent manner and showed that lactic acid demonstrate the greatest inhibition. It has been proposed that lactic acid not only has a direct effect on H. pylori but also inhibit its urease activity [169]. Thus, although *H. pylori* can counteract stomach acid by producing urease, inhibition of urease by some *Lactobacillus* species could render *H. pylori* susceptible to the lactic acid [169, 164]. Servin and co-workers [164] also showed that lactic acid can improve antibiotic activity by increasing the outer membrane permeability of gram-negative bacteria.

Bacteriocins, small, heat-resistant and dialysable peptidic structures, are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes that have a bactericidal or bacteriostatic effect on other species [170]. They are compounds with potential anti-*H. pylori* activity which synthesized by several bacterial species including lactic acid bacteria. The antimicrobial action of bacteriocins involves increased permeability of the

cytoplasmic membrane of the target cells, which leads to the release of small cytoplasmic particles, depolarization of the membrane potential and eventually to cell death [171].

Other studies have been demonstrated that in addition to SCFAs, especially lactic acid, and bacteriocins, *Lactobacillus* inhibitory factors including bacteriocins-like substances, peroxides, proteinases, exopolysaccharides, cell wall components and other bioactive substances can exert anti-*H. pylori* activity [172, 173, 174].

Another possible mechanism of probiotic action is inhibition of *H. pylori* adhesion. Adhesion of pathogens can also be inhibited by steric hindrance, where a large number of beneficial bacteria may cover receptor sites in a non-specific manner, or by competing for specific carbohydrate receptors that would otherwise be available to pathogens. Several probiotic species, such as *L. acidophilus*, *L. salivarius* and *L. gasseri*, have shown growth inhibition or anti-adhesion capacity against *H. pylori* in a gastric epithelial cell model [165, 168, 169]. In 2002, Mukai and colleagues [175] have also examined competition in the binding of nine *L. reuteri* strains and *H. pylori* to sulfatide and gangliotetraosylceramide (Asialo-GM1), which are putative glycolipid receptor molecules of *H. pylori*, and identified a possible sulfatide and Asialo-GM1 binding protein of the two *L. reuteri* strains (TM105 and JCM1081). In addition, mucins are high-molecular-weight glycoproteins secreted by epithelial cells and may bind to pathogens, thereby inhibiting their adherence to epithelial cells. Byrd, *et al*. [176] showed that *H. pylori* is able to down-regulate the release of gastric mucin. However, probiotic strains including *L. plantarum* 299V and *L. rhamnosus* GG also induced mucin gene expression to interfere with the actions of *H. pylori* [7, 8].

Moreover, several probiotic bacteria have been shown to prevent and repair mucosal damage by inhibiting damage to tight junction proteins [177]. The probiotic strains prevented the pathogen-associated disruption of the cytoskeletal and tight junction proteins in the epithelial cells, therefore improving the mucosal barrier function and preventing failure in the secretion of electrolytes [178]. In 2001, Gotteland, *et al.* [179] showed that regular ingestion of live *L. rhamnosus* GG protects the integrity of gastric mucosa, as evaluated by the sucrose permeability test, against alterations by indomethacin. Yan and Polk [180] also reported that *L. rhamnosus* GG is able to prevent cytokine-induced apoptosis in intestinal epithelial cell models through the inhibition of a TNF-α-induced activation of the pro-apoptotic p38/mitogen-activated protein kinase.

Finally, many reports suggest that probiotics have ability to differentially modulate innate immune responses in both anti-inflammatory and pro-inflammatory directions. In 2010, Megan and co-workers [181] reported that *L. plantarum* and *L. acidophilus* have the potential to be immunomodulatory probiotics, and their effects on cytokine expression are strain specific. By the IEC-6 rat intestinal epithelial cell line with and without LPS stimulation, *L. plantarum* 7-12 and *L. acidophilus* La-11 had an inflammatory effect on unstimulated cells as indicated by up-regulation of IL-18, and MIP-2 and IL-6 by *L. plantarum* 7-12, and *L. acidophilus* La-11, respectively. In contrast, *L. plantarum* 5-39 and *L. acidophilus* NCFM had an anti-inflammatory effect as indicated by down-regulation of IL-1β expression in LPS-stimulated cells. All strains except *L. plantarum* 7-12 also down-regulated expression of TNF-α in cells stimulated with LPS. In 2003, Pena, *et al*. [182] showed that *L. rhamnosus* GG is able to antagonize *H. pylori* LPS-induced TNF-α production by murine macrophages *in vitro* by a contact-independent mechanism. Chao, *et al.* [183] demonstrated that *H. pylori* LPS induces IL-8 production in SGC-7901 cells, human gastric adenocarcinoma cells, through activating TLR4 signaling and *L. bulgaricus* (LBG) prevents *H. pylori* LPS by down–regulation of TLR4 expression, inhibition of TAK1 and p38MAPK phosphorylation, prevention of NF-κB activation, and consequently suppression of IL-8 production. In addition to immunomodulatory abilities, several studies have been shown that *Lactobacillus* also has effective antioxidative [184, 185] and anti-apoptotic properties [180, 186].

The model used in this study

Because there is increasing evidence that *H. pylori* is a significant gastroduodenal pathogen, then searching for understand the pathogenic mechanism and efficient therapies against *H. pylori* infection are importance. In many previous studies, several animal species, such as gnotobiotic piglets, non human primates, pigs, dogs, cats, gerbils and mice, have been infected with *H. pylori* [187-189].

The rat is one of the most commonly used laboratory animals in gastrointestinal research, and its gastric physiology has been thoroughly investigated [190]. In several studies, *H. pylori* induced gastritis in rat model has been done but there used the expensive, difficult and long-period methods [190, 191].

In present study, we used *H. pylori*-infected rat model [192] that is a simple, but effective model to determined the *H. pylori*-induced gastric mucosal inflammation. In briefly, Sprague-Dawley rats were inoculated by *H. pylori*. Within two weeks after inoculation of *H. pylori*, the mild to moderate gastric inflammation were developed as confirmed by hematoxylin and eosin (H&E) staining. [192]. Additionally, it has been found that the histopathology did not become more progressive along with the time after two weeks of *H. pylori* inoculation [191]. The damaging histology of *H. pylori*-induced gastric inflammation was infiltrated by inflammatory cells into lamina propria, enhanced releases of proinflammatory cytokines, especially TNF-α and IL-1β, and generated reactive oxygen species (ROS) [193]. This infected rat model is considered to be an experimental model that closed resemblance to human gastritis.

CHAPTER III

MATERIALS AND METHODS

Chemical substances

95% Alcohol (Merck, Germany)

Absolute alcohol

ApopTag® plus peroxidase *In Situ* apoptosis detection kit (Chemicon, USA)

BCA Protein Assay Kit (Thermo Scientific, USA)

n-Butanol (Merck, Germany)

Diaminobenzidine (DAB) (DAKO, USA)

Distilled water

Eosin (C.V. Laboratories, Thailand)

30% Hydrogen peroxide (Merck, Germany)

Hematoxylin (C.V. Laboratories, Thailand)

IL-1β ELISA kit (R&D systems, Inc., USA)

NaOH (Merck, Germany)

Phosphate buffer solution pH 7.4

Proteinase K (DAKO, USA)

4% Paraformaldehyde in PBS

RIPA buffer (Cell Signaling Technology, Inc., USA)

Streptomycin (General Drug House Co., LTD., Thailand)

Sodium Thiopental (Abbott, Italy)

TBARS assay kit (Cayman, USA)

TNF-α ELISA kit (R&D systems, Inc., USA)

Xylene (Zenith Science Co., LTD., Thailand)

This experiment is divided into two parts: *in vitro* and *in vivo* study.

1. *In vitro* study to investigate the anti-*H. pylori* activity of *L. plantarum* B7 supernatants.

2. *In vivo* study to examine the effects of *L. plantarum* B7 on *H. pylori*-induced change of the followings:

- Stomach histopathology
- Serum TNF-α and IL-1β level
- Gastric MDA level
- Apoptosis in gastric epithelial cells

***In vitro* study**

A disk diffusion method was used to assess anti-*H. pylori* activity of *L. plantarum* B7 supernatants of intact pH and neutralized pH at the various concentrations of 1X, 5X and 10X against *H. pylori*.

Bacterial strains and culture conditions

H. pylori ATCC 43504 was grown on Columbia agar (Oxoid, Basingstoke, UK) containing 7% sheep blood and 7% horse serum. Plates were incubated at 37 °C under microaerophilic condition (10% CO_2, 5% O_2 and 85% N_2) produced by a gas generating system, AnaeroPack (MGC, Japan), for 72 h in anaerobic jar (Oxoid, Basingstoke, UK) (figure 13).

L. plantarum B7, isolated from Thai dyspeptic patients, was stored in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) with 20% glycerol at -80 °C. This strain was recovered from frozen stock and cultivated twice on MRS agar anaerobically (10% CO_2, 10% H_2 and 80% N_2) at 37 °C in anaerobic jar for 48 h (figure 14). After that a single colony of *L. plantarum* B7 was inoculated in 10 mL of MRS broth and grown at 37 °C under anaerobic condition for 24 h in 15 mL conical centrifuge tube (Corning, New York, U.S.A.). The OD_{600} of culture was determined by using spectrophotometer (Bio-Rad Smart Spec™ Plus), adjusted to OD_{600} of 0.1 in 10 mL of MRS broth and incubated for 48 h. After incubation, the culture supernatant was collected by centrifugation at 1,000 x g for 10 min at 4 °C (figure 15) and then filtered by using 0.22 µm pore size filter unit (Minisart, Germany) (figure 16). The supernatant of *Lactobacillus* without the cell pellet was called *Lactobacillus* condition media (LCM). The concentration and pH of LCM were adjusted to 1X, 5X and 10X by speed-vacuum drying (speed-vacuum, Savant instruments, U.S.A.) and resuspending in appropriate volume of intact pH 4 and

adjusted pH 7 of MRS broths (figure 16). Then sterile 6 mm-membrane disks (Whatman, Maidstone, UK) were dipped into resuspended LCM for at least 1 h at room temperature (figure 17).

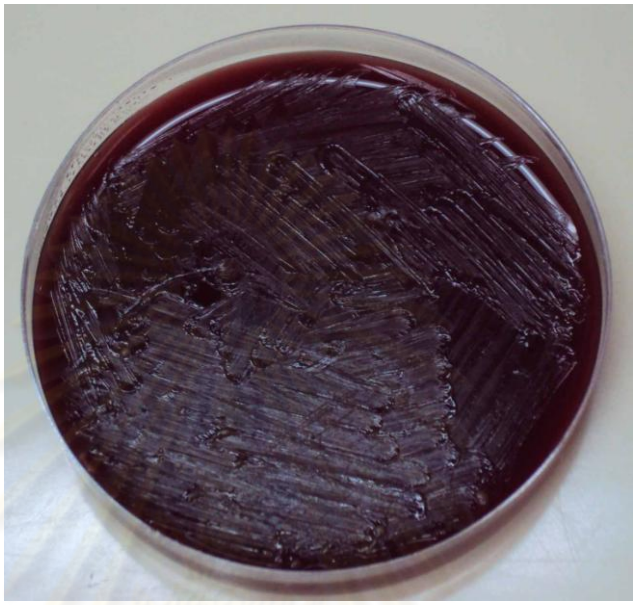


Figure 13 Show the *H. pylori* colonies on blood agar plate.

Figure 14 Show the *L. plantarum* B7 colonies on MRS agar plate.

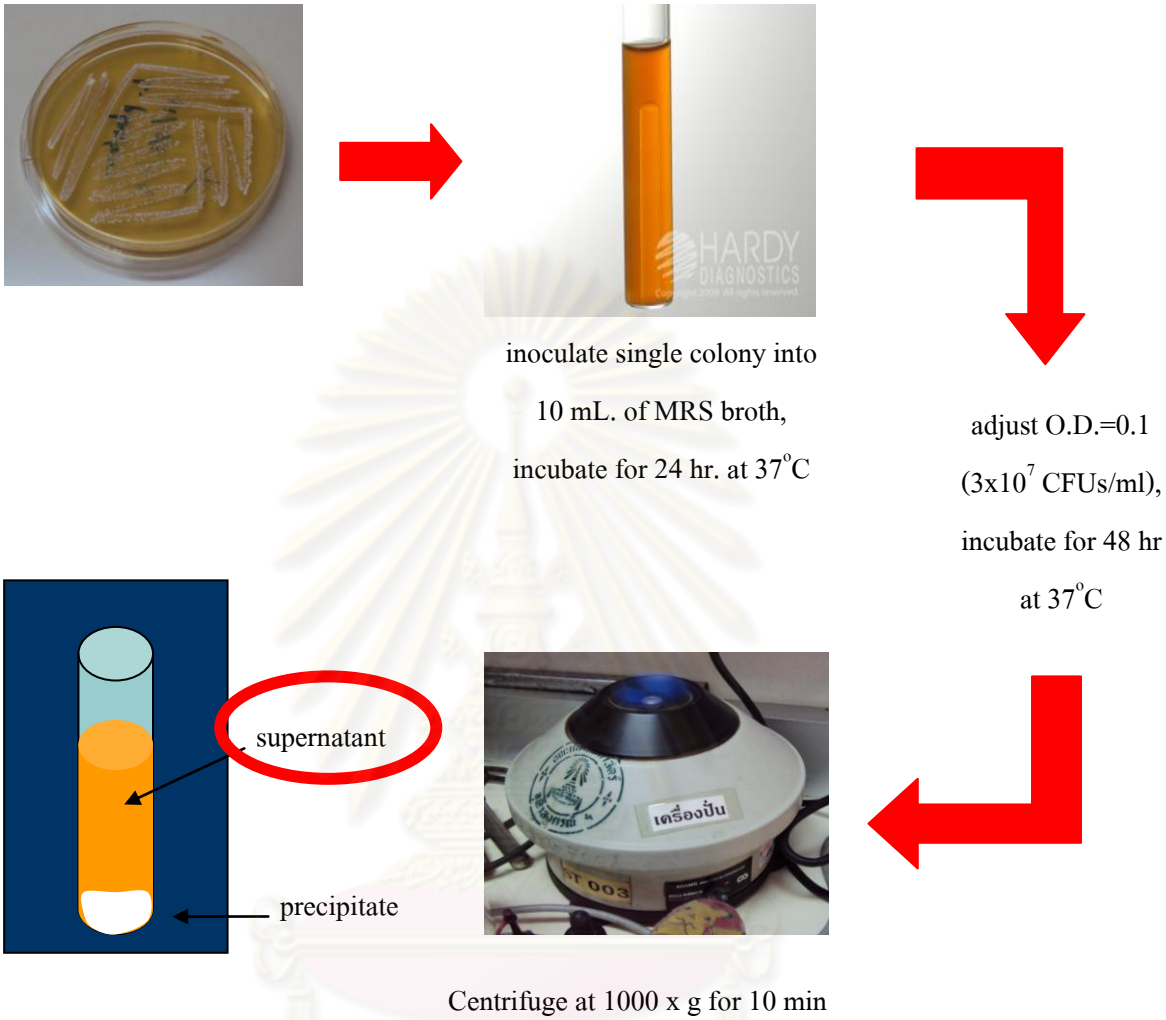


Figure 15 Show the *L. plantarum* B7 supernatant preparation.

filter sterilize supernatant by using 0.22 um membrane

Aliquot 500 uL / microcentrifuge tube

concentrate by speed-vacuum drying

resuspend in appropriate volume of MRS broth (intact pH 4 and adjusted pH 7)

1X resuspend **500 uL** of MRS broth

5X resuspend **100 uL** of MRS broth

10X resuspend **50 uL** of MRS broth

Figure 16 The adjustment of pH and concentration of *L. plantarum* B7 supernatant.

