การควบคุมการสร้างอินเตอร์ลิวคิน-8 ของเซลล์เยื่อบุกระเพาะอาหารที่ถูกกระตุ้นด้วย เฮลิโคแบคเตอร์ ไพโลไร โดยแลคโตบาซิลลัส

นางสาววิมลรัตน์ ปานเพ็ชร

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LACTOBACILLUS - MEDIATED MODULATION OF IL-8 PRODUCTION IN *HELICOBACTER PYLORI* - STIMULATED GASTRIC EPITHELIAL CELLS

Miss Wimonrat Panpetch

A Dissertation Submitted in Partial Fulfillment of the Requirements
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By	Miss Wimonrat Panpetch
Field of Study	Medical Microbiology
Thesis Advisor	Associate Professor Somying Tumwasorn, Ph.D.
Thesis Co-Advisor	Professor James Versalovic, M.D., Ph.D.
	Jennifer K. Spinler, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Graduate School
(Associate Professor Sunait Chutintaranond, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Anan Chongthaleong, M.D.)

.....Thesis Advisor
(Associate Professor Somying Tumwasorn, Ph.D.)

.....Thesis Co-Advisor
(Professor James Versalovic, M.D., Ph.D.)

.....Thesis Co-Advisor
(Jennifer K. Spinler, Ph.D.)

.....Examiner
(Associate Professor Tanapat Palaga, Ph.D.)

.....Examiner
(Assistant Professor Kanitha Patarakul, M.D., Ph.D.)

.....External Examiner
(Associate Professor Ratha-korn Vilaichone, M.D., Ph.D.)

วิมลรัตน์ ปานเพ็ชร : การควบคุมการสร้างอินเตอร์ลิวคิน-8 ของเซลล์เยื่อบุกระเพาะอาหารที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร โดยแลคโตบาซิลลัส (*LACTOBACILLUS* - MEDIATED MODULATION OF IL-8 PRODUCTION IN *HELICOBACTER PYLORI* - STIMULATED GASTRIC EPITHELIAL CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สมหญิง ธัมวาสร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ดร. เจมส์ เวอร์ซาโลวิค, ดร. เจนนิเฟอร์ เค. สพินเลอร์, 117 หน้า.

เฮลิโคแบคเตอร์ ไพโลไร เป็นสาเหตุของกระเพาะอาหารอักเสบ แผลเพ็ปติก และเป็นปัจจัยเสี่ยงสำคัญของมะเร็งกระเพาะอาหาร การติดเชื้อเฮลิโคแบคเตอร์ ไพโลไรกระตุ้นให้มีการปล่อยอินเตอร์ลิวคิน-8 (IL-8) จากเซลล์เยื่อบุกระเพาะอาหารทำให้เกิดการอักเสบและทำลายเนื้อเยื่อ การศึกษาก่อนหน้านี้แสดงให้เห็นว่า แลคโตบาซิลลัส แพลนทารัม สายพันธุ์ XB7 (LP-B7) แลคโตบาซิลลัส ซาลิวาเรียส สายพันธุ์ B37 (LS-B37) และ B60 (LS-B60) สามารถลดการสร้าง IL-8 ของเซลล์เยื่อบุกระเพาะอาหาร AGS ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร และ LP-XB7 ลดการแสดงออกของ IL-8 mRNA โดยการลดการกระตุ้น NF-kB และ c-Jun การศึกษานี้มีวัตถุประสงค์เพื่อหากลไกการควบคุมการสร้าง IL-8 ของเซลล์ AGS ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร โดยแลคโตบาซิลลัสเหล่านี้ ทดสอบการควบคุมการถอดรหัสของ IL-8 ของน้ำเลี้ยงเชื้อที่ปราศจากเซลล์ (LCM) โดยวิธี quantitative real-time PCR การเปลี่ยนแปลงวิถีสัญญาณที่เกี่ยวข้องกับการสร้าง IL-8 โดยวิธี western blot การแสดงออกของยีน *cag*A และ *cag*E โดยวิธี quantitative real-time PCR ตรวจพิสูจน์คุณสมบัติของ Immunomodulatory factors ที่ลดการสร้าง IL-8 โดยการทดสอบการทนต่ออุณหภูมิ การหาขนาดของสาร และการไวต่อเอนไซม์ ผลการทดลองพบว่า LCM ของ LS-B37 และ LS-B60 ลดการแสดงออกของ IL-8 mRNA ในเซลล์เยื่อบุกระเพาะอาหาร AGS เช่นเดียวกับ LCM ของ LP-XB7 อย่างไรก็ตาม LS-B37 และ LS-B60 รบกวนการสร้าง IL-8 mRNA โดยการลดการกระตุ้น NF-kB ขณะที่ LP-XB7 ลดการกระตุ้น NF-kB และ c-Jun การลดการสร้าง IL-8 ของแลคโตบาซิลลัสเหล่านี้ไม่มีผลจากการกดการแสดงออกของยีน *cag*A และ *cag*E สารที่ยับยั้งการสร้าง IL-8 ของแลคโตบาซิลลัสเหล่านี้มีคุณสมบัติทนต่อความร้อนและมีขนาดมากกว่า 100 kDa นอกเหนือจากนี้ สารที่ยับยังการสร้าง IL-8 ของ LS-B37 จะไวต่อเอนไซม์อไมเลส ส่วนสารที่ยับยั้งการสร้าง IL-8 ของ LP-XB7 และ LS-B60 จะไวต่อเอนไซม์ อะไมเลส ไลเพส โปรตีนเนสเค และทริปซิน จึงเป็นไปได้ว่าสารโพลีแซคคาไรด์ใน LCM ของ LS-B37 สามารถลดการสร้าง IL-8 ในเซลล์ AGS ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร และ immunomodulatory substance(s) ใน LCM ของ LP-XB7 และ LS-B60 เป็นสารประกอบที่แตกต่างกันมีองค์ประกอบเป็น โพลีแซคคาไรด์ ลิพิด และโปรตีน ผลการวิจัยนี้แสดงให้เห็นว่าแลคโตบาซิลลัสที่แยกได้จากกระเพาะอาหารของมนุษย์สร้างสารหลายชนิดที่สามารถลดการสร้าง IL-8 ในเซลล์เยื่อบุกระเพาะอาหารที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ ไพโลไร และสนันสนุนศักยภาพของสายพันธุ์เหล่านี้ในการใช้เป็นโพรไบโอติกส์สำหรับเสริมการรักษาการติดเชื้อเฮลิโคแบคเตอร์ ไพโลไร

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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WIMONRAT PANPETCH: *LACTOBACILLUS* - MEDIATED MODULATION OF IL-8 PRODUCTION IN *HELICOBACTER PYLORI* - STIMULATED GASTRIC EPITHELIAL CELLS. ADVISOR: ASSOC. PROF. SOMYING TUMWASORN, Ph.D., CO-ADVISOR: PROF. JAMES VERSALOVIC, M.D., Ph.D., JENNIFER K. SPINLER, Ph.D., 117 pp.

Helicobacter pylori causes gastritis, peptic ulcers, and is considered an important risk factor of gastric cancer. Infection with *H. pylori* induces the release of interleukin (IL)-8 from gastric epithelial cells leading to tissue inflammation and damage. *Lactobacillus plantarum* strain XB7 (LP-XB7), *Lactobacillus salivarius* strains B37 (LS-B37), and B60 (LS-B60) have been shown to suppress *H. pylori*-induced IL-8 production from AGS gastric epithelial cells. LP-XB7 was also shown to decrease IL-8 transcription via the suppression of NF-κB and c-Jun activation. This study aimed to investigate the mechanisms of IL-8 suppression in *H. pylori*-stimulated AGS cells by these lactobacilli. The effects of *Lactobacillus* conditioned media (LCM) on IL-8 transcription were tested by quantitative real-time PCR. Changes in the signaling pathway associated with IL-8 production were determined by western blot analysis. Expression of genes *cag*A and *cag*E was determined by quantitative real-time PCR. Immunomodulatory factors responsible for suppressing IL-8 production in *H. pylori*-induced AGS cells were characterized by testing thermal stability, size estimation, and enzyme sensitivity. LCM of LS-B37 and LS-B60 attenuated IL-8 mRNA expression ($p < 0.01$ and $p < 0.001$, respectively) like that of LP-XB7. However, LS-B37 and LS-B60 interfered with IL-8 mRNA transcription via the suppression of NF-kB activation ($p < 0.01$), while LP-XB7 did via NF-κB and c-Jun. Expression of *H. pylori* virulence genes *cag*A and *cag*E was not affected by LCM of these strains. IL-8 inhibitory substances of LP-XB7, LS-B37, and LS-B60 are heat-stable and more than 100 kDa in size. Additionally, IL-8 inhibitory substance of LS-B37 was sensitive to amylase, whereas those of LP-XB7 and LS-B60 were sensitive to amylase, lipase, proteinase K, and trypsin. It was thus suggested that the polysaccharide in LCM of LS-B37 is responsible for the inhibition of NF-κB activation in AGS cells stimulated with *H. pylori* and IL-8 inhibitory effect of LP-XB7 and LS-B60 is probably due to different complex components of polysaccharides, lipids and, proteins. These results showed that human gastric-derived *Lactobacillus* strains produce multiple immunomodulatory factors capable of suppressing *H. pylori*-induced IL-8 production from gastric epithelial cells and supported the potential of these strains as probiotics for adjunctive therapy of *H. pylori* infection.

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

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LIST OF ABBREVIATIONS

AP-1	Activator protein1
ATCC	American Type Culture Collection
BabA	Blood group antigen-binging adhesion
BCA	Bicinchoninic acid
BHI	Brain heart infusion
bp	Base pairs
BSA	Bovine serum albumin
^{o}C	Degree Celsius
Cag	Cytotoxin-associated gene
cDNA	Complementary Deoxyribonucleic acid
CFU	Colony-forming unit
CO_2	Carbon dioxide
DDW	Double-distilled water
DMSO	Dimethyl sulfoxide
DI	Deionised water
DNA	Deoxyribonucleic acid
DW	Distilled water
DupA	Duodenal ulcer promoting A
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
et al.	et alii
g	Gram
G+C content	Guanine+cytosine content
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
H_2	Hydrogen
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

H_2SO_4	Sulfuric acid
*ice*A	Induced-by-contact-with-epithelium gene A
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-12	Interleukin-12
JAM	Junctional adhesion molecules
JNK	c-Jun N terminal kinase
kb	kilobase
KC	Kerainnocyte-derived cytokine
KCl	Potassium chloride
kDa	kilodalton
KH_2PO_4	Potassium phosphate monobasic
LCM	*Lactobacillus* conditioned media
LPS	Lipopolysaccharide
µg	Microgram
µL	Microliter
µm	Micrometer
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Mililiter
mm	Millimeter
mM	Milimolar
MIP-2	Macrophage inflammatory protein-2
MRS	deMan Rogosa Sharpe
N_2	Nitrogen
NaCl	Sodium chloride