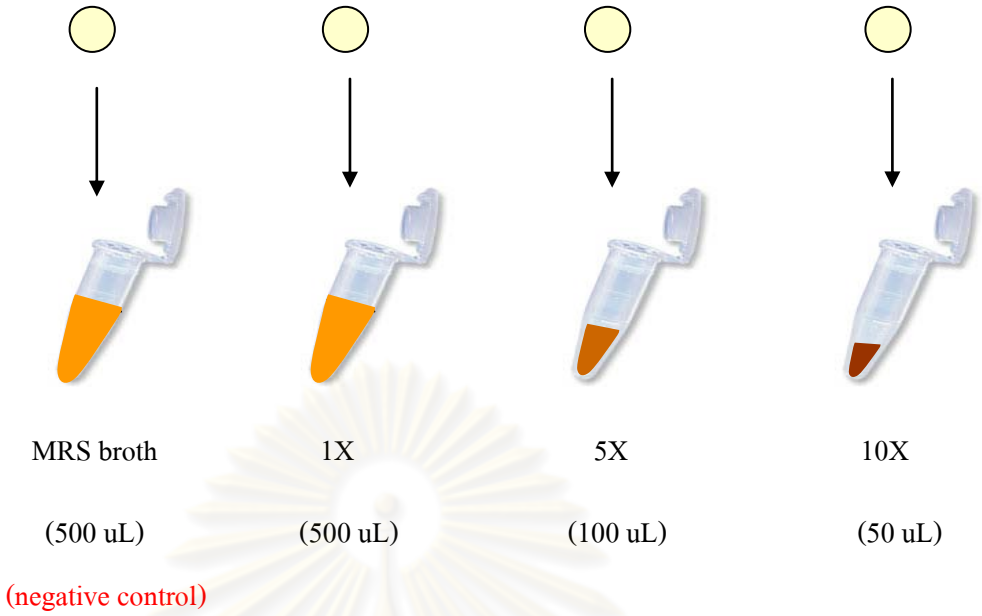


Figure 17 Preparation of paper disks. The sterile 6 mm-membrane disks were dipped into resuspended LCM for at least 1 hr at room temperature.

Disk diffusion method

The various concentrations of *L. plantarum* B7 supernatants were evaluated at two pH values, their intact pH 4, and adjusted pH 7 with NaOH, to detect the roles of concentration and lactic acid (pH), moreover, the possible involvement of additional inhibitory factors in the anti-*H. pylori* activity.

H. pylori was spreaded on Columbia blood agar plates, and *L. plantarum* B7 (LCM) disks were placed directly on the surface of the agar. The plates were incubated under microaerophilic conditions at 37°C for 72 h (figure 18), after which the diameters of the inhibition zones were measured in millimetres. In this study, the MRS broth was used as negative control. The experiments were tested in duplicate and mean values of growth inhibition zone were measured.

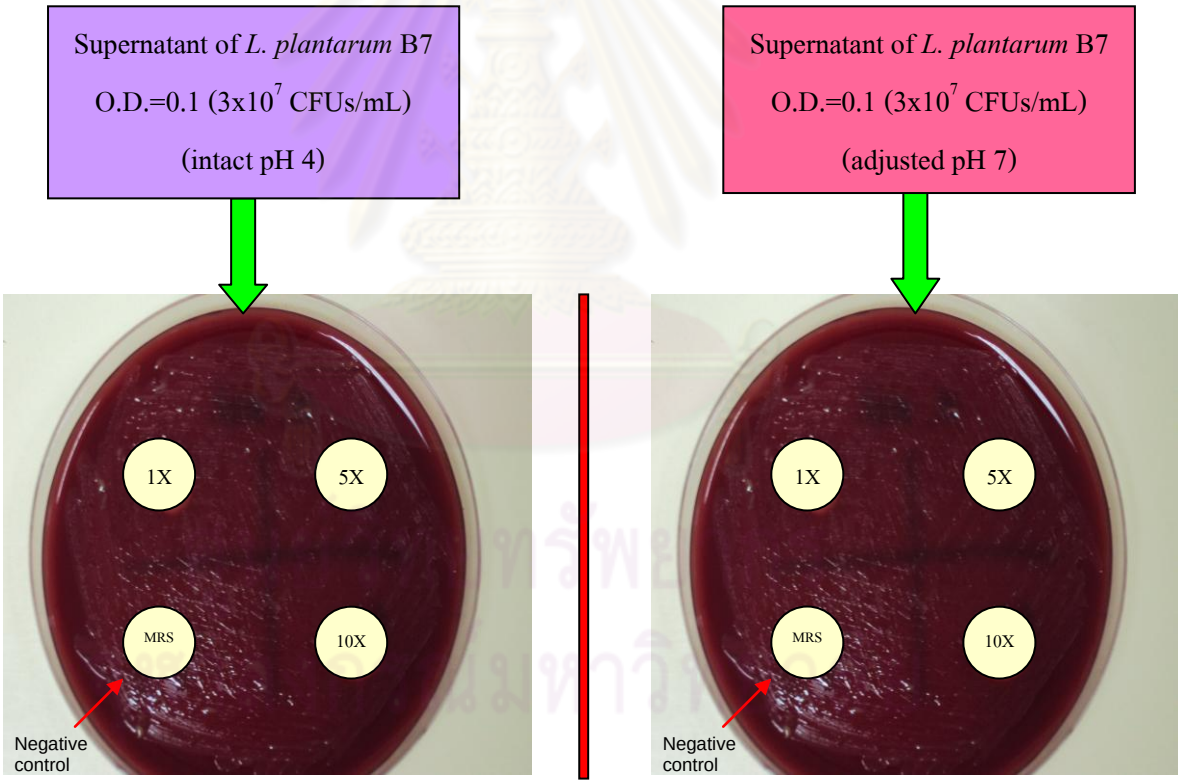


Figure 18 Show the disk diffusion method.

***In vivo* study**

Bacteria and animal preparation

H. pylori was subcultured twice on Columbia blood agar. Plates were incubated at 37 °C under microaerophilic condition for 72 h.

L. plantarum B7 was originally obtained from Thai dyspeptic patients who visited at the King Chulalongkorn Memorial hospital. This strain was cultivated twice on MRS agar anaerobically at 37 °C for 48 h.

Thirty-two male Spraque-Dawley rats (Salaya research animal center, Mahidol University, Bangkok, Thailand), weighed about 150-250 grams at the beginning of the experiment, were used. The experimental protocol was approved by the Ethical Committee of Medicine Faculty, Chulalongkorn University, Thailand. The animals were in Macrolon cages (5 animals per cage), given food and tap water ad libitum in a room temperature 18-22 ºC, humidity 55%, and 12/12 hours-light/dark cycle. The rats were divided into four groups of eight rats each as the following:

1. Control with vehicle (Control group)

The animals were received phosphate buffered saline (PBS) 1 mL/rat by gavage twice a day at an interval of four hours for three consecutive days. Then, they were housed with freely access to water and standard food for one week. After that, the animals were treated with PBS 1 mL/rat by gavage once daily for one week.

2. *H. pylori* infection with vehicle (*H. pylori* infected group)

Rats were inoculated with *H. pylori* by using model of Thong-Ngam *et, al*. [192]. Briefly, the *H. pylori* suspension (5×10^{10} CFUs/mL) in PBS was administered to the rats (1mL/rat) by gavage twice daily at an interval of four hours for three consecutive days. One week after the inoculation, the animals were treated with PBS (1 mL/rat) by gavage once daily for one week.

3. *H. pylori* infection with *L. plantarum* B7 10^6 CFUs/mL treatment (*L. plantarum* B7 10^6 CFUs/mL treated group)

In order to make the *H. pylori* infection, the same protocol was performed as the previous model. After one week of *H. pylori* inoculation, the rats were treated orogastrically with *L. plantarum* B7 10^6 CFUs/mL suspended in PBS once daily for one week [165].

4. *H. pylori* infection with *L. plantarum* B7 10^{10} CFUs/mL treatment (*L. plantarum* B7 10^{10} CFUs/mL treated group)

The identical protocol of *H. pylori* inoculation was performed. After one week, the rats were treated orogastrically with *L. plantarum* B7 10^{10} CFUs/mL suspended in PBS once daily for one week [165].

The procedures of animal preparation were concluded in the diagram as shown below.

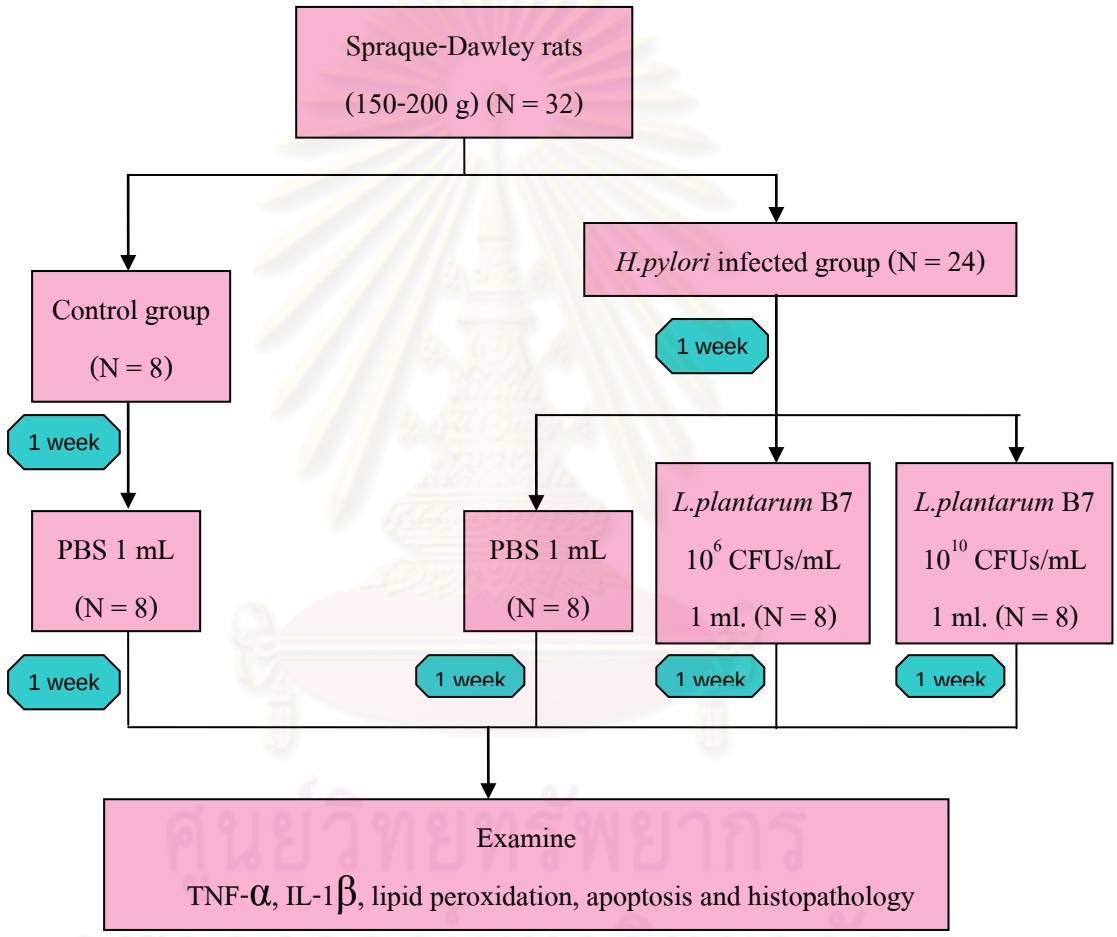


Figure 19 Diagram of experimental groups

***H. pylori* inoculation in the rat stomach** [192]

Streptomycin suspended in tap water (5 mg/mL) was pre-treated for three days before *H. pylori* inoculation. Then, the animals were fasted for 18 hours but can access to tap water ad libitum. The *H. pylori* suspension (5×10^{10} CFUs/mL) in PBS was administered to the rats (1 mL/rat) by intragastric tube twice daily at an interval of four hours for three sequential days. After the last inoculation, these animals were housed and given standard food and tap water ad libitum for one week.

Detection of *H. pylori* infection in gastric tissues

After finishing the experiment, rats were sacrificed. Antral tissues from the rats' stomach were taken for detecting the *H. pylori*. The present of *H. pylori* was determined by either positive rapid urease test or positive histopathology

Enzymatic test by rapid urease test

The stomach was removed and longitudinally dissected along the greater curvature from the anesthetised rat. The remained food in stomach was gentle removed. The 2 mm^2 of gastric mucosa from antrum, the area that *H. pylori* most colonized [191], was immediately cut and placed in the urease test tube in order to examine the urease activity. The gastric tissues were contacted with a gel containing urea and phenol red, a pH indicator in a tube. In the case of *H. pylori* infection, the enzyme activity converting urea into ammonia will be occurred. The increase of ammonia causes increase pH, so that the yellow of indicator in urease tube will be changed to pink as shown in figure 20. The results of rapid urease tests are read within 24 hours. The urease test has 86% to 97% sensitivity and 86% to 98% specificity [194].

Positive urease test

$(NH_2)_2CO + H_2O \xrightarrow{\textit{H. pylori}\ \text{urease}} CO_2 + 2NH_3$ (increase pH)

Figure 20 Rapid urease test: the presence of *H. pylori* urease is indicated by ammonia production.

Histopathology

After gastric antrum biopsy was taken for urease test, the remaining was fixed in 4% Paraformaldehyde in phosphate buffer solution, pH 7.4 at room temperature. Gastric tissue was processed by standard methods, embedded in paraffin, cut at 5μm, picked up on glass slides, and stained with hematoxylin-eosin (H&E). The slides were observed on light microscopy. However, the presence of *H. pylori* was detected by Warthin-Starry staining in the unclear cases. A grading system was adapted to evaluate the level of bacterial colonization.

The percentage of *H. pylori*-infected rate was calculated from following equation: 100 x (number of infected-rats / number of inoculate rats). Number of infected rats was identified by positive test either urease test or histopathology. The positive urease test must detect the color change of pH indicator in 24 hours. The antrum samples that give pink in the urease test tube were *H. pylori* positive. The remained stomach samples were detected for *H. pylori* colonization by the pathologist using scoring system [195] as follows, score 0: no bacteria detected; score 1: mild colonization in some gastric crypts; score 2: mild colonization in most gastric crypts; score 3: moderate colonization in all gastric crypts. The score 0 means no *H. pylori* detection. The stomach samples that were given score 1 or more were *H. pylori* positive.