$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Sodium phosphate dibasic, anhydrous
NaOH	Sodium hydroxide
NFAT	Nuclear factor of activated T-cells
NF- κB	Nuclear factor κB
NO	Nitric oxide
Nod1	Nucleotide-binding oligomerisation domain protein 1
O_2	Oxygen
OipA	Outer inflammatory protein A
OMP	Outer membrane protein
PAI	Pathogenicity island
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Picomol
PPI	Proton pump inhibitor
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RpoB	RNA polymerase β-subunit
sec	Second
SabA	Sialic acid-binding adhesion
SD	Standard deviation
16SrRNA	Sixteen subunit ribosomal ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBE	Tris-Boric Acid-EDTA
TBS	Tris-buffered saline
T4SS	Type 4 secretion system
Th0 cells	T-helper 0 cells

Th1 cells	T-helper 1 cells
Th2 cells	T-helper 2 cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
U	Unit
µg	Microgram
µl	Microliter
µM	Micromolar
V	Volt
VacA	Vacuolating cytotoxin A
v/v	Volume/volume
WHO	World Health Organization
w/v	Weight/volume
ZO-1	Zona occludens protein-1

CHAPTER I
INTRODUCTION

Helicobacter pylori is a gram-negative bacterium colonizing the stomach of approximately half of the world's population. *H. pylori* causes gastritis, peptic ulcer, and is considered an important risk factor of gastric carcinoma, and gastric lymphoma [1]. Only 15% of all infected people develop disease, and pathogenesis depends upon the virulence of the infecting strain, host genetic susceptibility, and environmental cofactors [1]. Gastric carcinoma is the second highest cause of cancer deaths worldwide owing to a combination of high incidence rate, aggressive disease course, and lack of effective treatment [1]. Moreover, infection of *H. pylori* is the strongest known risk factor for the development of gastric carcinoma [2]. *H. pylori* persistence, the most important event in pathogenesis, results in gastric inflammatory response, leading to the development of gastroduodenal diseases [3]. After many years of *H. pylori* infection and gastric mucosa inflammation, ulcer can happen mainly in mild-or late adulthood. Ultimately, gastric adenocarcinoma occurs in late adulthood after a longer period of chronic inflammation and epithelial damage [1].

The key pathophysiological event in *H. pylori* colonization is the start of an inflammatory response. Bacterial components or their virulence factors can induce inflammatory process and main mediators are proinflammatory cytokines and chemokines which recruit an infiltration of neutrophils, macrophages, eosinophils, and lymphocytes to the site of inflammation [4, 5]. Gastric infection with *H. pylori* is known to induce several cytokines, such as interleukin (IL)-1β, IL-2, IL-6, IL-12, tumor necrosis factor (TNF)-α and IL-8 production [6-9]. IL-8 is a potent neutrophil chemotactic and activating agent [10-14] and associated with *H. pylori* associated-disease activity [11, 12]. Several cell types such as monocytes, fibroblasts, endothelial, and epithelial cells can produce IL-8 [15]. It has been reported that *H. pylori*-infected and gastric cancer patients have increased levels of IL-8 in gastric epithelium [16]. Some studies demonstrated the stimulation of IL-8 production in gastric epithelial cell lines stimulated by *H. pylori*. [17, 18]. *H. pylori* infection also causes an increased induction of IL-8 mRNA expression and protein production in gastric epithelial cells

in vitro and *in vivo* [17, 19-21]. These evidences suggest that IL-8 is an essential chemokine induced by *H. pylori*.

H. pylori possesses several virulence factors that induce IL-8 production. Infections with strains carrying the cytotoxin-associated gene pathogenicity island (*cag* PAI) induce gastric epithelial cells to express large amounts (1,000-2,000 pg/mL) of IL-8 *in vitro* [22, 23]. Several reports indicated that IL-8 secretion is dependent upon the *cag* PAI gene and correlated with greater levels of gastric tissue inflammation, associated with enhancing mucosal levels IL-8 expression, and increased risk of peptic ulceration in humans [24, 25]. Epidemiological studies have been shown that subjects infected with *H. pylori* strains containing the cytotoxin-associated antigen A (*cag*A) gene are at considerably increased risk of gastric cancer than *cag*A-negative strains [26, 27]. Patients infected with *cag*A-positive *H. pylori* strains are associated with an increased risk for the eventual development of atrophic gastritis and intestinal metaplasia [28]. The CagA protein activates nuclear factor kappaB (NF-κB), via extracellular signal-regulated kinase (ERK), and induces IL-8 secretion [29]. AGS gastric epithelial cells transfected with CagA also induces IL-8 secretion [30]. CagA protein is translocated into gastric epithelial cells through a type 4 secretion system (T4SS) [31]. Besides CagA, T4SS can deliver peptidoglycan components of *H. pylori* into host gastric epithelial cells and then bind with the nucleotide-binding oligomerisation domain protein 1 (Nod1). Nod1 acts an intracellular pathogen-recognition molecule and leads to the activation of NF-κB-dependent proinflammatory responses, such as IL-8 secretion [2, 32, 33]. *cagE* is genes located within the cag-pathogenicity island shown to stimulate the production of IL-8, from gastric epithelial cells induced by *H. pylori*. *cag*E is involved in the activation of NF-κB leading to the production of IL-8 production. The correlation between *cag*E gene and the endoscopic diagnosis of erosive gastritis has been reported in *H. pylori*-infected patients and the presence of *cag*E is also associated with *cag*A [34]. Therefore, this gene is possibly connected with the increase of IL-8 production in gastric cells and the induction of epithelial cells damage. In addition, it was found that *cag*E-positive *H. pylori* is associated with the presence of duodenum ulcer in children [35] and infection of AGS gastric epithelial cells with *H. pylori*-containing *cag*E resulted in higher amount of IL-8 as compared with *cag*E-negative strain [35]. Moreover, a mutant *H. pylori* strain

constructed by deleting *cag*E (*cag*E-KO) could not induce MKN45 gastric epithelium cells to produce IL-8 [36]. Specific region of *H. pylori* outer membrane protein (OMP) which was called outer inflammatory protein (OipA) played a crucial role in the induction of gastric epithelial cell lines to produce IL-8 [34]. OipA is significantly associated with duodenal ulceration [37, 38] and gastric cancer [38]. Another virulence factor, VacA has been demonstrated to induce IL-8 production in eosinophils [39] and monocytes [40]. Several lines of evidence showed that major transcription factors responsible for IL-8 activation by *H. pylori* involved NF-κB and/or activator protein 1 (AP-1) [40-42]. Other transcription factors correlated with IL-8 production are p38 mitogen-activated protein kinase (MAPK) [40, 43], ERK [40], and c-Jun N terminal kinase (JNK) [43]. Therefore, the inactivation of the NF-κB and/or AP-1 pathway and its downstream immune cascades may be helpful in preventing severe *H. pylori*-associated diseases.

The treatment of choice for *H. pylori*-infected patients is a combination of a proton pump inhibitor (PPI) and two antibiotics, mainly amoxicillin and clarithromycin for at least one week [44-46], resulting in the eradication rates of about 80% [47]. Nevertheless, the eradication rates following standard therapies are reducing on account of antibiotics resistance, especially clarithromycin and poor compliance due to many side effects of the medications as well as expenses of therapy [48-50]. Probiotics have been recommended as a useful adjunctive treatment to improve the rates of eradication and reduce antibiotic side effect [51-55]. Probiotics are defined by the Food and Agricultural Organization as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [56]. There are evidences demonstrating that probiotics bacteria could prevent and treat *H. pylori* infection [55, 57]. Several studies showed that *Lactobacillus* can inhibit the adherence of *H. pylori* to mammalian epithelial cells [49, 58, 59] and inhibit pro-inflammatory cytokines both *in vitro* and *in vivo* [36, 49, 60]. Previous studies demonstrated the potential use of probiotic lactobacilli in the treatment of *H. pylori*-related diseases. *Lactobacillus gasseri* OLL2716 (LG21) is effective in both suppressing *H. pylori* colonization and reducing gastric mucosal inflammation in humans [61]. Viable LG21 can also suppress *H. pylori*-induced IL-8 production by MKN45 gastric epithelial cells *in vitro*, and also in human gastric mucosa of *H. pylori* infected patients [36]. *Lactobacillus salivarius*

UCC118 could inhibit *H. pylori*-stimulated production of IL-8 by gastric epithelial cell *in vitro* and also modulate the expression of *Cag* PAI genes of *H. pylori* [49]. *L. salivarius* WB1004 protects the colonization of *H. pylori* in the stomach of gnotobiotic BALB/c mice [62]. *Lactobacillus johnsonii* La1 attenuates *H. pylori*-associated gastritis and reduces the levels of proinflammatory chemokines including macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived cytokine (KC) in sera and gastric tissues of C57BL/6 mice [63]. Both viable cells and supernatant of *Lactobacillus bulgaricus* have been demonstrated to inhibit *H. pylori* LPS-stimulated IL-8 production by inhibition of the Toll-like receptor (TLR) 4 pathway *in vitro* [64]. It has been reported that *Lactobacillus acidophilus* pre-treatment reduced *H. pylori*-induced inflammation in gastric epithelial cells through the inactivation of the Smad7 and NF-κB pathway [65]. Recently, Thiraworawong et al. (2014) showed that conditioned media from *Lactobacillus plantarum* XB7, *L. salivarius* B101, and *Lactobacillus rhamnosus* B103 suppressed IL-8 mRNA expression and protein secretion in *H. pylori*-infected AGS cells without inhibiting the growth of *H. pylori* [66]. Moreover, *L. salivarius* strain B37 (LS-B37) and B60 (LS-B60) have been shown to suppress IL-8 production in *H. pylori*-infected AGS gastric epithelial cells (50, 54) and also suppress TNF production from THP-1 monocytoid cell stimulated with LPS (51). However, the inhibitory substance (s) produced by these gastric derived-*Lactobacillus* strains are unidentified.

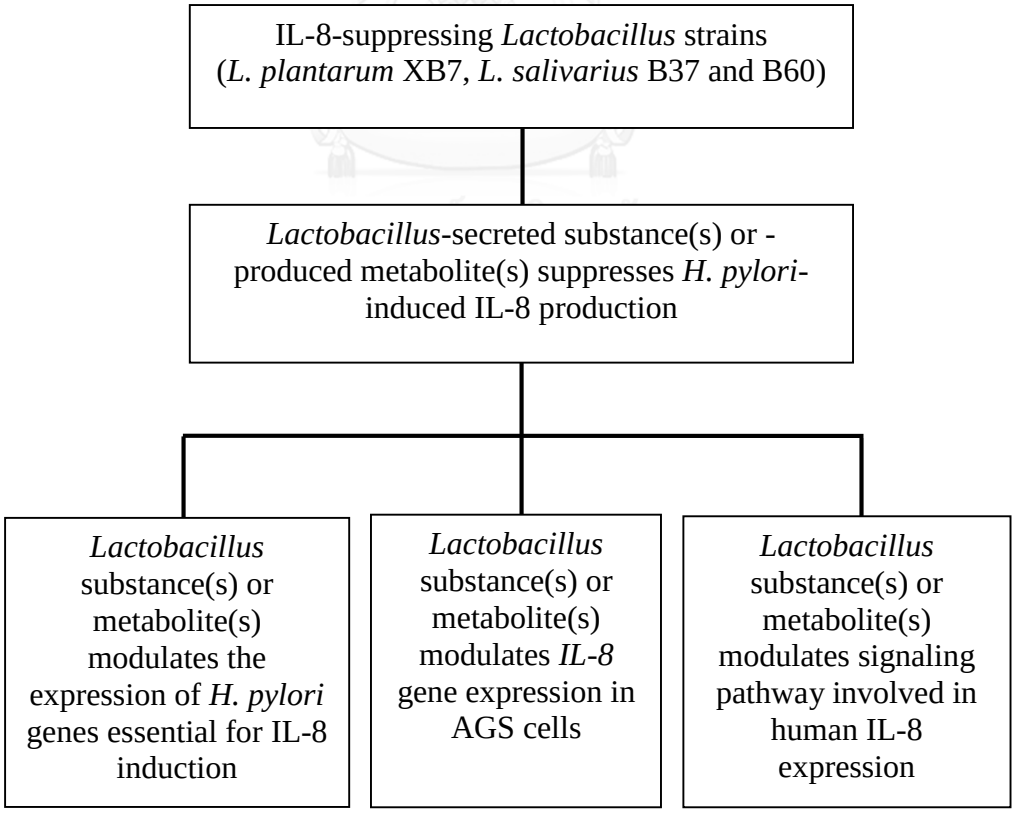
Our group has searched for gastric-derived *Lactobacillus* spp. suppressing IL-8 production stimulated by *H. pylori* and at present, eight *Lactobacillus* spp. are found. Of these 8 strains, *L. plantarum* strain XB7 (LP-XB7), *L. salivarius* strains B37 and B60 (LS-B37 and LS-B60) are selected for further investigation in this study. LP-XB7 decreases IL-8 production via the suppression of NF-κB and c-Jun activation and decreases inflammatory cytokine levels as well as inflammation in *H. pylori*-infected rat [66]. LS-B37 antagonizes the growth of *H. pylori* and 5% of its conditioned medium, which has no antagonistic effect, suppresses IL-8 production in *H. pylori*-induced AGS cells [66]. LS-B60 suppresses IL-8 production in *H. pylori*-induced AGS cells without antagonistic effect against *H. pylori* [66, 67]. In this study, we aimed to investigate in more details a number of aspects relating to the suppression of *H. pylori*-stimulated IL-8 production by these lactobacilli.

CHAPTER II
OBJECTIVES

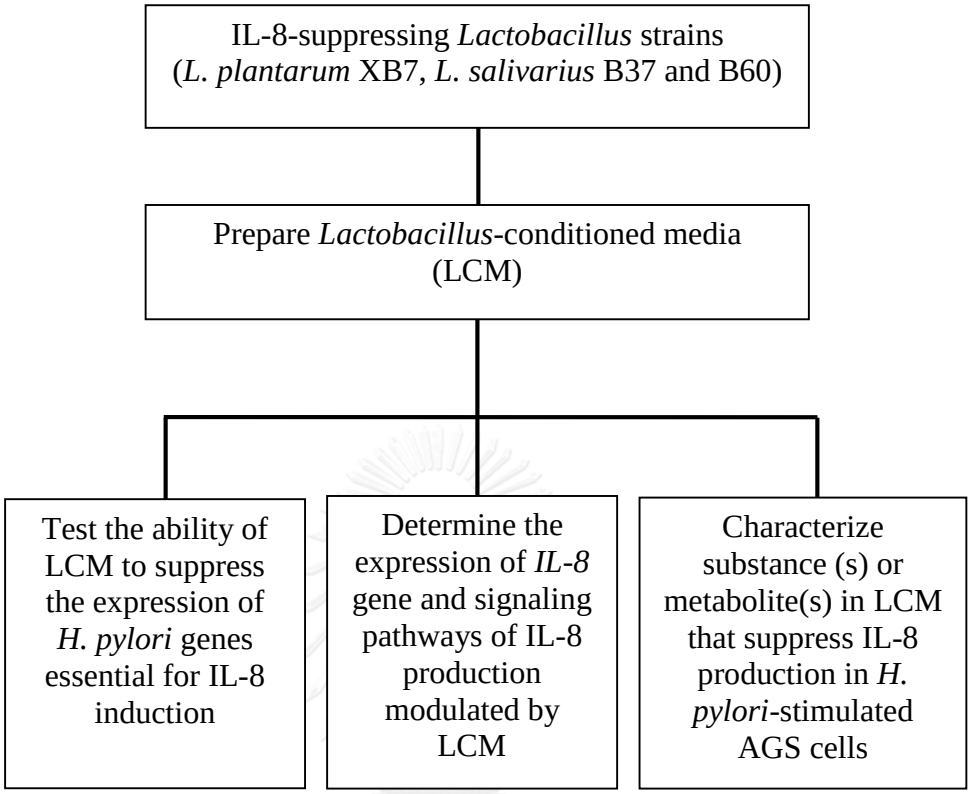
THE OBJECTIVES OF THIS STUDY WERE

1. Determine signaling pathways of IL-8 production modulated by *Lactobacillus* spp.
2. Investigate the suppression of *H. pylori* genes essential for IL-8 induction in human gastric epithelial cells by *Lactobacillus* spp.
3. Characterize substance(s) in LCM that suppress IL-8 production in *H. pylori*-stimulated human gastric epithelial cells

Conceptual framework



Methodology work flow



CHAPTER III
LITERATURE REVIEW

Helicobacter pylori is a stomach pathogen which causes gastritis, peptic ulcers, and has been linked to the development of gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (MALT) [68, 69]. The bacterium is gram-negative microaerophilic, spiral shaped, urease-, oxidase-, and catalase positive, and possesses 3-5 flagella which are important for motility. The ability of *H. pylori* to cause diseases depends on host immune response, bacterial virulence factors, and environmental factors. Bacterial factors including flagella, adhesins, urease, and delta-glutamyl transpeptidase are essential for *H. pylori* colonization [69]. Acidic pH < 2 is one of the most essential antimicrobial properties of the stomach for preventing bacterial growth and bacterial proliferation within the gastric lumen [70]. However, *H. pylori* is able to colonize in the highly acidic environments due to the secretion of urease to catalyze the breakdown of urea to ammonia and carbon dioxide [70].

H. pylori infects more than 50% of the world's population [71, 72]. Infection rates are increasing as more people live to an old age. It also occurs frequently in young people in the developing countries, in countries with poor sanitation. Infection by this bacterium causes the patients suffering from chronic gastritis, gastric ulcer, and duodenum ulcer. In peptic ulcer patients, 80% of duodenal ulcer patients and > 60% of gastric ulcer patients are infected with *H. pylori.* However about < 20% of individuals infected with *H. pylori* develop peptic ulcer [73].

History of *H. pylori*

In the time of the late 19th and early 20th centuries, many researchers had demonstrated the spiral shape microorganisms present in the animal stomachs. At the moment, similar spiral-shaped bacteria were observed in humans, and some of them occurred peptic ulcer disease (PUD) or gastric cancer. Later, the etiological role of these bacteria was considered for the progress of peptic ulcer disease and gastric cancer [3]. The bacteria indicated in stomach of humans were considered as food contaminants or bacterial overgrowth until the early 1980s. At this time, Warren and Marshall performed the experiments and definitively identified by culturing a spiral shape bacterium from gastric biopsy specimen of 58 of 100 patients [74, 75]. The microorganism was originally named "*Campylobacter*-like organism", "gastric *Campylobacter*-like organism", "*Campylobacter pyloridis*", and "*Campylobacter pylori*". However, now it was called *Helicobacter pylori* because this organism is distinct from the genus *Campylobacter* members. Since, it has been reported that after eradication of *H. pylori* in peptic ulcer patients, the rate of peptic recurrence are diminished [76, 77]. In a short time, it is crystal cleared that the spiral shape bacterium leads to chronic active gastritis, PUD, distal gastric adenocarcinomas, and gastric lymphomas. In 1994, *H. pylori* was designed as a type I carcinogen for stomach cancer, and at presence *H. pylori* is pondered the most common etiologic agent of infection-related cancers representing 5.5% of the global burden of cancer [78]. In 2005, Marshall and Warren were awarded the Nobel Prize of Medicine for their seminal discovery of this bacterium and its role in peptic ulcer disease.

Genus *Helicobacter*

The genus *Helicobacter* belongs to a subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. The genus *Helicobacter* comprises of more than 20 recognized species which are all microaerophilic [79]. Most of the genus *Helicobacter* are oxidase- and catalase positive, and many but not all species are urease positive. *H. pylori* is a gram-negative bacterium that usually appears spiral-shaped or a rod with multiple polar flagella [3]. *H. pylori* genome is heterogeneous, suggesting an absence of clonality [80]. They can be divided into two main lineages, the gastric

Helicobacter species and the enterohepatic (nongastric) *Helicobacter* species. In the first group, gastric *Helicobacter* species are able to colonize and persist to the inhospitable environments within the gastric mucosal surface. It is recently found that the stomachs of all mammals can be colonized by genus *Helicobacter* members. Moreover, species in the members of gastric *Helicobacter* are urease positive and exceptionally motile by flagella [81, 82]. This clarifies why both factors are important for gastric mucosa colonization of *H. pylori* [83-85]. Urease is essential for *H. pylori* colonization and survival in the highly acidic gastric lumen [83], whereas motility is thought to allow rapid movement toward the more neutral pH of the gastric mucosa [84]. Gastric *H. pylori* species grow in the viscous mucus layer due to the more neutral pH conditions caused by *H. pylori* urease. The members of gastric *Helicobacter* species are composed of *H. pylori, H. felis, H. mustelae, H. acinonychis,* and *H. heilmannii* [3]. In the second group, enterohepatic *Helicobacter* species can infect the lower gastrointestinal tract, including the ileum, colon, and biliary tree of humans and other animals. Persistent infections are contributed with chronic inflammation and hyperproliferation of epithelial cell that can lead to neoplastic disease, and are connected with human hepatobiliary ailment [81, 86]. Members of enterohepatic *Helicobacter* species consist of many species with different morphology, growth conditions, and the presence or absence of the urease [81]. The murine pathogen, *H. hepaticus*, has been superficially characterized, and was reported to cause chronic active hepatitis, hepatic tumors, and proliferative typhlocolitis [87, 88]. Even though *H. hepaticus* was originally found in the liver, the intestinal tract was the first site of its colonization and it was not found in the stomach [81]. *H. hepaticus* has similar morphology with *Campylobacter* species, with bipolar sheathed flagella [89]. *H. hepaticus* is urease-, oxidase-, and catalase- positive [3].