In addition to the *H. pylori* colonization, the gastric inflammation level was estimated and scored by the pathologist following the updated Sydney System [195]. The infiltration of polymorphonuclear leucocyte in the gastric mucosa defining the inflammatory scores was recorded. Scored from 0 to 3 represented normal, mild, moderate, and marked histopathology changes, respectively.

TNF-α and IL-1β assay

Enzyme-linked immunosorbent assay (ELISA) is a useful method in evaluating the concentration of proteins in a sample such as serum, plasma, or cell culture supernatant. ELISA kit is performed in 96 well plates which allow high throughput results. In principle, a monoclonal antibody has been pre-coated onto a microplate. Standards control, and samples are pipetted into the wells and any antigen present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The

intensity of color measured is in proportion to the amount of antigen bound in the initial step. The sample values are then read off the standard curve.

At the day of study, the Quantikine® rat TNF-α and IL-1β (R&D systems, USA) were used to determine level of TNF-α and IL-1β, respectively, in rat serum. The assay procedures were performed as protocol descriptions from the company and explained as following.

Procedure

1. For sample preparation, blood was collected by cardiac puncture to measure TNF-α and IL-1β levels in serum. The blood sample was allowed to clot for two hours at room temperature before centrifuging for 20 minutes at approximately 1000 x g. Then, the serum was removed and stored at -80˚C until the day of analysis. The samples were not allowed to repeat freeze-thaw cycles.

2. For standard curve preparation, the standard was diluted with 2 mL of calibrator diluent to obtain a stock solution of 800 and 2,000 pg/mL of TNF-α and IL-1β, respectively. After producing the stock solution, the standard was gently mixed at least 5 minutes prior to making dilutions. The polypropylene tubes were labeled and added the stock solution and calibrator diluent to each tube as described below.

Vial	Stock Solution Volume (µL)	Calibrator Diluent Volume (µL)	TNF-α Concentration (pg/mL)
A	200 of stock	0	800
B	200 of vial A	200	400
C	200 of vial B	200	200
D	200 of vial C	200	100
E	200 of vial D	200	50
F	200 of vial E	200	25
G	200 of vial F	200	12.5
H	0	200	0

Table 2 The TNF-α concentration (pg/mL) was prepared by adding the stock solution and calibrator diluent. The undiluted rat TNF-α standard was served as the high standard (800 pg/mL). Moreover, calibrator diluent was served as the zero standard (0 pg/mL).

Vial	Stock Solution Volume (μL)	Calibrator Diluent Volume (μL)	IL-1β Concentration (pg/mL)
A	200 of stock	0	2,000
B	200 of vial A	200	1,000
C	200 of vial B	200	500
D	200 of vial C	200	250
E	200 of vial D	200	125
F	200 of vial E	200	62.5
G	200 of vial F	200	31.2
H	0	200	0

Table 3 The IL-1β concentration (pg/mL) was prepared by adding the stock solution and calibrator diluent. The undiluted rat IL-1β standard and calibrator diluent were served as the high standard (2,000 pg/mL) and the zero standard (0 pg/mL), respectively.

3. For performing the assay, all samples, standards, and control were prepared as in the reagent preparation part of the protocol and assayed in duplicate. Next, 50 μL of assay diluent was added to each well and followed by 50 μL of standards, control, or samples to each well. Plate was gently tapped, covered with the adhesive strip and incubated for 2 hours at room temperature. Next, each well was aspirated and washed for five times. Then, 100 μL of TNF-α and IL-1β conjugate were added to each well. Plate was covered and incubated again for 2 hours at room temperature. After two hours, each well was aspirated and washed for five times. Then, 100 μL of substrate solution was added to each well. Incubation was performed for 30 minutes and must protect from light. After that, 100 μL of stop solution was added to each well and gently tapped the plate to ensure through mixing. The last step, the optical density (O.D.) was determined within 30 minutes by using a microplate reader (Model 680, Bio-Rad Laboratories, Inc., California, U.S.A.) set to 450 nm and wavelength correlation to 540 nm.

Calculation

To calculate results, the duplicated readings for each standard, control, and samples were averaged and subtracted the average zero standard optical density. Next, the standard curve was

created by using computer software of generating a linear curve-fit. The measured O.D. of each unknown was converted to its corresponding concentration by standard curve.

The data were linearized with computer software and the best fit line can be determined by regression analysis. The sample concentrations were calculated from the linear equation of standard curve.

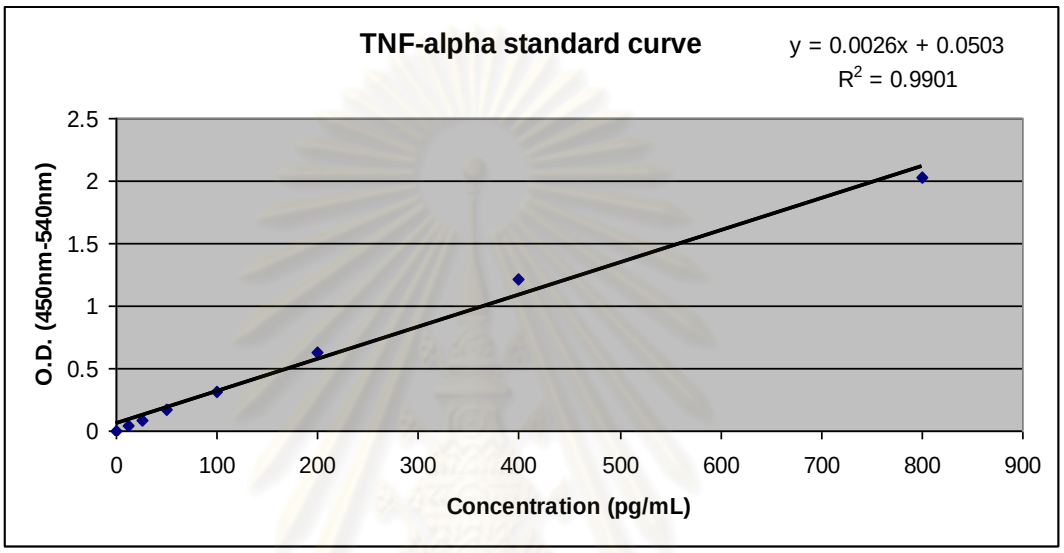


Figure 21 A standard curve of TNF-α: The x-axis and y-axis of the standard curve represented standard TNF-α concentrations (pg/mL) and O.D., respectively. Note: O.D. = Obtical density; pg/mL = pictogram per millilitre; nm = nanometer

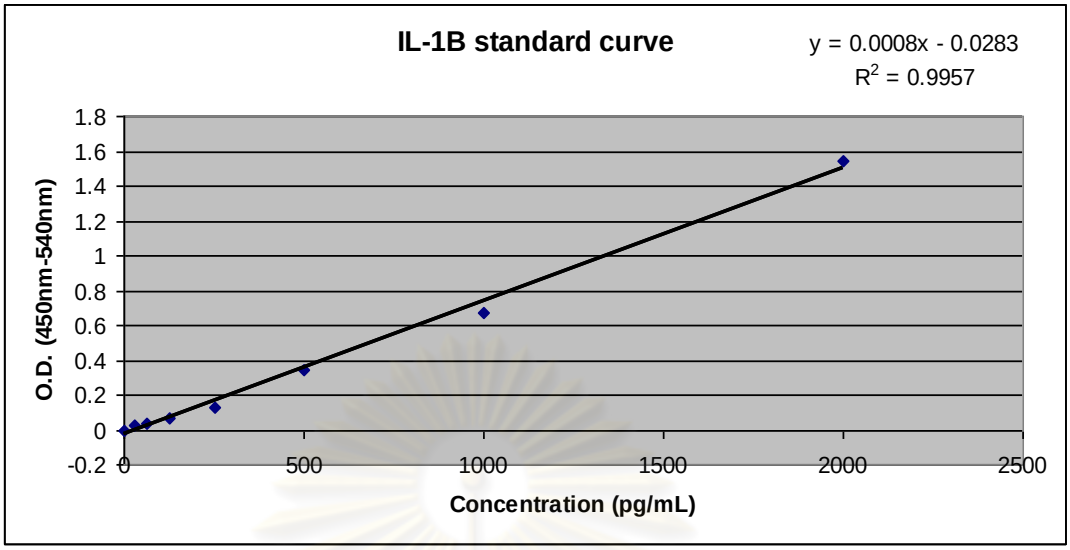


Figure 22 A standard curve of IL-1β: The x-axis and y-axis of the standard curve represented standard IL-1β concentrations (pg/mL) and O.D., respectively.

Gastric Malondialdehyde (MDA) Determination

MDA was measured from the homogenized tissue by using thiobarbituric acid reactive substances (TBARS) assay. Basically, principle of the method is the reaction of one molecule of MDA and two molecules of TBA to form a red MDA-TBA complex under high temperature (90-100 °C) and acidic conditions, which can be quantitated spectrophotometrically at 532 nm.

At the day of study, the TBARS assay kit (Cayman, USA) were used to determine level of MDA in gastric tissue homogenates. The assay procedures were performed as protocol descriptions from the company and described below.

Procedure

1. For sample preparation, 50 mg of gastric tissue was placed into a 1.5 mL centrifuge tube containing 500 µL of RIPA buffer (Cell Signaling Technology, Inc., USA) and sonicated for 15 seconds at 40V over ice. The supernatant was collected by centrifugation at 1,600 x g for 10 minutes at 4 °C, aliquot into sterile microcentrifuge tubes for detecting MDA and protein assay and stored at -80˚C until the day of analysis.

2. For standard curve preparation, 250 µL of the MDA standard was diluted with 750 µL of water to obtain a stock solution of 125 µM. The test tubes were labeled and added the amount of 125 µM MDA stock solution and water to each tube as described below.

Tube	MDA (µL)	Water (µL)	MDA Concentration (µM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

Table 4 Preparation of MDA concentration (µM).

3. For performing the assay, the tubes with screw caps were used and added 100 µL of samples or standards. Next, 100 µL of SDS solution and 4 mL of the color reagent were added to each tube. The tubes were capped and heated in the water-bath at 95 °C for one hour. After cooling the tubes by immersion in ice bath and incubation on ice for 10 minutes, they were centrifuged at 1,600 x g for 10 minutes at 4 °C and then placed at room temperature for 30 minutes. After loading 150 µL of each tube to plate, the absorbance was measured at 532 nm.

Calculation

By plotting the mean absorbance for each standard on the y-axis against the concentration of MDA (µM) on the x-axis, a standard curve was created to calculate the results. MDA levels of the samples were examined from the linear regression equation of a standard curve. The content of lipid peroxide is expressed in term of nmol of MDA/gram of wet weight and the total protein was determined by the BCA protein assay kit (Thermo Scientific, USA) to correct the MDA level which is expressed in terms of nmol/mg protein.

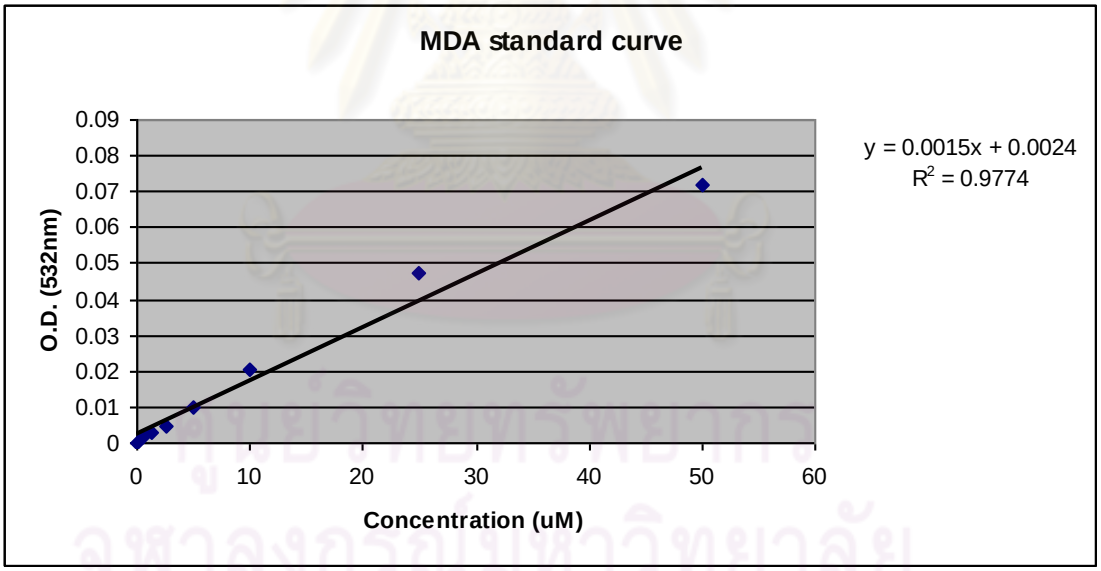


Figure 23 A standard curve of MDA: The x-axis and y-axis of the standard curve represented standard MDA concentrations (µM) and O.D., respectively.

BCA Protein Assay

BCA was measured from the homogenized tissue by using BCA protein assay. In principle, the amino acids (cysteine, cystine, tryptophan and tyrosine) and the peptide bonds of protein react with the Cu^{2+} (Cu^{2+}-protein complex) under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. Interestingly, the amount of reduction is proportional to the protein present. Then, the cuprous cation (Cu^{1+}) is colorimetrically detected by reaction with bicinchoninic acid (BCA). The Cu^{1+}-BCA complex results in the purple-colored reaction product which is analyzed by visible spectrophotometer at 562 nm.

At the day of study, the BCA protein assay kit (Thermo Scientific, USA) was used to assess the total protein of gastric tissue homogenates in terms of mg protein. The assay procedures were performed as protocol descriptions from the company and explained below.

Procedure

1. For sample preparation, the same sample, aliquot into microcentrifuge tubes and stored at -80˚C until the day of analysis, as the MDA detection was used.

2. For standard curve preparation, Albumin Standard (BSA) was diluted with RIPA buffer. Use the following table as a guide to prepare a set of standards (assay range = 125-2,000 µg/mL).

Vial	RIPA buffer Volume (µL)	BSA Source and Volume (µL)	Concentration (µg/mL)
A	0	200 of stock	2,000
B	66	200 of stock	1,500
C	100	100 of vial A	1,000
D	100	100 of vial B	750
E	100	100 of vial C	500
F	100	100 of vial E	250
G	100	100 of vial F	125

Table 5 Preparation of BCA concentration (µg/mL).

3. For performing the assay, 9 µL of each replicate of control, standard and unknown sample was pipetted to the center of the microplate well. Then 4 µL of compatibility reagent solution was added to each well. The plate was covered, mixed on a plate shaker at medium speed for one minute and incubated at 37 °C for 15 minutes. Next, 260 µL of the BCA working reagent was added to each well. The plate was covered and mixed on a plate shaker for one minute again. After cooling the plate at room temperature for 5 minutes, the absorbance of the standards, controls and unknown samples were measured at 562 nm on a plate reader.