Prevalence and geographical distribution

Approximately 50% of the world's population is infected with *H. pylori* and the infection prevalence demonstrates large geographical variations. The infection is generally acquired during early childhood and usually persists for life if left untreated. The prevalence of *H. pylori* infection in childhood can vary greatly by nation and regions in same country. Auspiciously, the prevalence rates worldwide are showing decrease

even in children. Over 80% of the population including young ages is *H. pylori* positive in developing countries [90]. The prevalence of *H. pylori* infection in the United States and other industrialized countries is generally under 40% and demonstrates considerably higher in adults and elderly people than in children and adolescents [91]. It has been suggested that *H. pylori* infection are associated with age, gender, ethnicity, socio-economic status, low level of education, consumption of alcohol, sanitary environments, and lifestyle [92]. Among geographical areas, the prevalence of *H. pylori* infection shows inversely correlation with socioeconomic status, especially related with the living circumstance during childhood [93]. The improvement of standard of living is followed by decreasing in the prevalence rates of *H. pylori* infection in children in a developing country such as Brazil [94]. The *H. pylori* infection prevalence is frequently higher between among first- and second-generation immigrants in the Western counties from the developing nations [95, 96]. In developing countries, *H. pylori* prevalence demonstrates relatively constant, while it is rapidly declining in the industrialized world [97]. Although, there is a decrease in the prevalence of *H. pylori* infection in northern and western European countries, the *H. pylori* prevalence is still common in southern and eastern parts of Europe and Asia [98]. This phenomenon can explain due the improvement of hygiene and sanitation reduced chances of childhood infection and the elimination of *H. pylori* by using antimicrobial treatment. The prevalence of *H. pylori* infection in developing countries rapidly increase in the first 5 years of life, after that it is constantly high unless specifically treated. It is suggested that acquisition of *H. pylori* is mainly occurred during early childhood [99]. However, *H. pylori* infection rate is low in early childhood and gradually increases with rising age in industrialized nations. In Western countries, the incidence of new case of *H. pylori* infection among adults is less than 0.5% per year. The higher *H. pylori* prevalence among the elderly indicates that a birth cohort effect with higher infection rates in the past [100]. A lower infection rate in children resulted from the eradication of *H. pylori* from the population and improvement of hygiene and housing conditions [101-103].

Source of infection, transmission and epidemiology

H. pylori has narrow host ranges in humans and some nonhuman primates. It has been rarely isolated from pet animals suggesting that the presence of pets is a risk factor for *H. pylori* infection [104-106]. However, conclusive evidence for zoonotic transmission of *H. pylori* is not proposed. *H. pylori* can be detected in saliva, vomitus, gastric refluxate, and feces [107-111]; however, there is no conclusive data of transmission by these products. There was no precise evidence of increasing risk for being a carrier of *H. pylori* in dentists, gastroenterologists, nurses, couple of *H. pylori*-positive patient, or visitors in clinic of sexually transmitted diseases [112]. Recently, Moreno and Ferrus (2012) were able to culture *H. pylori* from 6 of 45 wastewater samples [113]. Another study showed the isolation of *H. pylori* from five water samples from a river in Isfahan and Iran [114]. It is thought that acquisition of *H. pylori* takes place in early childhood, from close relatives. [115-117]. *H. pylori* infection prevalence is positively associated with childhood crowding in and outside the family [118, 119] while crowding of adults are less important [120, 121].

The primary modes of *H. pylori* transmission is considered as a direct contact of human-to-human thought to be fecal-oral and oral-oral or both routes but some indirect evidence has been published for *H. pylori* transmission via drinking water and other environmental sources [117]. Transmission of *H. pylori* may occur in a vertical mode (from parents to child) [116] or in a horizontal mode (across individuals or from environmental contamination). Studies supports both intrafamilial and extrafamilial transmission [117]. There have been demonstrated the high risk of intrafamilial infection in the previous studies [122-124]. It is believed that in the majority of infected individuals, infection is acquired during early childhood [98, 125-127]. Some previous studies demonstrated that having infected family members is highly mediated with the infection in children [122-124]. The mother probably plays a major role in *H. pylori* transmission in their children [123, 124, 128]. The clarification of the mode of spread of this pathogen is very important due to design strategies to prevent *H. pylori* infection. The main reservoir of *H. pylori* is the human stomach, and the bacteria are most likely spread from person to person [129]. *H. pylori* infection is frequently correlated with poor sanitation, crowded living conditions, and poor water supplies [130]. According to Konno el al. (2008), 76% of children showed the pattern of DNA fingerprints of isolated

H. pylori identical to those from at least one family member. The rates of identity in the mothers' patterns significantly higher than rate of identity in the fathers' patterns. Mother-to-child transmission was suggested as the most probable route of transmission of *H. pylori* [122]. The mothers taking care of children is possible play a key role in transmission [123, 124, 128]. Gastroenteritis, especially with vomiting in the patient who infected with *H. pylori* markedly increase risk for new *H. pylori* infection in humans [131].

H. pylori-associated disease

H. pylori colonization in gastric mucosa induces gastric inflammation or histologic gastritis in all infected persons, but only a minority of people with *H. pylori* progress to any obvious clinical signs. In *H. pylori*-infected patients, it has been estimated that there is lifetime risk about 10 - 20% to develop ulcer disease and a 1 - 2% risk of progress to distal gastric cancer [132-134]. The risk of *H. pylori*-positive patients to develop gastric mucosa-associated disease depends on a variety of virulence factors, host genetic susceptibility, and environmental cofactors (Figure 1) [2].

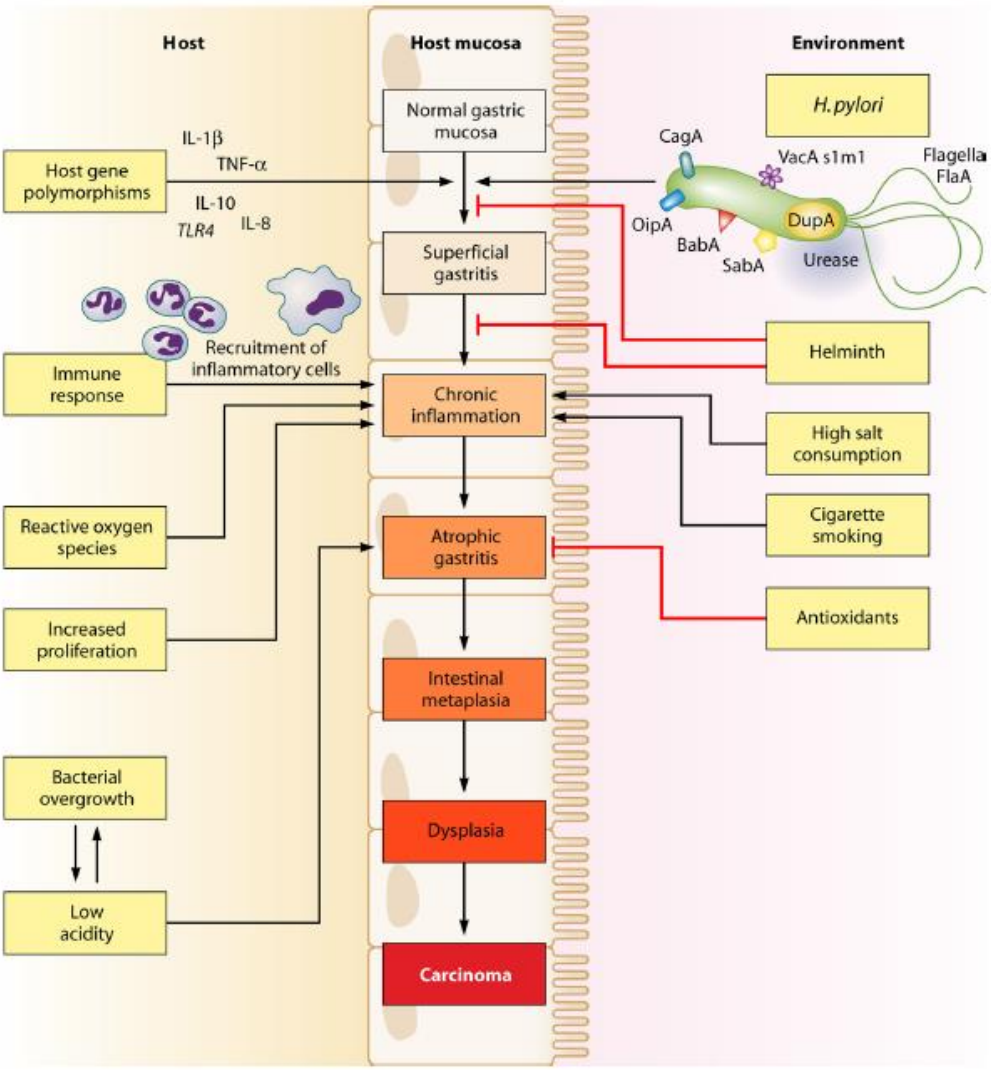


Figure 1: Multifactorial pathway leading to gastric carcinoma. Bacterial virulence, host factors, and environmental factors act in combination to contribute to the precancerous cascade leading to development of gastric cancer [2].

Acute and chronic gastritis

H. pylori infection result in infiltration of gastric mucosa in both antrum and corpus with neutrophils and mononuclear cells. This is initial step associated with the colonization and other *H. pylori* associated disease due to chronic inflammation course. Several reports have been showed that the acute phase of infection resulting from the subjects coincidentally or intentionally ingested *H. pylori* or underwent procedures with contaminated material [135-137]. *H. pylori* infection was demonstrated in human

challenge model which allowed controlled studies of the acute phase of infection in healthy volunteers by a well-studied laboratory strain of *H. pylori* [138]. Taken together, these reports demonstrated that *H. pylori* colonization with acute phase may be linked with transient nonspecific dyspeptic symptoms, including fullness, nausea, and vomiting. Additionally, it is correlated with ample inflammation both proximal and distal of stomach mucosa, or pangastritis which frequently mediated with hypochlorhydria. Persistance of *H. pylori* infection have been indicated a close correlation between the distribution of gastritis and the level of acid secretion (Figure 2). This relationship occurs from the effects of gastric acid on the *H. pylori* growth and gastric mucosa inflammation on acid regulation and secretion. *H. pylori* colonizes the gastric antrum in the subject with intact acid secretion, where few acid-secretory parietal cells are present and showing it is associated with an antrum-predominant gastritis. Moreover, in the subjects impairing acid secretion have a more *H. pylori* distribution of *H. pylori* in corpus and antrum, and bacteria in the corpus are in closer contact with the mucosa, leading to a corpus-predominant pangastritis [139]. Atrophic gastritis result from the reduction of acid secretion, but it can occur when parietal cell function is prohibited by acid-suppressive drugs, in particular, proton pump inhibitors (PPIs) [139]. The active corpus mucosa inflammation further increases hypochlorhydria, paralleling the acute phase of bacterial infection, as local inflammatory mediators such as proinflammatory cytokines, including IL-1β. This cytokine have a robust inhibitory effect on parietal cell function which inhibits acid secretion from parietal cells. The acid-suppressive effects of active corpus gastritis showing that patients with proinflammatory genotypes have a higher risk of corpus-predominant pangastritis, predisposing them to atrophic gastritis, intestinal metaplasia, and gastric cancer [140].

Pattern of gastritis	Gastric histology	Duodenal histology	Acid secretion	Clinical condition
 Pan-gastritis	• Chronic inflammation • Atrophy • Intestinal metaplasia	• Normal	• Reduced	• Gastric ulcer • Gastric cancer
 Antral-predominant	• Chronic inflammation • Polymorph activity	• Gastric metaplasia • Active chronic inflammation	• Increased	• Duodenal ulcer

Figure 2: Acid secretion and relation of gastritis pattern in *H. pylori* associated disease. The figure exhibits the relations between the pattern of colonization of *H. pylori*, gastric mucosa inflammation, gastric and duodenal histology, acid secretion, and clinical condition [3].

Peptic ulcer disease

Peptic ulcer are usually comprise of gastric or duodenal ulcers which defined as the defection of gastric mucosal with at least 0.5 cm in diameter penetrating through the muscularis mucosa into the submucosa layer or deeper. Gastric ulcers mainly occur along the lesser curvature of the stomach, at the transition from corpus to antrum mucosa [141]. Duodenal ulcers normally take place in the duodenal bulb, which is the area most exposed to gastric acid. Ulcers are to be distinguished from erosions, which erosions is epithelial disruption within the mucosa but no breach of the muscularis mucosa [84, 142]. In Western countries, gastric ulcers are common while duodenal ulcers are about four fold more common than gastric ulcers. Gastric ulcers are predominantly in over 40 years old individual while duodenal ulcer occur between 20 and 50 years of age. Infection of *H. pylori* are greatly mediated by both gastric- and duodenal ulceration. It has been reported that approximately 80% of patients with duodenal ulcers and > 60% of the patient with gastric ulcers are infected with *H. pylori.* However, about < 20% of individuals infected with *H. pylori* develop peptic ulcer [73].

Non-ulcer dyspepsia

Non-ulcer or functional dyspepsia is defined as presenting of symptoms of upper gastrointestinal distress absence any identifiable structural abnormality during diagnostic by performing upper gastrointestinal endoscopy. Symptoms of dyspepsia may exhibit a reflux-like character, with heartburn, or may have dysmotility-like, with satiety and nausea; or may appear ulcer-like, with pain and vomiting [143, 144]. Generally, these symptoms occurred in approximately 20-40% of the adult population of the Western country. The prevalence of *H. pylori* infection from 30 to 60% of patients with non-ulcer dyspepsia; however, this prevalence is not much distinct with the unaffected people [145].

Atrophic gastritis, intestinal metaplasia, and gastric cancer

Chronic inflammation induced by *H. pylori* can eventually lead to abnormality of gastric mucosal structure as well as damage of gastric glands, and also following by substitution of fibrosis and intestinal-type epithelium. It has been reported that atrophic gastritis and intestinal metaplasia presents in about 50% of the *H. pylori*-infected persons [146]. The distribution and pattern of chronic active inflammation causing by *H. pylori* infection demonstrates the risk for atrophic gastritis. Patients decreasing with gastric acid show a rapid progression towards atrophic gastritis [147]. In addition, loss of gland and intestinal metaplasia increase the risk for gastric cancer between 5- and 90-fold relying on the magnitude and severity of atrophy [148]. There is evidence indicated that *H. pylori* infected subjects increases the risk of gastric cancer development more than uninfected controls [149]. There have been shown the incidence of gastric carcinoma associated with the prevalence of *H. pylori* infection [150]. The risk of gastric cancer can be increased by *H. pylori* infection about 10 fold. *H. pylori* was recognized as a cause of gastritis, peptic ulcer, and it has been designed as a class I carcinogen by World Health Organization (WHO) [151]. Previous case-control studies in gastric cancer subjects showed higher odds ratios for development of distal gastric cancer in the presence of *H. pylori* infection than control patients [152]. *H. pylori* possessing *cag*A possible increased the risk of atrophic gastritis and gastric cancer [26, 28]. This is supported by the results from *in vivo* studies, Mongolian gerbil infected with *H. pylori* induces atrophic gastritis and gastric cancer [153, 154]. Although, the

incidence of gastric cancer is decline in Western countries, gastric cancer is the fourth most common cancer in worldwide, the incidence of this disease still very high in large areas of the world, particularly in regions of East Asia and South America [155]. In Western countries, *H. pylori* positive subjects have lifetime of gastric cancer approximately 1-2 % [133]. 60% to 80% of gastric cancers are correlated to the long-term presence of *H. pylori* in the developed word.

Gastroesophageal reflux disease (GERD)

GERD has been considered to occur independently of *H. pylori* colonization due to the similar frequency and severity in *H. pylori*-positive and *H. pylori*-negative subjects. The evidence showed that the cross-sectional study in the prevalence of *H. pylori* infection among GERD patients was similar to that among controls [156]. However, further studies suggested that *H. pylori* infection might protect from development of GERD that benefit to their hosts. There have been reported that after *H. pylori* eradication is increasing incidence of GERD [157]**.** The data suggest that there may be inverse correlation between GERD and *H. pylori* infection. However, there is not conclusive evidence for the rate of *H. pylori* infection and GERD.

Gastric MALT lymphoma

Usually, the gastric mucosa does not contain lymphoid tissue, but MALT nearly appears in the stomach mucosa in response to *H. pylori* colonization. Rarely, a monoclonal B cells population may arise from gastric tissue and slowly proliferate to form a MALT lymphoma. The histological criteria for the diagnosis of MALT lymphoma remain controversial. Recently, all MALT lymphoma patients are *H. pylori* positive [158]. There is evidence showed that a significantly increased risk for the development of gastric MALT lymphoma [159] and also in the patients who infected with *H. pylori*, MALT lymphomas occurs in less than 1% [160]. *H. pylori*-positive subjects have a 2 - 6 fold increased risk of developing gastric cancer and mucosal associated-lymphoid-type (MALT) lymphoma compared with their uninfected group. Recent studies have shown a correlation between the development of gastric cancer and long-term *H. pylori* infection. Gastric cancer is the second most common cancer

worldwide; however, it is unclear whether *H. pylori* eradication will improve outcomes in patients with gastric cancer [161]

Iron deficiency

Acute infection with *H. pylori* is associated with transient hypochlorhydria. *H pylori*-associated atrophy, hypochlorhydria has a role in iron deficiency due to alter in the physiology of iron-complex absorption. Harris *et al.* (2013) suggested that hypochlorhydria in *H. pylori* infected children may be the role in the aetiology of iron deficiency [162]. A study by Queiroz et al. (2013) determined that the serum ferritin and haemoglobin concentrations were low in *H. pylori* infected children from Latin-America [163]. The mean serum Fe and ferritin levels of *H. pylori* positive group were significantly lower than those of *H. pylori* negative control [164] suggesting that low socioeconomic status appear to an important risk factor for *H. pylori* infection and also showing that exclusive breast-feeding at least for 4 months have a protective role against *H. pylori* infection [164].

***H. pylori* virulence factors**

1. ***H. pylori* adhesins**

Adhesins are factors that enable bacteria attach to cells. The initial step required for colonization and pathogenesis of *H. pylori* infection is the adherence of *H. pylori* to gastric mucosa. *H. pylori* adhesins are considered as bacterial virulence factors and involved in the process of infection. Many bacterial factors mediated the adhesion of *H. pylori* to gastric epithelial cells include:

- Blood group antigen binding adhesion A (BabA) (HopS)

The 78-kDa BabA protein presumably represents the best-characterized *H. pylori* adhesion protein. BabA mediates the binding of the bacterium to fucosylated Lewis B (Le^b) blood group antigen which expresses in gastric epithelial cells in some people [32]. There are two distinct *babA* alleles, *bab*A1 and *bab*A2, which *bab*A2 gene encodes the active form bacterial adhesion protein. It has been shown to be associated with up-regulated the level of IL-8 mRNA transcription in human gastric biopsy specimens [165]. However,

in vitro study indicated that babA2 was not correlated with increased IL-8 production [34].

- The sialic acid-binding adhesion (SabA) (HopP)

SabA mediates binding to sialic acid containing glycoconjugates. *H. pylori* induces gastric inflammation and gastric carcinoma associated with the replacement of nonsialylated Lewis antigen by sialylated Le^x and sialylated Le^a. Thus, the role of SabA is presumably during the chronic inflammation and atrophic disease [166]. Human granulocytes also carry sialylated glycan on the cell surface, which these cells are specific recognized by SabA. *In vitro* study, neutrophils were stimulated with isogenic mutants lacking SabA had no neutrophil-activating capacity [167]. This result, suggesting binding of *H. pylori* to sialylated neutrophil receptors plays a role in the adherence and phagocytosis of *H. pylori* and production of reactive oxygen species causing damage of the gastric mucosa. *H. pylori* infection and the resulting mucosal inflammation, the sialyl-dimeric-Lewis x (Lex) glycosphingolipid is up-regulated and SabA mediates the binding of the bacterium to the sialyl-dimeric-Lex [168].

- Outer inflammatory protein A (OipA) (HopH)

The outer inflammatory protein (OipA) is another member of the Hop protein family that initially identified as surface protein which promotes *in vitro* IL-8 secretion in a T4SS independent manner and heightens gastric inflammation *in vivo*. All *H. pylori* strains are express the gene that encode OipA protein and also significantly linked to duodenum ulcer and gastric cancer. Previously study showed that *oip*A gene was involved in the induction of IL-8 production in gastric cancer cell lines [34]. This gene has lately been discovered, there in currently not much data on its associate with a disease-specific marker [3]. A functional OipA was significantly correlated with high *H. pylori* density, severe neutrophil infiltration, and high mucosal IL-8 levels [37].

2. Cytotoxin-associated gene (*cag*) pathogenicity island (PAI)

The strains of *H. pylori* containing the *cag* PAI are more virulent than those that do not contain the *cag* PAI and are associated with increasing gastric inflammation and a higher incidence of gastroduodenal diseases [169]. The entire *cag* PAI was sequenced from *H. pylori* and found to be a 40 kb DNA insertion element, which carries up to 32 genes flanked by 31 bp direct repeats. Most of the *cag* PAI genes are presumably involved in the assembly of the type 4 secretion system (T4SS) called molecular syringe that translocates protein CagA, peptidoglycan, and possibly other bacterial factor into the gastric epithelial cells [3] (Figure 3). This secretory system involved in the induction of the proinflammatory cytokine, IL-8 and tyrosine phosphorylation of proteins in the gastric cells. *H. pylori* strains containing *cag* PAI stimulate epithelial cell lines to express large amount of proinflammatory cytokine and CagA positive strains are associated with high level IL-8 expression of epithelial cell *in vivo* [32].