Calculation

By using the control replicates from the 562 nm absorbance value as the blank, they were subtracted from the 562 nm absorbance value of all standard and unknown sample replicates. The standard curve was prepared by plotting the average blank-corrected 562 nm value for each BCA standard on the y-axis against its concentration (µg/mL) on the x-axis. To determine the protein concentration of each unknown sample, the linear regression equation of the standard curve was used.

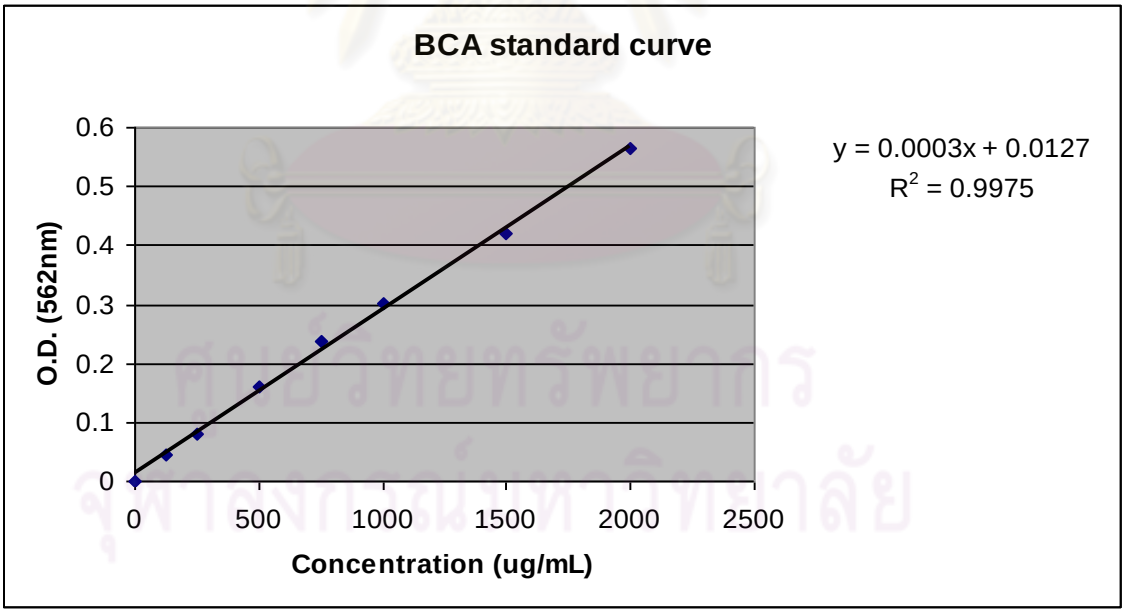


Figure 24 A standard curve of BCA: The x-axis and y-axis of the standard curve represented standard BCA concentrations (µg/mL) and O.D., respectively. Strong correlation (R^2 = 0.9975) was able to present by the linear equation of y = 0.0003x + 0.0127.

Gastric apoptosis determination

The DNA fragmentation is characteristic apoptotic cell which is detected by TdT-mediated X-dUTP nick end labeling (TUNEL) assay (ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon, USA). By definition, the formation of large numbers of DNA fragments leads to the presence of large numbers of exposed DNA 3'-hydroxyl (OH) ends. From this feature, DNA end-labeling or *in situ* end labeling assays have enabled to develop. One form of the end labeling assay is the TUNEL assay. In principle, the nucleotides are enzymatically added to the DNA by the terminal deoxynucleotidyl transferase (TdT). TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double-stranded or single-stranded DNA. The incorporated nucleotides form an oligomer composed of digoxigenin-conjugated nucleotide and unlabeled nucleotide in a random sequence. DNA fragments which have been labeled with the digoxigenin-nucleotide are then allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule. The bound peroxidase antibody conjugate enzymatically generates a permanent, intense, localized stain from chromogenic substrates, providing sensitive detection in immunohistochemistry.

Procedure

1. Deparaffinized Tissue Section

Stomach sections of 5 μm thick from paraffin-embedded blocks were put on slides and deparaffinized in 3 changes of xylene for 10 minutes. Next, the slides were hydrated in 3 changes of 95% alcohol for 5 minutes and washed in 3 changes of distilled water in a coplin jar for 5 minutes. The slides should not be allowed to dry out at any point during procedure.

2. Pretreatment of Tissue

Freshly Proteinase K (DAKO, USA) was applied on the slides (15 μL/cm^2) at room temperature for 10 minutes in order to suffice permeabilization of cells, so TUNEL reagents can reach nuclei. After incubation, the slides were washed in 3 changes of distilled water in a coplin jar for 3 minutes per wash and then gently tapped off excess distilled water.

3. Application of TUNEL Reagents

Equilibration buffer (15 μL/cm^2) was immediately applied directly on slides for at least 10 seconds at room temperature. Next, working strength TdT enzyme was applied on the slides (15 μL/cm^2). The slides were covered with plastic coverslips and incubated in a humidified chamber at 37^{o}C for 1 hour. After incubation, working strength stop/wash buffer was applied

directly on the slides (300 μL/cm^2). Then, the slides were agitated for 15 seconds and incubated for 10 minutes at room temperature. The slides were washed in 2 changes of PBS for 3 minute each wash and gently tapped off excess liquid. Anti-digoxigenin-peroxidase was applied on the slides (15 μL/cm^2) for 30 minutes at room temperature. After that, the slides were washed in 2 changes of PBS for 3 minutes per wash and gently tapped off excess liquid.

4. Color Development and Counterstain

3,3' diaminobenzidne (DAB) was applied on the slides (15 μL/cm^2) for 10 minutes at room temperature. Next, the slides were run with tap water for 3 minutes, immersed in hematoxylin for 10 seconds at room temperature and run with tap water again for 1 minute. Then, the slides were immersed in 0.1% lithium carbonate for 10 seconds, run with tap water for 1 minute and dehydrated by moving the slide through 2 chambers of 95% alcohol and 2 chambers of xylene, respectively for 10 seconds in each chamber. After dehydration, the slides were mounted with a mounting medium. The positive stained cells presented dark brown nuclei under light microscopy. To verify the incidence of apoptosis, the numbers of dark brown-stained cells were counted. One thousand of gastric epithelial cells were counted for each rat. The data were shown as percentage (%) of apoptotic cells calculating from this equation: the percentage of apoptotic cells (%) = (numbers of positive stained cells x 100) / 1000.

Statistical analysis

Data were expressed and analyzed as means ± standard deviation. Each parameter was compare among groups with One-way analysis of variance (One-way ANOVA), unpaired *t*-test, LSD, and Duncan (α=0.05) with SPSS program version 11.0

CHAPTER IV

RESULTS

In vitro study

Anti-*H. pylori* activity of intact pH 4 and adjusted pH 7 of *L. plantarum* B7 $3x10^7$ CFUs/mL supernatants at the various concentrations of 1X, 5X and 10X were tested using a disk diffusion method. In this study, the MRS broth was used as negative control. The inhibitory growth effect of *H. pylori* was interpreted by size of the inhibition zone. The experiments were tested in duplicate and mean values of growth inhibition were calculated.

In vitro experiment, both intact pH 4 and adjusted pH 7 of *L. plantarum* B7 supernatants showed anti-*Helicobacter* activity in a dose-dependent manner, but the supernatant of low pH *L. plantarum* B7 at the concentration of 10X showed the clearest inhibition (average diameter of the inhibition zone 13 mm).

At intact pH 4, mean inhibitory zone diameters of 8.5 and 13 mm were noted at concentrations of 5X and 10X of *L. plantarum* B7 supernatant disks, respectively. At adjusted pH 7, *L. plantarum* B7 supernatants at concentrations of 5X and 10X yielded mean inhibitory zone diameters of 6.5 and 11 mm, respectively. Both pH 4 and pH 7, MRS broth, used as negative control, and *L. plantarum* B7 supernatant at the concentration of 1X did not inhibit the growth of *H. pylori*. The averages of inhibition zone diameters (mm) of all concentrations of *L. plantarum* B7 supernatant at intact pH 4 and adjusted pH 7 were shown in table 6 and figure 25-27.

Concentration of *L. plantarum* B7 supernatant	Diameters of inhibition zone (mm)	
	L. plantarum B7 supernatant at intact pH 4	*L. plantarum* B7 supernatant at adjusted pH 7
MRS (negative control)	0	0
1X	0	0
5X	8.5 ± 0.7	6.5 ± 0.7
10X	13 ± 0	11 ± 1.4

Table 6 Means ± SD of inhibition zone diameters (mm) of all *L. plantarum* B7 supernatant concentrations at intact pH 4 and adjusted pH 7. (n = 2)

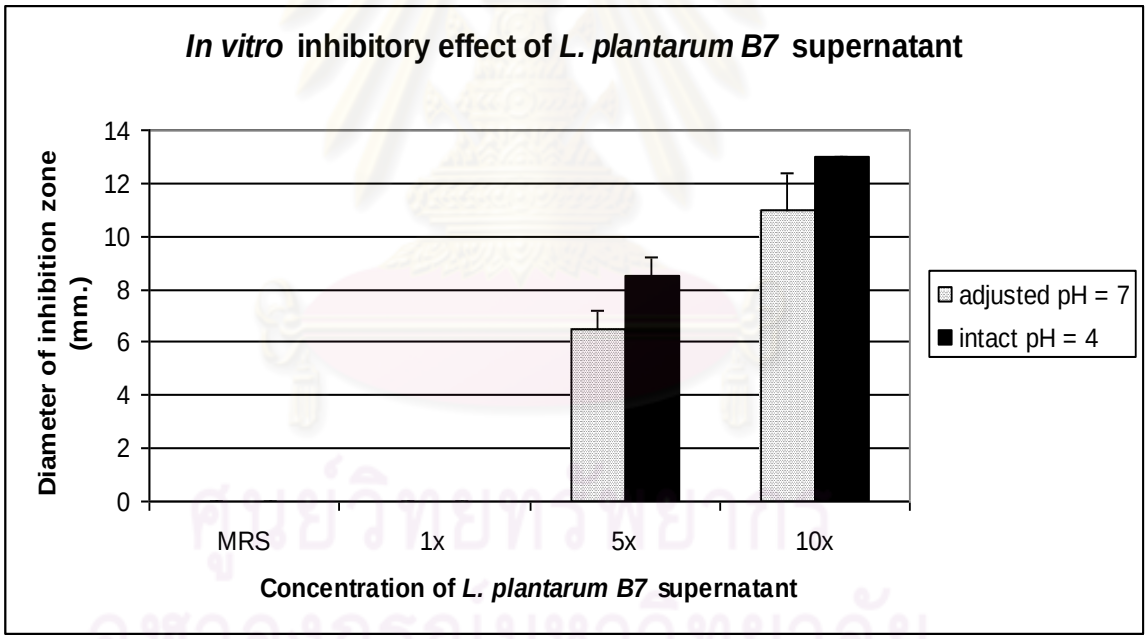


Figure 25 A bar graph shows the mean ± SD of inhibitory zone diameters (mm) of all *L. plantarum* B7 supernatant concentrations at intact pH 4 and adjusted pH 7. (n = 2)

intact pH 4

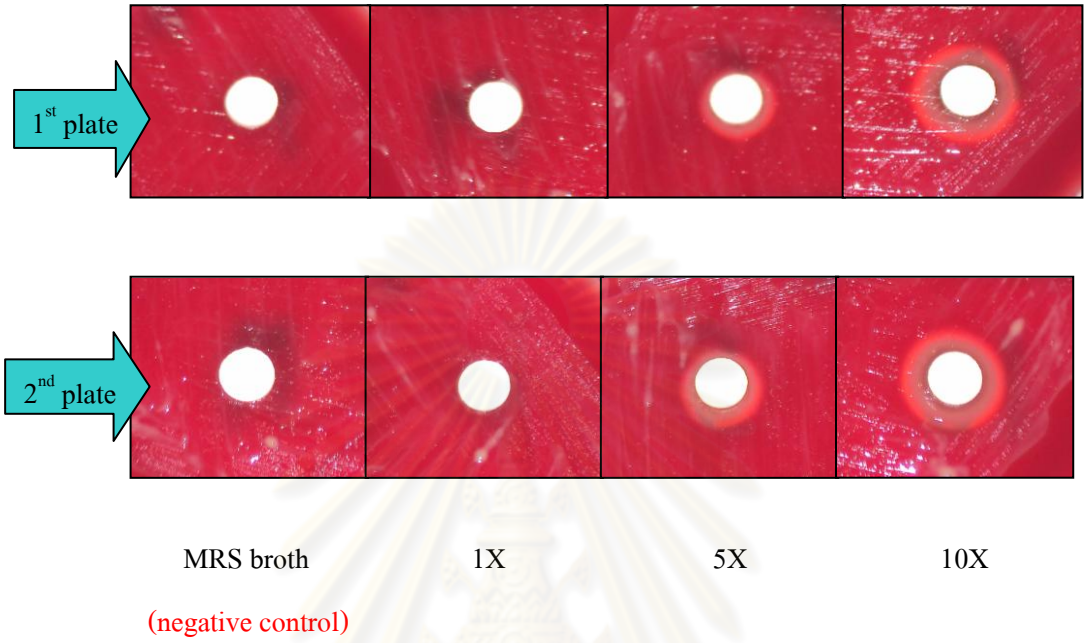


Figure 26 Show inhibition zone of all concentration of *L. plantarum* B7 supernatant disks at intact pH 4.

adjusted pH 7

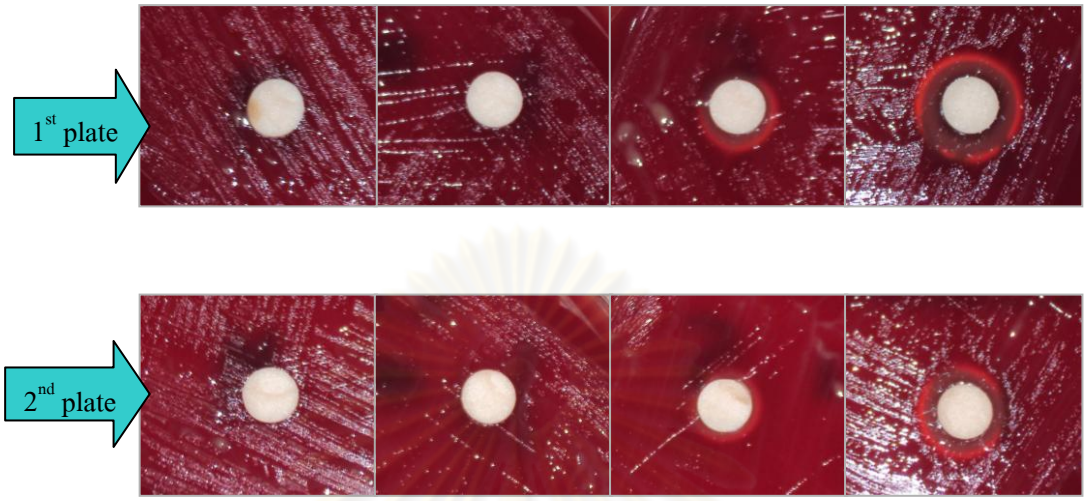


Figure 27 Show inhibition zone of all concentration of *L. plantarum* B7 supernatant disks at adjusted pH 7.