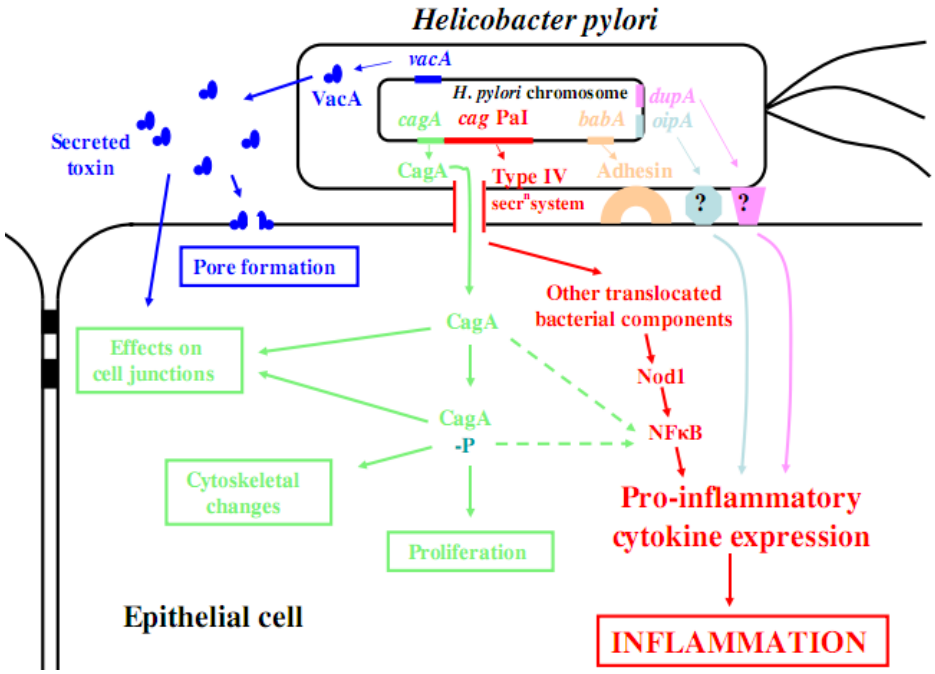


Figure 3: Diagrams showing virulence factor of *H. pylori* and their major effects. The Cag PAI gene encodes a type IV secretion system (T4SS) and the translocation of CagA into epithelium cells is the main cause for inducing to epithelium cells proinflammatory cytokine and inflammation [32].

3. Cytotoxin-associated gene A (CagA)

CagA protein is immunogenic protein encoded by *cag*A gene which expresses in approximately 50-70% of *H. pylori* stains [3]. The injected CagA protein is recognized by the epithelial cells as signaling molecules and activated by phosphorylation of tyrosine residues in EPIYA motifs by src kinases. Phosphorylated CagA then interacts with SHP-2 and induces MAP kinase signaling resulting in abnormal of cell proliferation and morphological changes in epithelial cells [32]. The variation in the number of the EPIYA tyrosine phosphorylation motifs among the CagA protein of different *H. pylori* isolates. Strains possessing CagA with greater numbers of these repeats induced morphological changes in cultured epithelial cells and associated with an increased risk of gastric carcinogenesis [3]. Patients infected with *cag*A-positive strains usually have a higher inflammatory response and are significantly more at risk for developing a symptomatic outcome [3, 170]. Infection by *cag*A-positive *H. pylori* is also associated with gastric carcinomas and gastric mucosa-associated lymphoid tissue (MALT) lymphomas [35]. Epidemiologic studies demonstrated that *cag*A positive is presented in the gastric mucosa of most gastric MALT lymphomas patients [171].

4. Cytotoxin-associated gene E (CagE)

cagE is genes located within the cag-pathogenicity island and also placed upstream of *cag*A gene, and encodes a protein involved in the translocation of CagA and in the process of chemokine expression. The *cag*E showed to stimulate the production of chemokine IL-8 from gastric epithelial cells induced by *H. pylori*. *cag*E is involved in the activation of NF-κB leading to the production of IL-8 production. There have been showed the correlation between the *cag*E gene and the endoscopic diagnosis of erosive gastritis in the patients who infected with *H. pylori* and also the present of *cag*E gene associated with the *cag*A gene [34]. Therefore, this gene is possibly connected with increasing of IL-8 production in gastric cells and inducing of epithelial cells damage. CagE positive *H. pylori* associated with the presence of duodenum ulcer in children [35]. Infection of AGS human gastric epithelial cells with *H. pylori*-containing *cag*E resulted in higher amount of IL-8 as compared with *cag*E-negative strain [35]. Moreover, According to Tamura, A. et al. (2006) the *cag*E mutant

H. pylori stains constructed by deleting the *cag*E (*cag*E-KO) could not induce MKN45 gastric epithelium cells to produce IL-8 [36].

5. Vacuolating cytotoxin (VacA)

The VacA protein is produced as a 140-kDa protoxin and cleavaged into the 95-kDa mature form when secreted from *H. pylori*. The s region of the *vacA* gene, which encodes the signal peptide exist as an s1 or s2 type, and the middle region, which contains the p58 cell binding domain, occurs as an m1 or m2 type [3]. VacA s1/m1 genotype is more frequently associated with peptic ulcer and carcinoma. VacA protein is an important role in the pathogenesis of peptic ulcer and gastric cancer [3]. The correlation between toxin activity and the *H. pylori* pathogenicity, with the s1/m1 type of VacA being the most virulent in Western population but not observed this correlation in Asian subjects. Despite, active forms of VacA are associated with increased cancer risk, suggesting that increased stimulation of local inflammation not only mechanism by *H. pylori* predisposes to gastric carcinoma [32]. This protein influences cellular processes via different rout such as surface-bound VacA directly delivered to the membrane, bind to cellular membrane receptor and initiate a proinflammatory response, taken up directly by the cell into the mitochondria and induce apoptosis, taken up by pinocytosis and induce vacuolization, and form membrane channel and induce leakage of nutrients to the extracellular space, and pass through the tight junctions and inhibit T-cell activation and proliferation [172].

6. Acid resistance

H. pylori moves rapidly toward the gastric mucosa layer by chemotactic motility using the urea and bicarbonate gradients in the gastric environment. The main component of *H. pylori* acid resistance is the urease converting urea into ammonia and carbon dioxide, which urease activity is present in all *H. pylori* isolates. However, urease activity is differ between strains of *H. pylori* and depend upon the growth condition [32]. The ammonia and bicarbonate have been associated in the pathogenesis of *H. pylori*. Ammonia is cytotoxic effect of gastric epithelial cells, while bicarbonate is thought to suppress the bactericidal effect of peroxynitrite, a nitric oxide metabolite [3].

7. Lipopolysaccharide (LPS)

H. pylori expresses LPS, which O polysaccharides of *H. pylori* contain Lewis antigens, mimicking glycan structures produced by host cells [3]. These bacterial antigens (Lewis antigen) can interact with human dendritic cells, so inducing a suppression of the immune response and facilitating a chronic *H. pylori* infection [173]. Bacterial expression of Lewis antigens similar to that of host $Lewis^X$ or $Lewis^Y$ is thought of a form molecular mimicry, contributing to bacterial persistence thought immune erosion. *H. pylori* LPS may have an important role in autoimmune immune response in gastric epithelial cells and the immunological activity of *H. pylori* LPS has been considered to be low. Previously showed that the production of chemokine IL-8 from neutrophils and TNF, IL-6 from human mononuclear cells after induced by *H. pylori* LPS was significantly lower than *Escherichai coli* and *Salmonella* LPS [174].

8. The RNA polymerase β–subunit, RpoB

Recently, the genetic diversity of *H. pylori* isolates were investigated by analyzing rpoB sequences. The rpoB sequences of Asian and non-Asian strains were found difference. There has been identified the polymorphism of *Rpo*B gene, which a nucleotide substitution at position 497 from an Alanine-Threonine. Interestingly, 67% of Asian countries *H. pylori* strains possessed $RpoB^{Thr}$ compared to only 2.9% of Western countries. $RpoB^{Thr}$ strains induced significantly more IL-8 secretion from MKN45 cells than $RpoB^{Ala}$, which these chemokine that plays an important role in chronic inflammation [175].

9. Duodenal ulcer promoting gene A (*dupA*)

Currently, a novel gene was identified in some strain of *H. pylori*, which associated with duodenum ulcers, but also with reduced risk for gastric atrophy and cancer [176]. dupA was indicted to be significantly mediated with the induction of IL-8 secretion *in vitro* and *in vivo*. Its association with duodenum ulcer-promoting and protective effects against atrophy/cancer was evident in both Asian and Western countries.

H. pylori factors that contribute to gastric colonization

H. pylori colonization of the gastric mucosa induces an acute inflammatory response and damage to the gastric mucosa. The ability of *H. pylori* to colonize in human gastric mucosa contributes to the production of colonization factors such as urease, flagella, and adhesins [70, 177]. Urea can be hydrolyzed by *H. pylori* urease to carbon dioxide and ammonium, which neutralizes acid in stomach environment [178]. The flagella confer motility and allow rapid movement in the mucus layer overlying the gastric epithelial cells [70]. Outer membrane proteins of *H. pylori*, including BabA (HopS), SabA (HopP), AlpA (HopC), AlpB (HopB), HopZ, and OipA (HopH) are associated with the adherence of *H. pylori* to epithelial surface of the gastric mucosa (Figure 4).

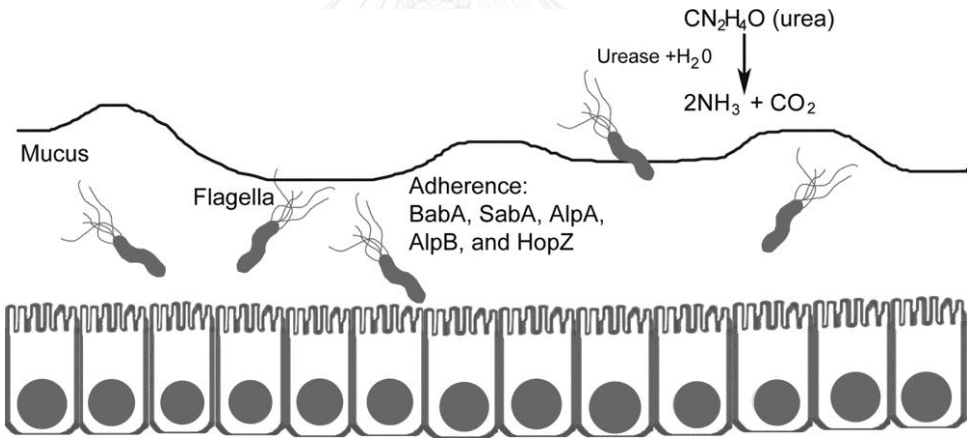


Figure 4: Colonization factors of *H. pylori*. Many bacterial factors contribute to the ability of *H. pylori* to infect the human stomach. Flagella confer bacterial motility and urease enzyme contributes to the acid resistance. Several outer membrane proteins, including BabA, SabA, AlpA, AlpB, and HopZ, can mediate bacterial adherence to gastric epithelial cells [70].

Interactions of host and bacterial factors in pathogenesis of *H. pylori* infections

H. pylori attachment to the human gastric epithelial cells involves specific bacterial adhesion molecules such as BabA, BabB, HopZ, and other adhesins. Cag PAI-positive strain allows translocation of CagA into the host cells by type 4 secretion system, resulting in the production of IL-8 and other chemokines by gastric epithelial cells. The secreted chemokines cause of the recruitment of polymorphonuclear cells (PMNs), and neutrophils into the site of infection resulting in gastric tissue inflammation. CagA of *H. pylori* disrupts gastric epithelial barrier and alters the morphology of epithelial cells. The evidence shows that cagA positive strain colonizes with the tight junction protein such as Zona occludens protein (ZO-1) and Junctional adhesion molecules (JAM) as contribute to the disruption of the barrier function of tight junctions in polarized epithelial cells [179, 180]. When tight junction disruption, VacA translocates into the submucosa and induces gastric epithelial cells apoptosis by reducing mitochondrial transmembrane potential and stimulating cytochrome *c* release, which might also contribute to the disruption of gastric epithelial barrier. Moreover, TNF produced by macrophage mediated apoptosis may also bring to disruption of the epithelial barrier. The chronic gastritis infected by *H. pylori* connects an adaptive immune response with the initial innate response. IL-12, produced by macrophage stimulates helper T cells, responding with a biased TH1 response. Cytokines may also change the mucus secretion contributing to *H. pylori*-induced disruption of the mucous layer, as they induce changes in gastric-acid secretion and homeostasis. *H. pylori* suppresses the host immune responses by blocking nitric oxide (NO) production by macrophages and the capability of VacA to interfere with the IL-2 signaling pathway in T cells which blocks the expression of IL-2 and IL-2 receptor resulting in inhibit T cell activation and proliferation (Figure 5). This is evasive mechanisms which *H. pylori* used to evade the host immune responses [181].

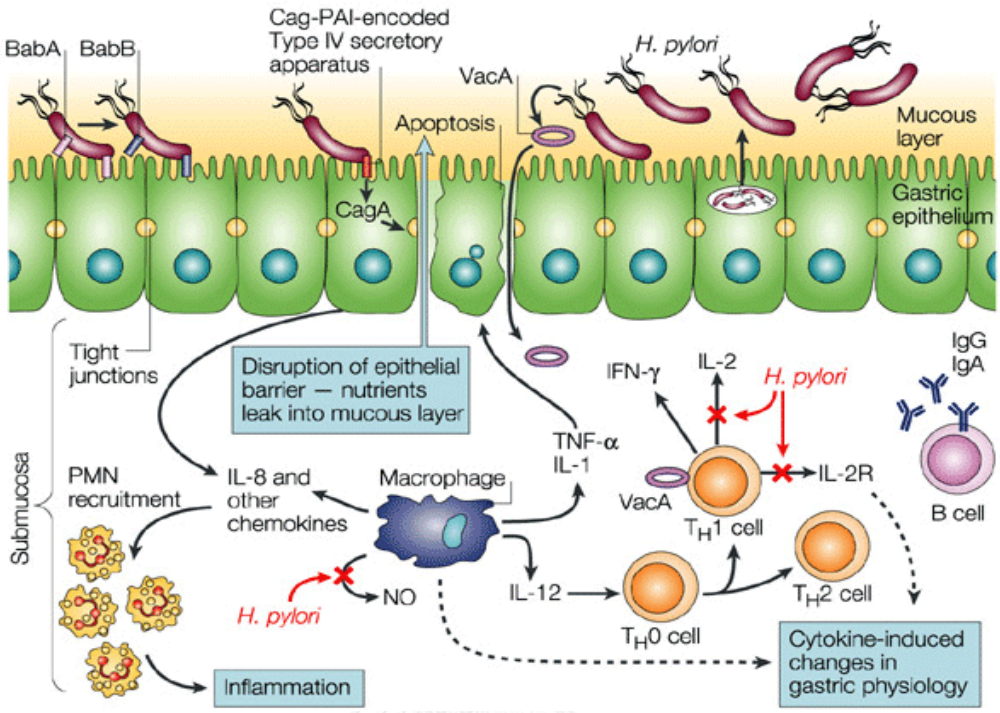


Figure 5: Interaction between *H. pylori* factors and the host response leads to chronic gastritis and persistent colonization [181].

H. pylori-induced cytokines and chemokines

H. pylori induces inflammation through direct contact with gastric epithelial cells and stimulation of cytokine release [177]. The gastric inflammatory response induced by *H. pylori* composed of neutrophils, macrophages, lymphocytes (T and B cells), mast cells, and along with varying degrees of epithelial cells degeneration and injury. Gastric mucosal levels of the proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, TNF, and IL-8 have been reported to be increased in *H. pylori*-infected subjects. The concentrations of cytokines TNF, IL-2R, IL-6, and IL-8 in gastric fluids and serum of *H. pylori*-positive were higher than *H. pylori*-negative control [182]. The mucosal levels of IL-1β and IL-8 were significantly higher in specimens infected with *cag*A positive strains than in those infected with *cag*A negative strains [173]. The chemokine IL-8 is an important mediator of neutrophils infiltration and associated with the chronic inflammation. IL-8 producing cells are gastric epithelial cells, monocytes, macrophage, lymphocytes, and endothelial cells [183]. *H. pylori* can induce the

production of IL-8 in human gastric mucosa resulting in the accumulation of neutrophils in gastric tissues and initiates inflammatory damage in gastric mucosa, which plays a major role in the pathogenesis of *H. pylori* infection [36].

Factors of *H. pylori* involved in IL-8 production

There are several virulence factors of *H. pylori* that induce IL-8 production. Infections with strains carrying the cytotoxin-associated gene pathogenicity island (*cag* PAI) induce gastric epithelial cells to express large amounts (1,000-2,000 pg/mL) of IL-8 *in vitro* [22, 23], showing that IL-8 secretion is dependent upon the *cag* PAI gene. *cag* PAI is associated with higher grades of gastric inflammation, correlated with enhanced mucosal levels IL-8 expression, and increased risk of peptic ulceration in humans [24, 25]. Epidemiological studies have shown that persons infected with *H. pylori* strains containing the cytotoxin-associated antigen A (*cag*A) gene are at considerably increased risk of gastric cancer than *cag*A negative strains [26, 27]. Infection with *cag*A-positive *H. pylori* strains is associated with an increasing risk for the eventual development of atrophic gastritis and intestinal metaplasia [28]. The CagA protein activates NF-kB, via ERK, and induces IL-8 secretion [29]. Transfection of AGS gastric epithelial cells with *cag*A also induces IL-8 secretion [30]. CagA protein is translocated into host epithelial cells via a type 4 secretion system (T4SS) after bacterial attachment [2]. Besides CagA, T4SS can deliver components of *H. pylori* peptidoglycan into host cells which interacts with the nucleotide-binding oligomerisation domain protein 1 (Nod1). Nod1 acts an intracellular pathogen-recognition molecule and leads to the activation of NF-kB-dependent proinflammatory responses, such as secretion of IL-8 [2, 32, 33]. *cagE* is genes located within the cag-pathogenicity island shown to stimulate the production of chemokines, such as IL-8, from gastric epithelial cells induced by *H. pylori*. *cag*E is involved in the activation of NF-κB leading to the production of IL-8 production. Thus disruption of the *cag*E gene encoding the homologue of the *Agrobacterium* VirB4 ATPase which powers the translocation process, or deletion of the entire cag PAI, obliterates *H. pylori*-induced IL-8 synthesis [184]. Ramis et al. (2013) showed the correlation between the *cag*E gene and the endoscopic diagnosis of erosive gastritis in the patients who infected with *H. pylori* and also the present of *cag*E gene associated with the *cag*A gene [34].

Therefore, this gene is possibly connected with increasing of IL-8 production in gastric cells and inducing of epithelial cells damage. CagE positive *H. pylori* associated with the presence of duodenum ulcer in children [35]. Infection of AGS gastric epithelial cells with *H. pylori*-containing *cag*E resulted in higher amount of IL-8 as compared with *cag*E-negative strain [35]. Moreover, According to Tamura et al. (2006) the *cag*E knockout mutant *H. pylori* strain cannot stimulate gastric epithelial cell lines to produce IL-8 could not induce MKN45 gastric epithelium cells to produce IL-8 [36]. Specific region of *H. pylori* outer membrane protein (OMP) which was called outer inflammatory protein (OipA) played an important role in induction of gastric epithelial cell lines to produce IL-8 [34]. OipA is significantly associated with duodenal ulceration [37, 38] and gastric cancer [38]. Another *H. pylori* virulence factor, VacA has been demonstrated that can induce IL-8 production in eosinophil [39] and monocyte [40].

The pathogenesis of *H. pylori*-related signaling and inflammation

H. pylori-infected gastric epithelial cells elicits a variety of phenotypic responses including the expression of proinflammatory genes and change in the actin cytoskeleton resulting in phenotypic alternation in gastric epithelial cells. These responses are mediated with the type 4 secretion system encoded by the *cag* PAI gene [184, 185]. According to Guillemin et al. (2002) the transcriptional profiles by using human cDNA microarrays of *H. pylori* strain G27-infected AGS cells showed *H. pylori* G27 induces expression of genes which involves in cells shape regulation, innate immune response, and signal transduction [184]. Infection with *H. pylori* strains containing the *cag* PAI induces the signaling pathway and the release of proinflammatory cytokines and chemokines [186]. The *cag* PAI is involved with the activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), which is key regulators of the expression of various inflammatory genes [41, 186]. The colonization of *cag*A positive *H. pylori* to AGS cells induces the rapid activation of extracellular signal-regulated kinases (ERK), p38, c-Jun N-terminal kinase (JNK), and mitogen-activated protein kinase (MAPK) [43]. Translocation of CagA induced the phosphorylation of ERK and p38 MAPK in BJAB human B cell lines [187].

The peptidoglycan-derived muropeptides (GM) are released into gastric epithelial cells through the type 4 secretion system (T4SS) of *H. pylori*. These muropeptides are recognized by the host pattern recognition receptor (PRR), intracellular nucleotide-binding oligomerisation domain protein 1 (Nod I) and induced signaling transduction leading to NF-κB activation and induction of gene expression encoding proinflammatoty cytokine [32]. *H. pylori* containing *cag* PAI direct signaling cascades involves PAK1, NIK, and IKK complex. IκB was phosphorylated by the IKK complex leading to IκB ubiquitination, degradation, and translocation of NF-κB into the specific promoter. Activation of JNK mediated with p21-activated kinase (PAK1), unknown mitogen-activated protein kinase (MAP) kinase kinase kinase (MKKK), MKK4 [41], and JNK leading to AP-1 activation and translocation into the promoter. Furthermore, p38 kinase is induced by *H. pylori* strains possessing T4SS. NF-κB and/or AP-1 are key transcription factors of inflammatory process induced by *H. pylori* (Figure 6) [32]. Several kinds of evidence showed that major transcription factors responsible for IL-8 activation by *H. pylori* involved NF-κB and/or AP-1 [40-42]. Other signaling proteins correlated with IL-8 production are p38 mitogen-activated protein kinase (MAPK) [40, 43], extracellular signal-regulated kinase (ERK) [40], and c-Jun N-terminal kinases (JNK) [43]. Therefore, the inactivation of the NF-κB and/or AP-1 pathway and its downstream immune cascades may be helpful in preventing severe *H. pylori*-associated diseases.