***In vivo* study**

***H. pylori* infection in rat stomach**

In this experiment, the method of *H. pylori* inoculation was using according to the method explained by Thong-Ngam *et al*. [192]. It was found that *H. pylori* was detected by positive either urease test or histopathology. For this experiment, in *H. pylori* inoculation group, there were 27 of 29 rats (93.1 %) were infected using this technique.

When the rat was terminated by injection of an overdose of thiopental, the 2 mm^2 of gastric antrum mucosa was immediately cut and examined the *H. pylori* urease activity. After that, the remaining stomach was fixed in 4% buffer paraformaldehyde at room temperature for 24 h. The gastric tissue was processed, embedded in paraffin and cut at 5μm thickness. The sections were picked up on glass slides, stained with H&E and microscopically examined.

By histological examination, there were no *H. pylori* found in the control group as shown in figure 28. In *H. pylori* infected group, the infiltration of inflammatory cells were detected, and *H. pylori* were observed especially in the antrum of stomach as shown in the figure 29a and 29b, respectively.

The histopathology in control group was normal (figure 30a and 31a), while, in *H. pylori* infected group revealed mild to moderate *H. pylori* colonization and inflammation (figure 30b and 31b). *L. plantarum* B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups reduced *H. pylori* colonization and improved stomach inflammation (figure 30c and 31c, and figure 30d and 31d, respectively). Histological score of *H. pylori* colonization and gastric inflammation were summarized in table 7.

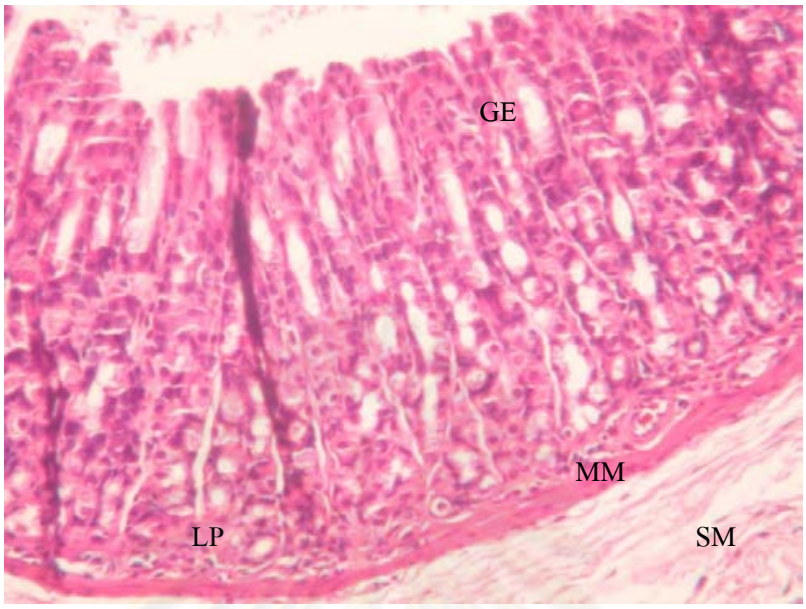
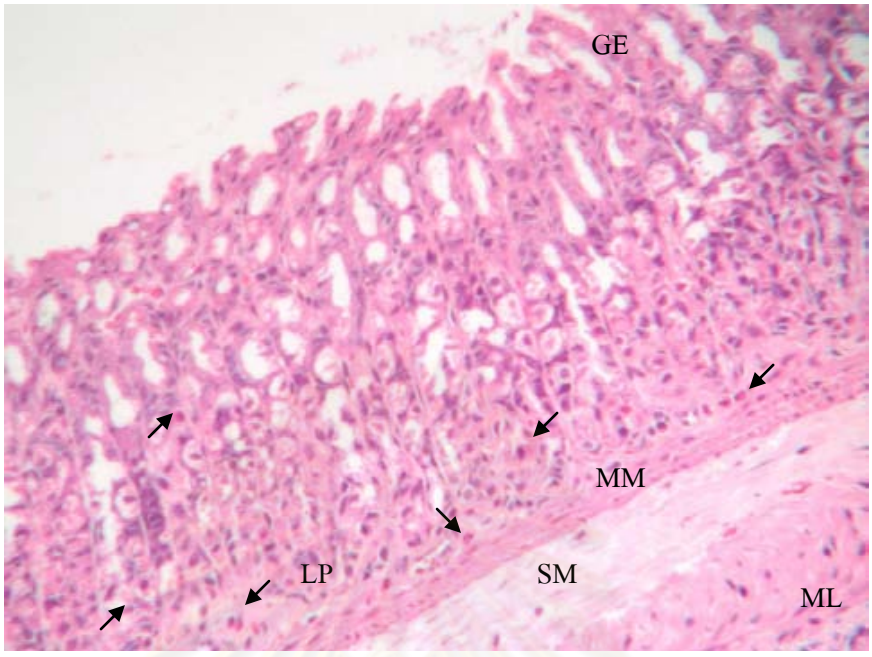
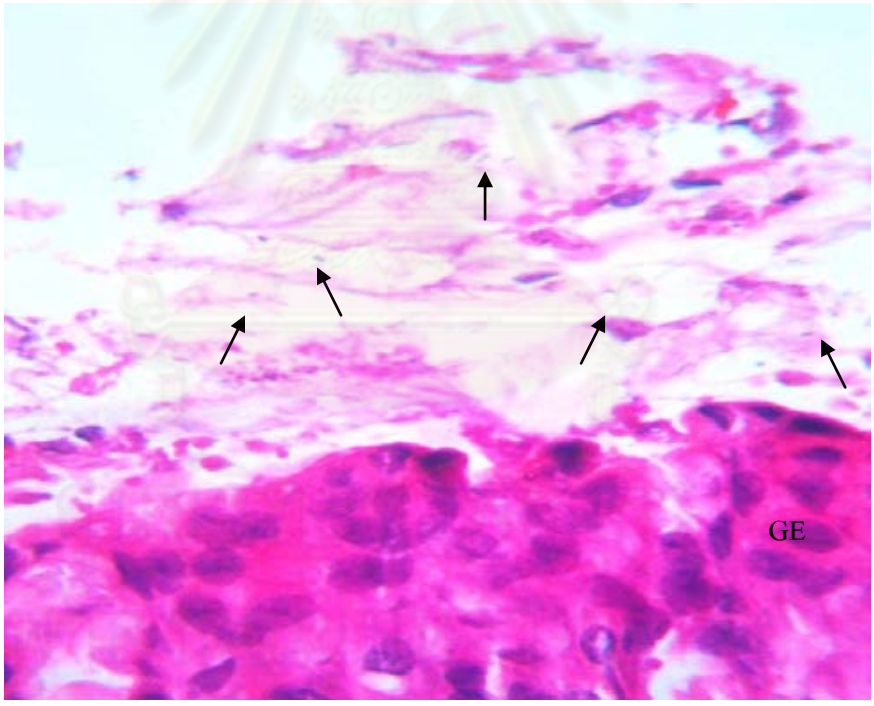


Figure 28 Histopathology of control group (H&E straining, x20); GE: gastric epithelium, LP: laminar propria, MM: muscularis mucosae, and SM: submucosa.



(a)



(b)

Figure 29 Histopathology of *H. pylori* infected group; GE: gastric epithelium, LP: laminar propria, MM: muscularis mucosae, SM: submucosa, and ML: muscularis. Gastric mucosa and histology of rat in *H. pylori* infected group; (a) note the infiltration by inflammatory cells (arrows) in LP (H&E straining, x20). (b) *H. pylori* clumped on gastric epithelium (arrows) (H&E straining, x40).

Group	Number	Level of *H. pylori* Colonization				Gastric Inflammation			
		0	1	2	3	0	1	2	3
Control group	8	8	-	-	-	8	-	-	-
H. pylori infected group	8	1	5	2	-	-	3	5	-
L. plantarum B7 10^6 CFUs/mL treated group	8	4	4	-	-	-	8	-	-
L. plantarum B7 10^{10} CFUs/mL treated group	8	3	5	-	-	-	8	-	-

Table 7 Summary of the scores of the bacterial colonization levels and gastric inflammation in all groups.

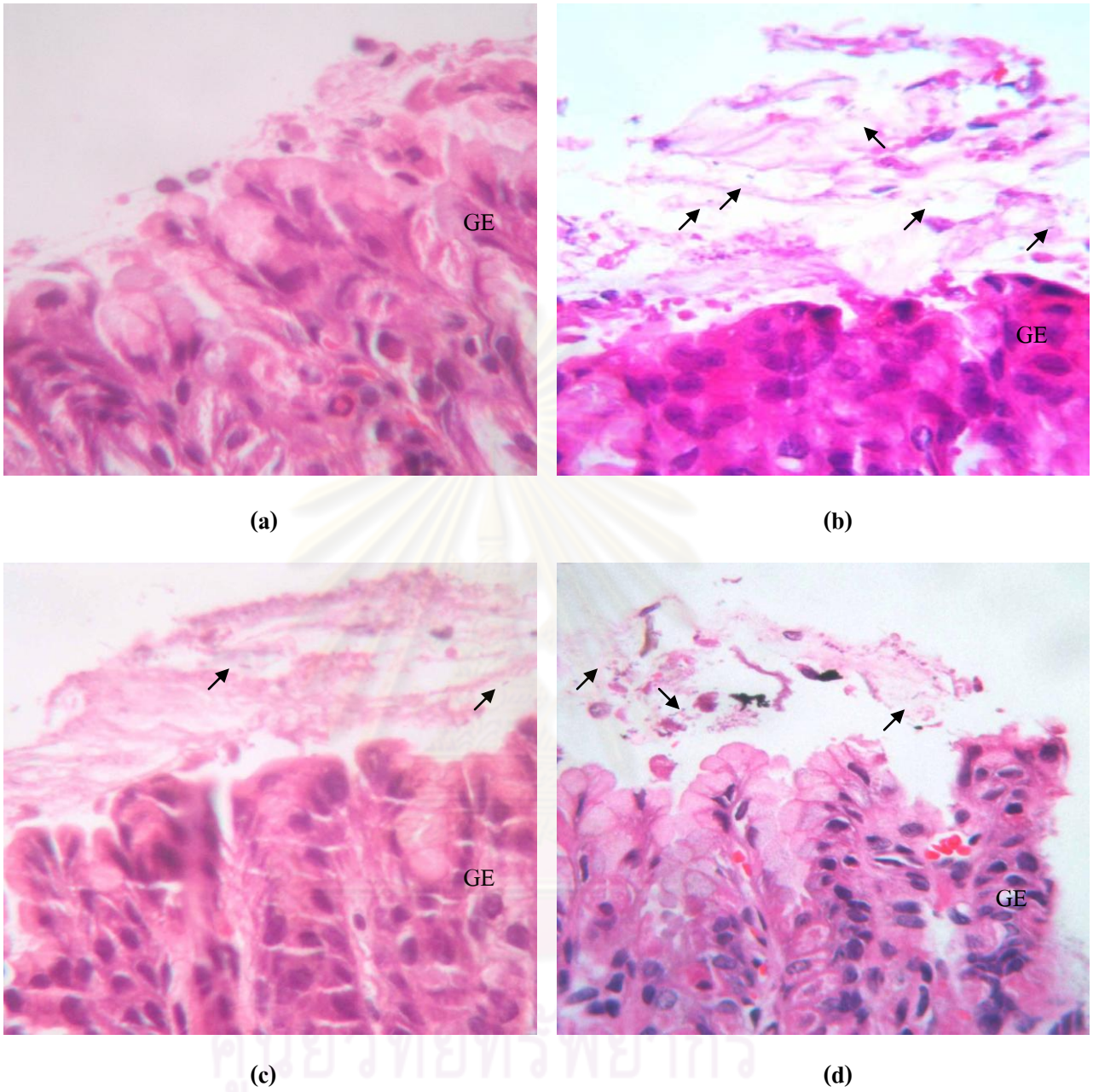


Figure 30 Hematoxylin-eosin stained gastric sections (x40), GE: gastric epithelium. (a) control group showed no *H. pylori* detection. (b) *H. pylori* infected group showed colonization of *H. pylori*. (c) and (d) *L. plantarum* B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed decrease of *H. pylori* colonization.

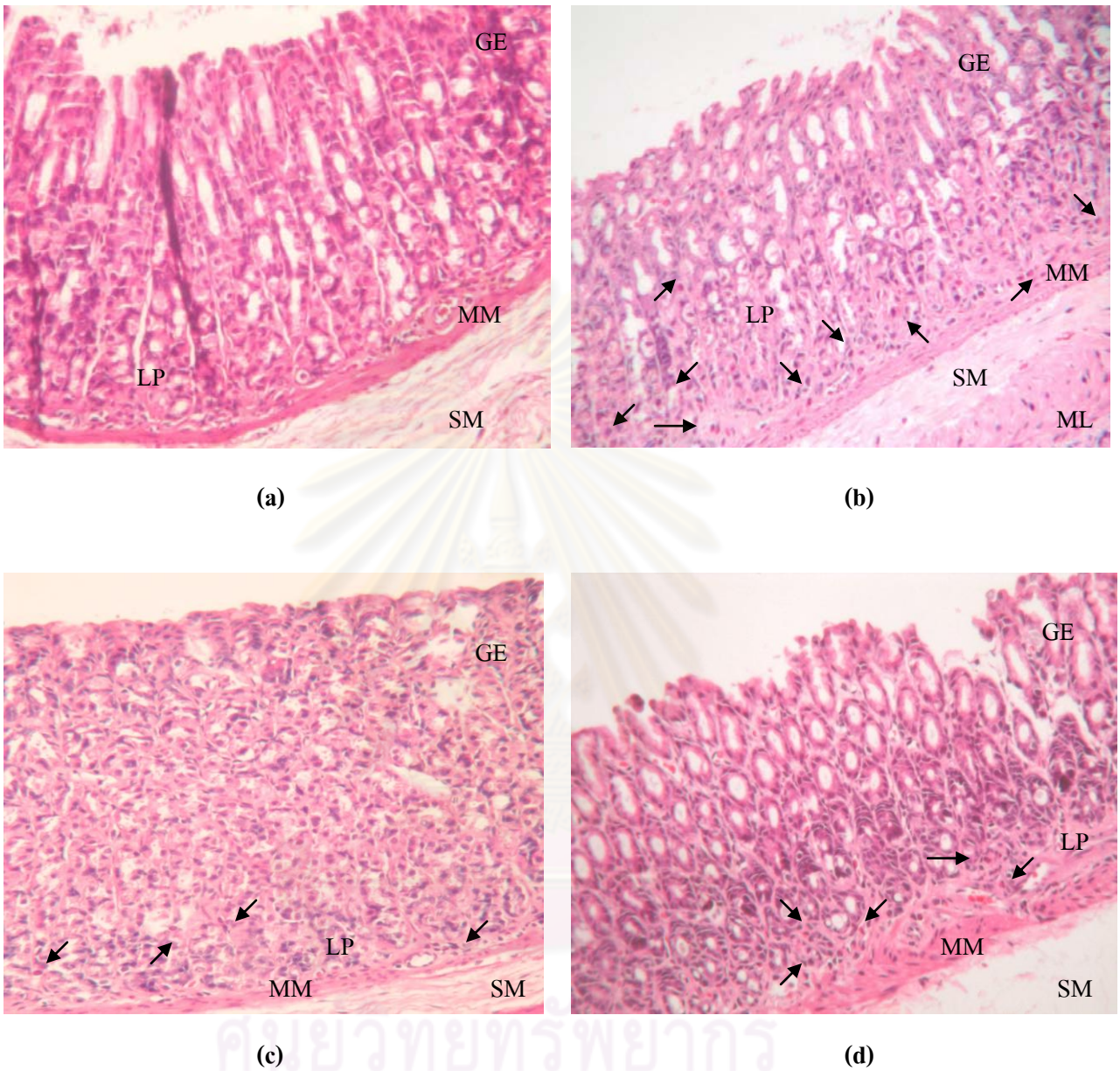


Figure 31 Hematoxylin-eosin stained gastric sections (x20), GE: gastric epithelium, LP: laminar propria, MM: muscularis mucosae, SM: submucosa, and ML: muscularis. (a) control group showed normal gastric histopathology. (b) *H. pylori* infected group showed infiltration of inflammatory cells (arrow). (c) and (d) *L. plantarum* B7 10^{6} CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed examples of improvement in gastric inflammation.

Effects of *H. pylori* infection on serum TNF-α level and role of *L. plantarum* B7

The serum TNF-α level examined by using ELISA assay. From the results, the serum TNF-α levels were not significantly different between control group and *H. pylori* infected group. However, in *L. plantarum* B7 10^6 CFUs/mL treated group, there was significant decrease of serum TNF-α level compared with *H. pylori* infected group (p = 0.019). The average concentrations of serum TNF-α were 17.22 ± 0.63 pg/mL, 18.05 ± 1.94 pg/mL, and 16.52 ± 0.84 pg/mL in control, *H. pylori* infected, and *L. plantarum* B7 10^6 CFUs/mL treated group, respectively. TNF-α concentration tended to decrease in *L. plantarum* B7 10^{10} CFUs/mL treated group but no significant difference ($p > 0.05$). The averages of serum TNF-α level of all groups were shown in table 8 and figure 32.

Group (n = 8)	Serum TNF-α level (pg/mL)	p-value (compared with control group)	p-value (compared with *H. pylori* infected group)
control	17.22 ± 0.63	-	0.189
H. pylori infected	18.05 ± 1.94	0.189	-
L. plantarum B7 10^6 CFUs/mL treated	16.52 ± 0.84 #	0.261	0.019
L. plantarum B7 10^{10} CFUs/mL treated	17.26 ± 1.06	0.949	0.211

Table 8 Means ± SD of serum TNF-α level (pg/mL) in all groups

is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.

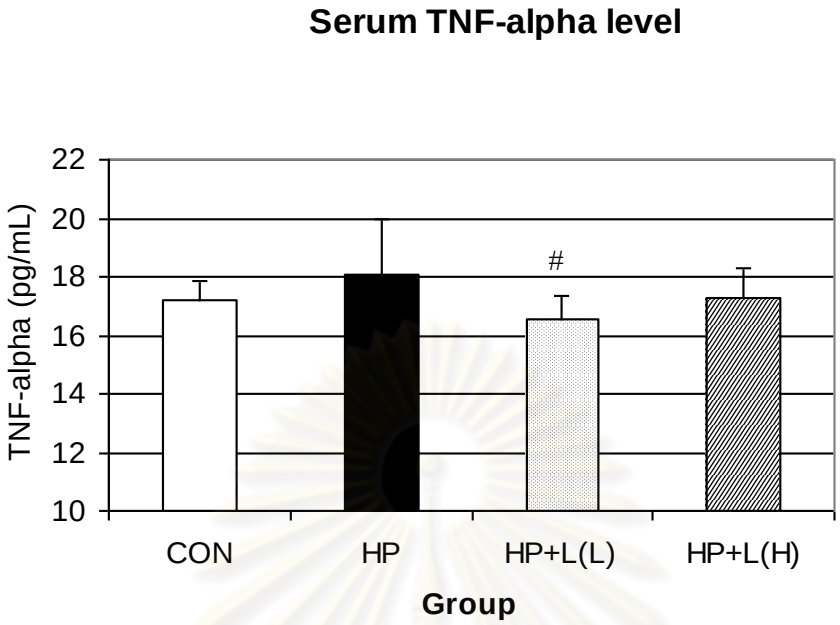


Figure 32 A bar graph shows the mean ± SD of serum TNF-α level (pg/ml) in all groups, CON: control group, HP: *H. pylori* infected group, HP+L(L): *L. plantarum* B7 10^6 CFUs/mL treated group, and HP+L(H): *L. plantarum* B7 10^{10} CFUs/mL treated group. Each group is represented by the mean of 8 rats.

[#] is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.

Effects of *H. pylori* infection on serum IL-1β level and role of *L. plantarum* B7

The serum IL-1β level of the control group was 102.65 ± 7.25 pg/mL, while that of the *H. pylori* infected group was 105.00 ± 13.98 pg/mL. For *L. plantarum* B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups, the serum IL-1β level were 98.78 ± 3.67 and 100.17 ± 5.40 pg/mL, respectively. There was no significant difference among groups as shown in table 9 and figure 33.

Group (n = 8)	Serum IL-1β level (pg/mL)	*p*-value (compared with control group)	*p*-value (compared with *H. pylori* infected group)
control	102.65 ± 7.25	-	0.585
H. pylori infected	105.00 ± 13.98	0.585	-
L. plantarum B7 10^6 CFUs/mL treated	98.78 ± 3.67	0.372	0.155
L. plantarum B7 10^{10} CFUs/mL treated	100.17 ± 5.40	0.565	0.267

Table 9 Means ± SD of serum IL-1β level (pg/mL) in all groups

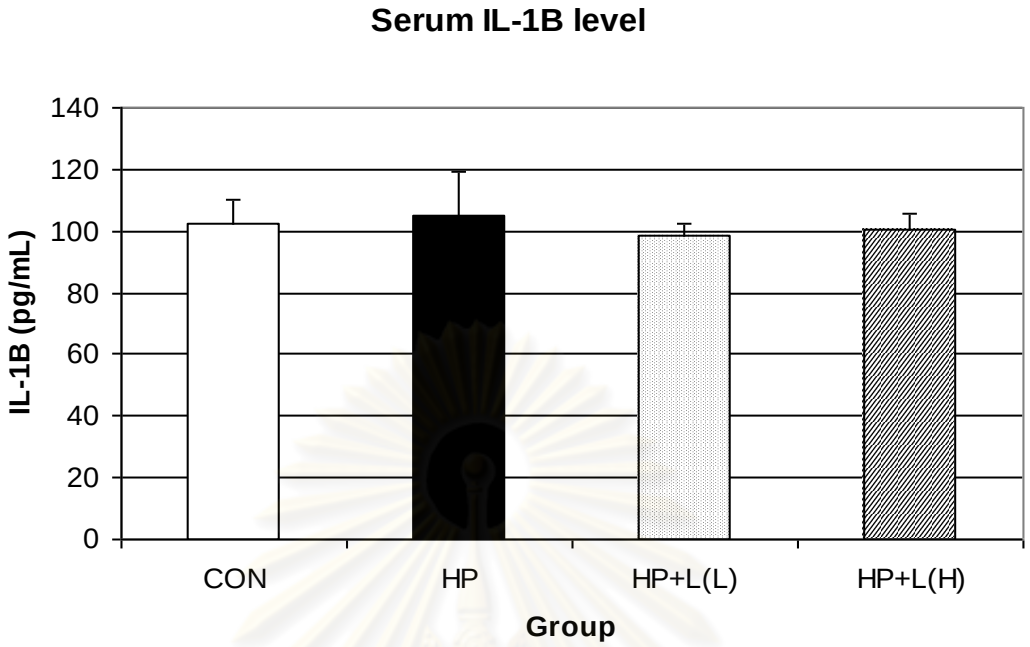


Figure 33 A bar graph shows the mean ± SD of serum IL-1β level (pg/mL) in all groups, CON: control group, HP: *H. pylori* infected group, HP+L(L) and HP+L(H): *L. plantarum* B7 10^6 CFUs/mL and *L. plantarum* B7 10^{10} CFUs/mL treated group, respectively. Each group is represented by the mean of 8 rats.

Effects of *H. pylori* infection on lipid peroxidation in gastric tissue and role of *L. plantarum* B7

The level of gastric MDA increased significantly in *H. pylori* infected group compared with control group (3.46 ± 1.25 vs. 1.05 ± 0.41 nmol/mg protein, $p = 0.000$, respectively). After treating one week with 10^6 CFUs/mL or 10^{10} CFUs/mL of *L. plantarum* B7 suspension, there were significant decrease of elevated gastric MDA level in both of *L. plantarum* B7 treated group compared with *H. pylori* infected group (1.28 ± 0.69, 1.37 ± 0.66 vs. 3.46 ± 1.25 nmol/mg protein, $p = 0.000$, respectively) (table 10 and figure 34).

Group (n = 8)	Gastric MDA levels (nmol/mg protein)	*p*-value (compared with control group)	*p*-value (compared with *H. pylori* infected group)
control	1.05 ± 0.41	-	0.000
H. pylori infected	3.46 ± 1.25 *	0.000	-
L. plantarum B7 10^6 CFUs/mL treated	1.28 ± 0.69 #	0.573	0.000
L. plantarum B7 10^{10} CFUs/mL treated	1.37 ± 0.66 #	0.437	0.000

Table 10 Means ± SD of gastric MDA level (nmol/mg protein) in all groups

* is represented significant difference ($p < 0.05$) compared with control group.

is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.

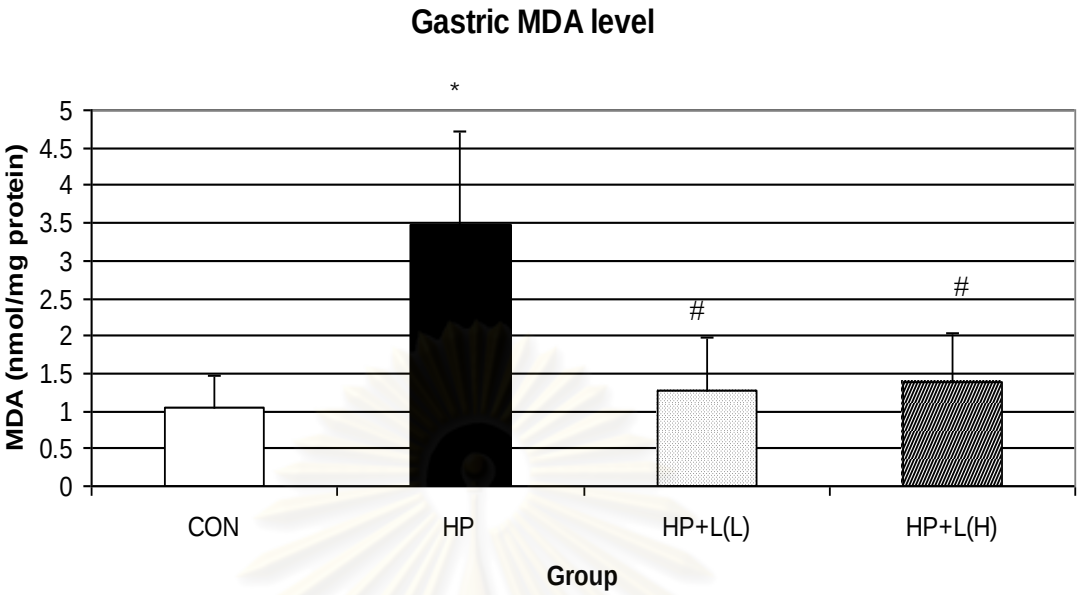


Figure 34 A bar graph shows the mean ± SD of gastric MDA levels (nmol/mg protein) in all groups, CON: control group, HP: *H. pylori* infected group, HP+L(L): *L. plantarum* B7 10^6 CFUs/mL treated group, and HP+L(H): *L. plantarum* B7 10^{10} CFUs/mL treated group. Each group is represented by the mean of 8 rats.

* is represented significant difference ($p < 0.05$) compared with control group.

is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.

Effects of *H. pylori* infection on apoptosis in gastric tissue and role of *L. plantarum* B7

H. pylori-induced apoptosis was studied by using the TUNEL assay. The positive stained cells presented dark brown nuclei under light microscopy. To verify the incidence of apoptosis, the numbers of positive stained cells were counted. One thousand of gastric epithelial cells were counted for each rat. The data were shown as percentage (%) of apoptotic cells calculating from this equation: the percentage of apoptotic cells (%) = (numbers of positive stained cells x 100) / 1000. From the results, the percentage of apoptotic cells was significantly increased in *H. pylori* infected group when compared with control group (7.44% ± 2.65 vs. 0.58% ± 0.13, $p = 0.000$, respectively). After treatment with 10^6 CFUs/mL or 10^{10} CFUs/mL of *L. plantarum* B7 suspension, the percentage of apoptotic cells was significantly decrease in 10^6 CFUs/mL ($p = 0.027$) and 10^{10} CFUs/mL ($p = 0.038$) compared with *H. pylori* infected group. The average percentages of apoptotic cells of all groups were shown in table 11 and figure 35. The figure 36 showed gastric sections processed for apoptosis assay by TUNEL reaction.

Group (n = 8)	Apoptotic cells (%)	*p*-value (compared with control group)	*p*-value (compared with *H. pylori* infected group)
Control	0.58 ± 0.13	-	0.000
H. pylori infected	7.44 ± 2.65 *	0.000	-
L. plantarum B7 10^6 CFUs/mL treated	5.63 ± 1.18 *#	0.000	0.027
L. plantarum B7 10^{10} CFUs/mL treated	5.75 ± 1.09 *#	0.000	0.038

Table 11 Means ± SD of apoptotic cells (%) in all groups.

* is represented significant difference ($p < 0.05$) compared with control group.

is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.

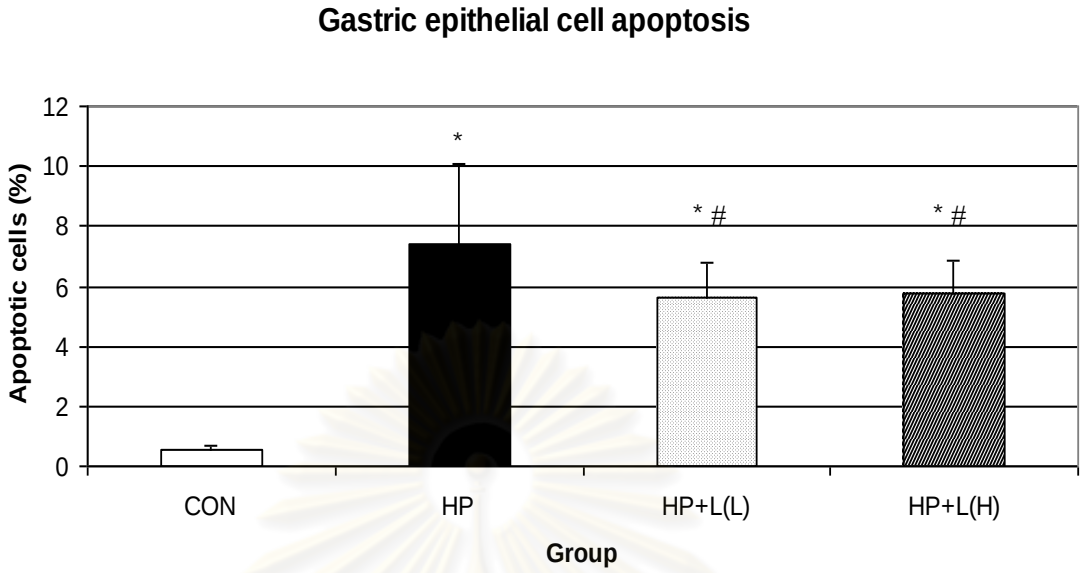
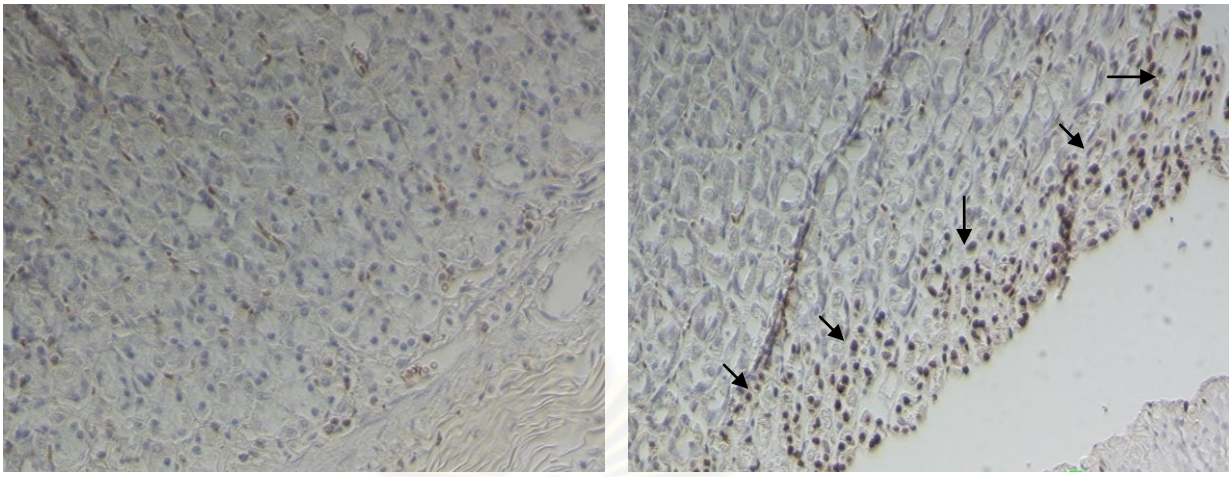


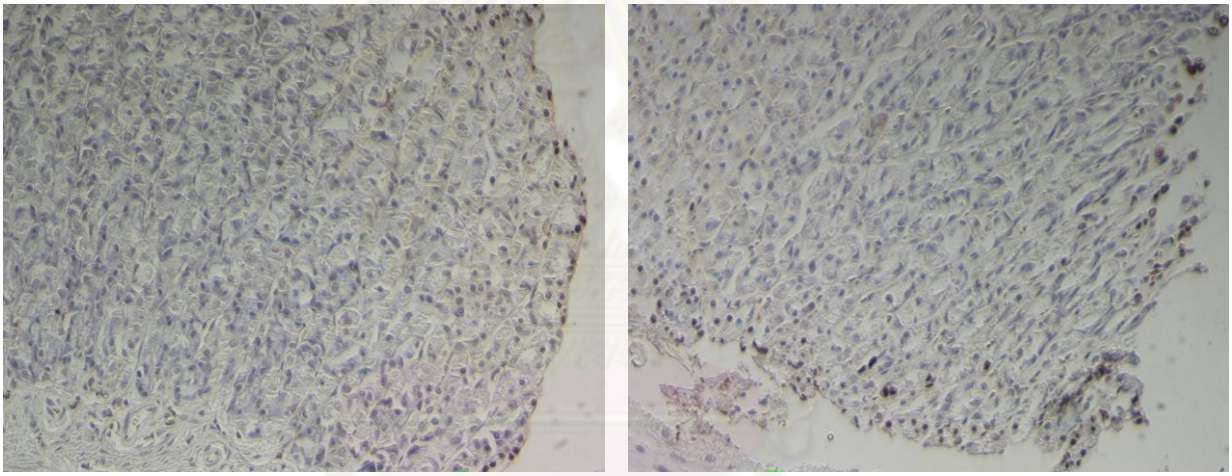
Figure 35 A bar graph shows the mean ± SD of apoptotic cells (%) in all groups, CON: control group, HP: *H. pylori* infected group, HP+L(L): *L. plantarum* B7 10^6 CFUs/mL treated group, and HP+L(H): *L. plantarum* B7 10^{10} CFUs/mL treated group. Each group is represented by the mean of 8 rats.

* is represented significant difference ($p < 0.05$) compared with control group.

is represented significant difference ($p < 0.05$) compared with *H. pylori* infected group.



(a) **(b)**



(c) **(d)**

Figure 36 Representative gastric sections processed for apoptosis assay by TUNEL reaction (x20). (a) control group. (b) *H. pylori* infected group, the arrows indicated a TUNEL positive gastric epithelial cell apoptosis. (c) and (d) *L. plantarum* B7 10^6 CFUs/mL treated and *L. plantarum* B7 10^{10} CFUs/mL treated groups showed a decrease of gastric epithelium apoptosis.

CHAPTER V

DISCUSSIONS

In vitro study

Anti-*H. pylori* activity of *L. plantarum* B7 supernatant *in vitro*

According to Sgouras, *et al*. [169] and Ryan, *et al*. [196] only viable cells of *L. casei* Shirota and *L. salivarius* have inhibited *H. pylori* growth. On the other hand, Lorca, *et al*. [197] has found the lactic acid and pH of *L. acidophilus* cultures to be important for the activity. To confirm previous data, in 2010, study has shown that all culture supernate of *Lactobacillus* strains, isolated from goat's milk, inhibit *H. pylori* growth but this inhibitory effect is lost after neutralization of *Lactobacillus* supernate [198].

In 2009, Boyanova, *et al*. [199] reported the anti-*Helicobacter* activity of *L. delbrueckii* subsp. *bulgaricus* cultures is strains-dependent and better at their native pH. Similarly, in this study, both intact pH 4 and adjusted pH 7 of *L. plantarum* B7 supernatants showed anti-*H. pylori* activity in a concentration dependent manner, but the culture supernatants of intact pH 4 showed the higher inhibition. These imply that low pH values were important for anti-*H. pylori* activity.

It is known that *Lactobacillus* secretes metabolic products such as lactic acid exerting activity against *H. pylori* [168]. In order to determine if the lactic acid associated with the growth inhibition of *H. pylori* in this study, an additional experiment was conducted. Our data showed that using disk diffusion method, no inhibition zones were found when testing with paper disks containing L- or DL-lactic acid at pH 4. These results suggest that the substance produced by the *L. plantarum* B7, which develops anti-*H. pylori* activity, was not involved in lactic acid.

Even though lactic acid inhibits the urease activity and viability of *H. pylori*, several studies have reported that *Lactobacillus* inhibitory factors other than lactic acid, including bacteriocins, peroxides, proteinases, exopolysaccharides and cell wall components could exert antibacterial effects [172, 173, 162]. Also, Coconnier, *et al*. [165] showed that a heat-stable antimicrobial substance secreted by *L. acidophilus* LB is active against *H. pylori* infection.

In summary, this *in vitro* study found that *L. plantarum* B7 supernatant inhibited *H. pylori* growth in a dose dependent manner and better at intact pH 4 indicating the amounts of antimicrobial substance released by *L. plantarum* B7 correlated with the intensity of their inhibitory effect against *H. pylori*. Furthermore, the anti-*H. pylori* activity of this substance was supported by low pH values.

***In vivo* study**

The present study found that the stomach histopathology in *H. pylori* infected group revealed mild to moderate *H. pylori* colonization and inflammation as well as increased gastric MDA and gastric epithelial cell apoptosis. However, the results of serum TNF-α and IL-1β levels showed no significant change in *H. pylori* infected group. *L. plantarum* B7 treatments resulted in improving stomach pathology, decreasing of serum TNF-α level, gastric MDA level, and apoptotic epithelial cells, and providing a trend of decreased IL-1β concentration.

Effect of *H. pylori* infection on histopathological change in rats

Using *H. pylori* cagA$^+$, vacA$^+$ strains, Li, *et al*. [191] investigated responses from the gastric mucosa of rats during long-term *H. pylori* infection. By microscopic examination, they found that *H. pylori* are mainly observed in gastric pits of the antrum, both in the lumen and on the surface of epithelial cells. Following the longer periods of infection, the density of *H. pylori* colonization in the antrum is increased. Regarding *H. pylori*-induced gastritis, they showed that the infiltration of inflammatory cells into the lamina propria is increased to a degree between mild and moderate at two weeks after *H. pylori* infection. In addition, they found that the gastric inflammation do not become more progressive along with the time after two weeks of *H. pylori* inoculation. Thong-Ngam D, *et al*. [192] showed that *H. pylori*, obtained from peptic ulcer patient, can induce mild to moderate gastric inflammation, confirmed by H&E staining, in *H. pylori* inoculated rats. This finding was observed at two weeks after *H. pylori* infection. Similarly, from histopathology in our experiment, rats infected with *H. pylori* cagA$^+$, vacA$^+$ strains revealed mild to moderate *H. pylori* colonization and gastric inflammation. These imply that the strain of *H. pylori* may be important for the pathogenesis of *H. pylori*-associated gastroduodenal disease. Several studies have been shown that *H. pylori* strains that have virulent factors namely *cag* PAI gene encoded protein CagA, the cytotoxin VacA, or the adhesion BabA induce peptic ulcer and gastric adenocarcinoma in long-term of infection [200, 201]. Moreover, lysates of toxic strain expressing CagA and VacA, but not those of non-toxic strain that do not express CagA or VacA, cause gastric damage in mice and that purified VacA cytotoxin causes gastric injuries when administered orally [202]. However, host immune response may also be associated with disease outcomes or histopathology change in *H. pylori* infection.

Effect of *H. pylori* infection on serum TNF-α and IL-1β levels in rats

H. pylori-induced gastroduodenal disease depends on the production of specific virulence factors such as CagA, VacA and urease that cause damage to gastric epithelial cells and disruption of the gastric mucosal barrier, and on the inflammatory response of the host [203]. The presence of genes in the *cag* PAI, containing approximately 30 genes, has been linked to variation in the ability of *H. pylori* strains to trigger inflammatory mediators from gastric epithelium [203]. One of the PAI genes, *cagA*, has been used as a marker of the *cag* PAI [35]. Recently, study reported that infection by $cagA^+$ strains is associated with enhanced inflammatory mediators and cellular responses *in vivo* and an increased risk of peptic ulceration, gastric atrophy and gastric cancer [204].

H. pylori induces host inflammatory response including production of cytokines, resulting in mucosal damages. Produced cytokines lead to infiltration of inflammatory cells, namely PMNs, lymphocytes and macrophages, to the site of infection [205]. These inflammatory cells then release large amounts of reactive oxygen species (ROS), causing tissue injuries. Wilson, *et al*. [139] showed that the gastric mucosal levels of the proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, are significantly higher in *H. pylori* positive patients than in negative patients. Algood and co-workers [59] also reported that the local cytokine response to *H. pylori* infection is of the Th1 type, as increased expression of TNF-α and IFN-γ, but not IL-4, is detected in the stomachs of *H. pylori*-infected mice. In recent study, Crabtree, *et al*. [206] showed that increased gastric mucosal production of TNF-α and IL-6 is associated with *H. pylori* gastritis. Moreover, they implied that inflammatory cytokines generated locally within the gastric mucosa can be relevant to the gastric physiology of *H. pylori* infection.

As mentioned above, infection with *H. pylori* in gastric mucosa is known to activate the production of many proinflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8. The production of these proinflammatory cytokines are not limited at the local site of infection but are further produced in a numerously with contribute to the systemic circulation. In 2006, Thong-Ngam D, *et al*. [207] investigated the effects of chronic *H. pylori* infection on serum TNF-α level in rats. They found the significant increase of serum TNF-α in *H. pylori* infection groups when compared with the control groups. However, the results obtained in our study showed that there was no significant change of serum TNF-α and IL-1β levels in *H. pylori* infected group. These results might occur from the variation of cytokines in serum that may depend on the period of blood collection but not the severity of diseases.

Effect of *H. pylori* infection induced oxidative stress in rats

H. pylori infection induces production of proinflammatory mediators such as TNF-α, IL-1β, IL-2, IL-6 and IL-8, and infiltration of lamina propria with inflammatory cells as well as the generation of reactive oxygen species (ROS) [208]. However, these *H. pylori*-induced inflammatory responses do not appear to confer protective immunity, and may lead to the excess production of ROS, oxidative burst caused by phagocytic cells, and gastric tissue damages.

In this study, despite no significant increase in serum TNF-α and IL-1β levels in *H. pylori* infected group, infection with *H. pylori* cagA$^+$, vacA$^+$ strains contributed to significant increased gastric MDA level, a representative of oxidative stress.

Several studies have been shown that *H. pylori* strains having *cagA*$^+$*/vacAs1* genotype are more virulent than other genotypes [91]. Similarly, Azuma and colleagues [209] reported that *H. pylori* cagA$^+$ strains are involved in more intense tissue responses than cagA$^-$ strains. Moreover, Epidemiological studies have shown that colonization with cagA$^+$ *H. pylori* is associated with increased risk for development of both peptic ulcer disease and gastric cancer [204]. *In vitro* study, Zhang, *et al*. [117] demonstrated that *H. pylori* cagA$^+$ strains induce an increased oxidative burst in polymorphonuclear neutrophils (PMNs) with higher ROS production. Recently, studies have shown that ROS production in gastric mucosa is enhanced by the infection of cagA$^+$ *H. pylori* species with an extensive accumulation of neutrophils in both patients with chronic gastritis and gastric ulcer [210, 211].

IL-8 is a potent neutrophil-activating chemokine. Its expression results in migration and activation of neutrophils, induction of adhesion molecules including CD11b/CD18 expression, and production of ROS. Several studies have shown that *H. pylori* cagA$^+$ strains elicit a significantly higher IL-8 response than cagA$^-$ strains *in vitro* [200, 212]. *In vivo* investigations reported that infection with cagA$^+$ strains leads to increased mucosal IL-8 production and more intense gastritis [213]. Two independent studies recently showed that *H. pylori* CagA injection into the host gastric epithelial cell can induce IL-8 production through NF-ΚB activation [214, 215]. In 2000, Li and co-workers [216] reported that *H. pylori* cagA$^+$ gastric biopsies have significantly more severe infiltration by mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) and show more frequent coexpression of IL-8 and iNOS mRNAs than cagA$^-$ specimens. Also, in 2001, they demonstrated that levels of oxidized and nitrated proteins are increased in human gastric biopsies infected with *H. pylori*, especially cagA$^+$ strains. Their prevalence and levels were well correlated with infiltration by MNCs and PMNs and expression of IL-8 and iNOS mRNAs in the mucosa [217]. Additionally, in 2008, Marcelo, et al. [218] showed that higher levels of oxidative DNA damage are found in infected patients with *H. pylori cagA*$^+$,

vacAs1 m1 and *iceA1* genotype than infected patients with *H. pylori cagA*$^-$, *vacAs2 m2* and *iceA2* genotypes and uninfected patients. Thus, these mentioned results might reflect that infection with *H. pylori* cagA$^+$ strains can induce oxidative stress in stomach mucosa and involve in the pathogenesis of *H. pylori*-associated gastroduodenal diseases. In the present study, significant increased gastric MDA level was found in rats infected with *H. pylori* cagA$^+$, vacA$^+$ strains, suggesting that oxidative stress expression may associated with cagA$^+$ status of *H. pylori*.

Effect of *H. pylori* infection induced apoptosis in rats

H. pylori induces apoptosis both directly by the production of specific virulence factors, namely CagA, VacA [136], urease [137] and LPS [138], and indirectly via the host inflammatory response including release of proinflammatory cytokines [140] as well as production of ROS [144, 145].

In this study, we detected that *H. pylori* cagA$^+$, vacA$^+$ strains can induce epithelial cell apoptosis in rats. The *cagA* gene or expression of VacA might be involved in gastroduodenal diseases by affecting apoptosis. The *cagA* gene is a marker of the presence of the pathogenicity island that encodes disease-associated virulence factors [35] and is associated with the expression of VacA. Nevertheless, most *in vitro* and animal studies have shown that both cagA$^+$ and cagA$^-$ strains induce apoptosis, and that there is no association between CagA expression and apoptosis [219-221], other studies reported enhanced apoptosis by *cagA*$^+$ strains [222, 223]. In 2006, Monica, *et al*. [222] showed that pro-apoptotic proteins such as Bax and Bak expression are higher than anti-apoptotic proteins including Bcl-2 and Bcl-XL in most cases of gastric biopsies from patients with *H. pylori* gastritis and are significantly higher in patients infected by *cagA*$^+$ strains than in those infected by *cagA*$^-$. Moreover, they found that Bak expression is higher at the lesser curvature (antrum and incisura) than in the other regions and is correlated with atrophy. Peek, *et al*. [223] demonstrated that co-culture of *H. pylori cagA*$^+$ strains with AGS cells significantly decrease cell viability and increase apoptosis when compared with *cagA*$^-$ strains. They also showed that diminished AGS cell viability, and enhanced apoptosis associated with *cagA*$^+$ *H. pylori* strains are dependent upon expression of *vacA* and genes within the *cag* pathogenicity island. This result suggests that in addition to *cagA*, *vacA* plays a crucial role in induction of apoptosis.

Two independent groups have recently confirmed that VacA induces epithelial cell apoptosis [136, 224]. First, by co-culturing gastric AGS cells with either VacA producing or

isogenic *vacA*-null mutant *H. pylori* strains, Cover and co-workers. [136] demonstrated that only VacA producing bacteria can contribute to an elevated number of apoptotic cells. By transfecting DNAs encoding various parts of the VacA toxin into epithelial human cervix carcinoma (HEp-2) cells, the latter study reported that both the whole molecule and its N-terminal moiety (p37) can target mitochondria, leading to epithelial cell apoptosis through cytochrome c release [224]. Furthermore, in India study, Manisha, *et al*. [225] investigated the relationship between *vacA* genotypes and the severity of gastritis, and gastric epithelial cell apoptosis in *H. pylori*-infected children. They found that children infected with the s1a/m1 and s1a/m2 *vacA* genotypes had higher severity of chronic inflammation and gastric epithelial cell apoptosis than the s2/m2 genotype.

Recently, study in rats showed that at two weeks after *H. pylori* $cagA^+$, $vacA^+$ strains inoculation, apoptotic cells are significantly increased in both the gastric antrum and body compared with uninfected rats [191]. Neu and colleague [226] reported that apoptosis of rat gastric parietal cells is induced in a dose- and time-dependent manner by *H. pylori* $cagA^+$, $vacA^+$ strains but not by *H. pylori* $cagA^-$, $vacA^-$ strains. Their investigation also found that $cagA^+$, $vacA^+$ strains are capable to induce activation of NF-ΚB complexes in nuclear extracts of parietal cells, which are consisted of p65 and p50 subunits. Several studies have shown that *H. pylori*-induced apoptosis through NF-ΚB activation [227, 228]. One of these reported that *H. pylori* induces increment of apoptotic AGS cells by down-regulation of Bcl-2 level, which is mediated by NF-ΚB activation [228].

In present study, our data also showed that infection with *H. pylori* $cagA^+$, $vacA^+$ strains leads to elevated gastric MDA level, as previously mentioned. MDA, a major product of lipid peroxidation, can react with DNA to form MDA-DNA adducts, resulting in DNA damage [229]. In response to DNA damage and cell stress, several machines involve in apoptosis such as p53, p21 and pro-apoptotic proteins, including Bad, Bax and Bid are activated [230]. Thus, these results indicate that apoptosis in epithelial cells may occur in response to *H. pylori*-induced cell stress, or/and relate to infection with *H. pylori* $cagA^+$, $vacA^+$ strains.

Role of *L. plantarum* B7 on histopathological changes in *H. pylori* infected rats.

By histological examination, our results showed that the histopathology in control group was normal, while, in *H. pylori* infected group revealed mild to moderate *H. pylori* colonization and inflammation. Treatment with *L. plantarum* B7 at both concentrations of 10^6 and 10^{10} CFUs/mL resulted in decreasing *H. pylori* colonization and improving stomach inflammation. Interestingly, these results were concordance to the previous studies. According to the studied of Johnson-Henry, *et al*. [231] found that probiotic combination containing *L. rhamnosus* R0011 and *L. acidophilus* R0052 decrease the effects of *H. pylori* infection in a C57BL/6 mice model of infection through reducing *H. pylori* colonization and alleviating *H. pylori*-induced gastric mucosa inflammation. Studies by Sgouras, *et al*. [169, 232] in a C57BL/6 mice model reported that *L. casei* strain Shirota and *L. johnsonii* La1, both administered in drinking water, attenuate *H. pylori* infection-induced inflammation of the stomach. Nevertheless, only *L. casei* strain Shirota is capable to down-regulate the colonization of *H. pylori* to gastric mucosa. Moreover, Ushiyama and collogues [233] showed that *L. gasseri* is found to reduce clarithromycin resistant *H. pylori* colonization.

However, in this study, from the rapid urease test and histopathological results in *L. plantarum* B7 treated groups, there were positive results in both of tests suggesting that *L. plantarum* B7 has no effect to get rid of *H. pylori*.

Overall results of histological study, these indicate that *L. plantarum* B7 does not eradicate *H. pylori* but maintain lower levels of this pathogen in the stomach, leading to attenuation of gastric inflammation and improvement of stomach pathology.

Role of *L. plantarum* B7 on serum TNF-α and IL-1β levels in *H. pylori* infected rats.

Nonetheless, our results showed that there were no significant change of serum TNF-α and IL-1β levels in *H. pylori* infected group when compared with control group. *L. plantarum* B7 exerted to decrease these proinflammatory cytokine levels.

The information about effects of *L. plantarum* B7 on *H. pylori* is relatively rare. However, several previous investigations have been shown the anti-inflammatory properties of *Lactobacillus* [182, 183, 163, 234, 9]. In 2003, Pena, *et al*. [182] showed that *L. rhamnosus* GG is able to antagonize *H. pylori* LPS-induced TNF-α production by murine macrophages *in vitro* by a contact-independent mechanism. Chao, *et al.* [183] also demonstrated that *H. pylori* LPS

induces IL-8 production in SGC-7901 cells, human gastric adenocarcinoma cells, through activating TLR4 signaling and *L. bulgaricus* (LBG) prevents *H. pylori* LPS by down–regulation of TLR4 expression, inhibition of TAK1 and p38MAPK phosphorylation, prevention of NF-κB activation, and consequently suppression of IL-8 production.

Regarding *L. plantarum*, study by Ko, *et al*. [163] reported that *L. plantarum* is capable to inhibit epithelial barrier dysfunction and reduce IL-8 secretion induced by TNF-α. In 2008, Rokka and co-workers [234] showed that live lactic acid bacteria (LAB) including *L. plantarum* MLBPL1, *L. rhamnosus* GG and *L. lactis* have abilities to reduce the adhesion of *H. pylori* on AGS cells, and decrease *H. pylori*-induced IL-8 production. Interestingly, in same year, Panpetch and co-workers [9] reported that *L. plantarum* B7 show the highest *E. coli* LPS-induced TNF-α inhibitory activity *in vitro*.

Moreover, it has been proposed that *L. plantarum* also has immunomodulatory properties. Bin and colleagues [157] reported that *L. plantarum* L2 is found to induce a considerable level of IL-10 from PBMCs, but low levels of pro-inflammatory cytokines including TNF-α, IFN-γ and IL-12. Megan and co-workers [181] showed that *L. plantarum* and *L. acidophilus* have the potential to be immunomodulatory probiotics, and their effects on cytokine expression are strain specific. By the IEC-6 rat intestinal epithelial cell line with and without LPS stimulation, *L. plantarum* 7-12 and *L. acidophilus* La-11 had an inflammatory effect on unstimulated cells as indicated by up-regulation of IL-18, and MIP-2 and IL-6 by *L. plantarum* 7-12, and *L. acidophilus* La-11, respectively. In contrast, *L. plantarum* 5-39 and *L. acidophilus* NCFM had an anti-inflammatory effect as indicated by down-regulation of IL-1β expression in LPS-stimulated cells. All strains except *L. plantarum* 7-12 also down-regulated expression of TNF-α in cell stimulated with LPS.

Based on these previous findings, it might be concluded that decrease of serum TNF-α level and tendency to reduce of IL-1β concentration in *L. plantarum* B7 treatments may associated with the anti-inflammatory properties of *L. plantarum* B7.

Role of *L. plantarum* B7 on oxidative stress and apoptosis in *H. pylori* infected rats.

It has been proposed that *Lactobacillus* is able to attenuate oxidative stress and apoptosis. Previously, several studies have been shown the antioxidative ability of them. According to Truusalu, *et al*. [235] found that *L. fermentum* ME-3 suppresses the excessive oxidative stress-associated inflammation induced by *S. typhimurium* infection in mice model. Using the same

experimental typhoid fever model, they also showed that treatment with *L. fermentum* ME-3 alone or in combination with an antimicrobial quinolone (ofloxacin) leads to significant decrease in lipid peroxidation and glutathione redox ratio (GSSG/GSH) [236]. Lin, *et al*. [184] investigated antioxidative ability of nineteen strains of lactic acid bacteria including *L. acidophilus* B, E, N1, 4356, LA-1, and Farr, *L. bulgaricus* 12 278, 448, 449, Lb, 1006, and 11 842, *S. thermophilus* 821, MC, 573, 3641, and 19 987, and *B. longum* B6 and 15 708. They found that intracellular cell-free extract of all strains demonstrate antioxidative activity with inhibition rates of ascorbate autoxidation. Moreover, metal ion chelating ability, reducing activity and reactive oxygen species scavenging ability are observed in these 19 strains. In 2010, Zhang and colleague [237] reported that oral *L. plantarum* treatment of rats with obstructive jaundice increases GSH levels in liver and stimulates GSH biosynthesis, resulting in attenuated oxidative damage. Using TUNEL assay, they also showed that treatment with *L. plantarum* significantly decreases hepatic apoptosis.

Regarding apoptosis, study has been shown that *L. plantarum* is capable to inhibit epithelial barrier dysfunction, IL-8 secretion, ERK activation, and IκB-α degradation in TNF-α-stimulated Caco-2 cells [163]. This finding implies that *L. plantarum* may preserve epithelial barrier function and inhibit the inflammatory response by affecting the signal transduction pathway. Recently, *in vitro* study reported that pre-treatment of HT-29 cells with *L. plantarum* 299v prior to cytokine exposure contributes to substantial decrease in apoptosis, as measured by TUNEL positive cells, when compared to cells exposed to the cytokine mixture. Also, caspase-3, caspase-7 activity, and caspase positive cells in the multi-caspase activity assay are reduced by *L. plantarum* 299v pre-incubation [238]. Lam, *et al*. [239] showed that pre-treatment of rats with *L. rhamnosus* GG markedly reduces ethanol-induced mucosal lesion area and gastric cell apoptosis. Yan and Polk [180] demonstrated that *L. rhamnosus* GG is able to prevent cytokine-induced apoptosis in intestinal epithelial cell models through the inhibition of a TNF-α-induced activation of the pro-apoptotic p38/mitogen-activated protein kinase. Then, they purified two secreted *L. rhamnosus* GG proteins, p75 and p40, from *L. rhamnosus* GG broth culture supernatant and found that each of these purified protein preparations stimulates the anti-apoptotic Akt/protein kinase B, inhibits TNF-α-induced epithelial cell apoptosis and organ culture lesions, and promotes cell growth in human and mouse colon epithelial cells and cultured mouse colon explants [186].

Interestingly, all of these studies were concordance to our results. In current study, we found that *L. plantarum* B7 treatments resulted in decreasing of gastric MDA level and epithelial cell apoptosis. However, the mechanisms of action were unclear those need further investigations

CHAPTER VI

CONCLUSIONS

This study found that *L. plantarum* B7 supernatant inhibited *H. pylori* growth in a dose dependent manner and better at intact pH 4 *in vitro*.

In vivo study, this investigation found that *L. plantarum* B7 could attenuate *H. pylori*-induced gastric inflammation by the improvement of stomach pathology, and reduction of serum TNF-α level, oxidative stress and gastric epithelial cell apoptosis. Moreover, *L. plantarum* B7 might has a tendency to decrease IL-1β concentration.

Based on this finding, it implied that *L. plantarum* B7 may has the anti-*Helicobacter* activity *in vitro* and anti-inflammatory effect on *H. pylori* infection. Thus, it might be beneficial for clinical application, use as an adjunction to antibiotics to decrease gastric inflammation and reduce side effects of the triple therapy.

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