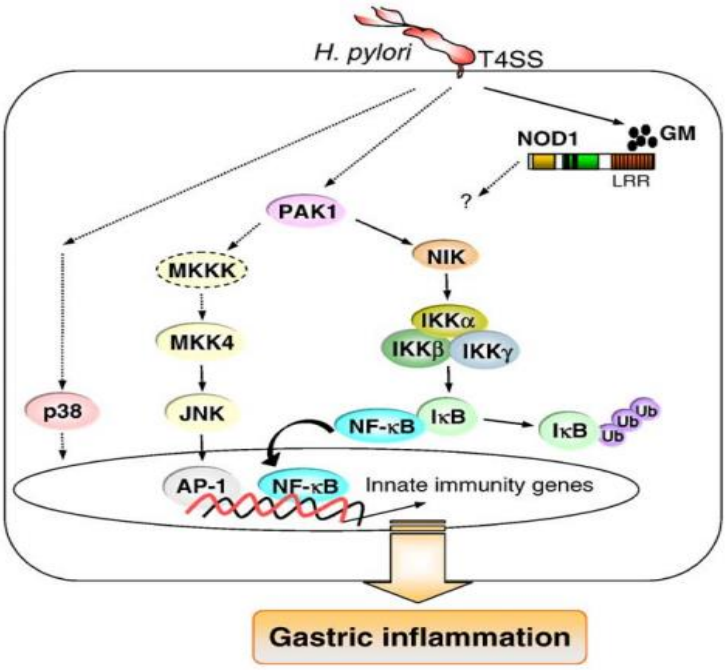


Figure 6: Host cell signaling activated with *H. pylori* and inducing to the gastric inflammation [188]

Treatment of *H. pylori*

One-week standard triple therapy, combining a proton pump inhibitor plus two antibiotics is recently considered as a first line therapy of choice of *H. pylori* eradication; however, the eradication rates about 80%. Poor patient compliance due to side effects and resistance to bacterial agent are the main factors contributing to treatment failure. Therefore, the improvements of drug compliance, increasing eradication rates and reducing side effects are need in the current used. Furthermore, the antibiotic treatment of the large dosages for 1 week may induce an alteration in bowel microflora and lead to gastrointestinal side-effects after triple therapy. Antibiotic treatments are often associated with gastrointestinal side-effects such as diarrhea, nausea, vomiting, bloating and abdominal pain. Probiotics supplementation might help to increase the eradication rates of *H. pylori* and prevent or reduce such drug-related manifestations.

Probiotics have been proposed as a useful adjunctive therapy to improve either side effect or the rates of eradication. According to Michetti et al. (1999), culture supernatant of *L. acidophilus* (*johnsonii*) La1 shown to be effective *in vitro* has a partial long-term suppressive effect on *H. pylori* infection in humans [189]. Canducci et al. (2000) reported that the supplementation of standard eradication therapy with a lyophilized and inactivated culture of *L. acidophilus* strain LB increased the eradication rates in *H. pylori*-positive dyspeptic volunteers. However, no differences in eradication rates between the patients with underlying duodenal ulcer [52]. According to Sheu et al. (2002), the supplementation of the standard triple therapy with *Lactobacillus* and *Bifidobacterium* containing yogurt increased the full drug compliance of patients and decreased side-effects such as vomiting, constipation, and diarrhea and also restored the depletion of *Bifidobacterium* in stool of patients after triple therapy [54]. Armuzzi et al. (2002), they reported that *Lactobacillus* GG supplementation reduced *H. pylori* standard triple therapy-related side-effects such as diarrhea, nausea and taste disturbance and also showed improvement in treatment tolerability [51]. Sgouras et al. (2003) reported that the levels of *H. pylori* colonization was reduced in the antrum and body mucosa in mouse model in the *L. casei* strain Shirota-treated group which associated with the reduction of chronic and active gastric mucosal inflammation [190] (Table 1).

Table 1: Clinical trials using probiotics with *H. pylori* eradication treatment

Eradication treatment	Probiotic strain (s)	Eradication rates	% side effect	Probiotic(s) efficacy	Reference
Esomeprazole 20 mg, levofloxacin 500 mg, amoxicillin 1 g, all of these *bid*, 7 days	10^8 CFU *L. reuteri*, during therapy and 7 days thereafter	80% (36/45)	66.7	Eradication rate ↑ Adverse effects ↓ (nausea and diarrhea)	44
	Control	60% (27/45)	100		
Esomeprazole 20 mg and amoxicillin 1 g, both *bid*, 5 days; then esomeoprazole 20 mg, clarithromycin 500 mg, and tinidazole 500 mg, all *bid*, 5 days (sequential therapy)	10^9 CFU *L. acidophilus*, 10^9 CFU *L. bulgaricus*, 5×10^8 CFU *Bifidobacterium bifidum*, 10^9 CFU *Streptococcus thermophilus*, during therapy	89% (65/73)	39.7	Eradication rate ↔ Adverse effects ↓ (metallic taste, abdominal/epigastric pain, diarrhea)	45
	Control	88.2% (67/76)	65.8		
Omeprazole 1 mg/kg (before breakfast), amoxicillin50 mg/kg *bid* (after meals), and clarithromycin 15 mg/kg *bid* (after meals), 7 days	5×10^9 CFU *L. plantarum*, 2×10^9 CFU *L. reuterii*, 2×10^9 CFU *L. casei subsp. rhamnosus*, 2×10^9 CFU *B.infantis* and *B. longum*, 109 CFU *L.salivarius*, 10^9 CFU *L. acidophilus*, 5×10^9 CFU *S. thermophilus*, 10^9 CFU *L.sporogenes*, during therapy	88.2% (30/34)	14.5	Eradication rate ↔ Adverse effects ↓ (epigastric pain, nausea, vomiting, diarrhea)	46
	Control	76.4% (26/34)	61.5		
Omeprazole 20 mg, clarithromycin 500 mg, and amoxicillin 1 g, all*bid*, 7 days	14 days pre-treatment with 3.0×10^7 CFU *L. acidophilus*, 1.5×10^7 CFU *S. faecalis* 3.0×10^4 CFU *B. subtilis* daily	81.6% (62/76)	85.5	Eradication rate ↑ Adverse effects ↔	47
	14 days post-treatment with 3.0×10^7 CFU *L. acidophilus*, 1.5×10^7 CFU *S. faecalis*, 3.0×10^4 CFU *B. subtilis* daily	82.4% (61/74)	89.2		
	Control	61.5% (48/78)	85.5		

Table 1: Clinical trials using probiotics with *H. pylori* eradication treatment (cont.).

Eradication treatment	Probiotic strain (s)	Eradication rates	% side effect	Probiotic(s) efficacy	Reference
Omeprazole 20 mg, bismuth subcitrate 240 mg, amoxicillin 1 g, clarithromycin 500 mg, all *bid*, 14 days (quadruple therapy)	viable count 10^8 CFU of each *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus* during therapy	82% (69/84)	18.8	Eradication rate ↔ Adverse effects ↔ (decrease of diarrhea but increase of abdominal pain)	48
	Control	84.8% (73/68)	16.6		
Standard triple therapy (details not disclosed)	3×10^9 CFU *B. infantis* 2036 during therapy	83% (83/100)	9	Eradication rate ↑ Adverse effects ↓ (diarrhea, loose bowel motion)	49
	3.0×10^9 CFU *B. infantis* 2036 (twice daily), 14 days before therapy, then during therapy	90.5% (86/95)	7.6		
a sequential regimen of therapy, 10 days	3.0×10^9 CFU *B. infantis* 2036 (twice daily), during therapy for 10 days	90.8% (69/72)	4		
	Control	68.9% (73/106)	34.9		
Amoxicillin 50 mg/kg, furazolidone 6 mg/kg, both *bid*, 7 days, plus omeprazole 1 mg/kg, 28 days	*L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. bulgaricus*, *B. infantis*, *B. breve*, *S.thermophilus*, total viable count 10^9 CFU, during therapy	90.1% (30/33)	21.2	Eradication rate ↑ Adverse effects ↓ (nausea, vomiting, diarrhea)	50
	Control	69.7% (23/33)	63.6		
Standard triple Therapy	*L. acidophilus*, *B. bifidum* during and after therapy	83.7% (36/43)		Eradication rate ↑	52
	Control	64.4% (29/45)			
Omeprazole 20 mg, amoxicillin 1 g, clarithromycin 500 mg, all *bid*, 14 days	2×10^8 CFU *L. reuteri*, during therapy and further 14 days	74.3% (26/35)	28.6	Eradication rate ↔ Adverse effects ↓	53
	Control	66% (23/35)	68.6		

Genus *Lactobacillus*

Lactobacillus is gram-positive anaerobic, facultative anaerobic rod shaped bacterium, and some strains might be microaerophilic. They are member of the lactic acid bacteria group. Lactobacilli are straight, curved rods with varying length and thickness. Cell varies from long rod to short rod, sometimes slender or bent rods [191]. Cell arrangement occurs as single, in pair, and in chain sometimes filamentous or pleomorphic without clubbing or bifid formation. They are non-spore-forming, non-motile but few strains are motile by peritrichous flagella. They do not produce catalase, oxidase, indole, and do not reduce nitrate. Most strains of *Lactobacillus* are vancomycin-resistant, but some strains including *L. gasseri* are vancomycin-susceptible [191].

Habitats

Lactobacilli are commonly associated with the body of humans and animals. They are microflora in the oral cavity, gastrointestinal tracts, and vagina [192, 193]. Members of several species of lactobacilli are found at each of these sites. In general, those most often present in the body microflora are in the oral cavity such as *L. acidophilus*, *L. casei*, *L. paracasei*, *L. plantarum*, *L. salivavius*, *L. fermentum*, *L. rhamnosus*, *L. buchneri*, and *L. brevis* [192, 194, 195]. *L. murinus* is found in the oral cavity of mice [196]. *L. crispatus*, *L. gasseri L. jensenii*, *L. rhamnosus*, and *L. vaginalis* are found in vagina [197]. *Lactobacillus* in gastrointestinal (GI) tracts is widely considered of having beneficial role including immunomodulation, interference with enteric pathogen, and plays an important role in normal gut function and maintaining host health. The GI tract of fetal baby is sterile, but colonization starts immediately after birth with bacteria from the mother and the environments, depending on the mode of delivery [198, 199], hygiene levels, type of feeding, the use of antibiotics or other medicine [200]. Difference in gut microflora composition occur between breast- and formula-fed infants [201, 202]. A study which found that *L. salivarius* CECT 5713 was isolated from feces of a one-month-old breast-fed infant as well as the mother's breast milk suggested that the gut microflora of breast-fed

infants reflects that of the maternal breast milk [203]. Various species of *Lactobacillus* in GI tracts consist of *L. salivarius*, *L. cesei*, *L. plantarum*, *L. fermentum*, *L. acodophilus*, *L. gasseri*, *L. brevis*, and rarely *L. reuteri* [204]. The gastrointestinal microflora are categorized into two types, autochthonous microflora (indigenous microflora) and allochthonous flora (transient microflora) [205]. Indigenous microflora is strain inhabiting a place from earliest times and colonize specific niches in human body. *L. crispatus*, *L. gasseri*, *L. reuteri* [206], *L. ruminis*, and *L. salivarius* are suggested to be truly indigenous microflora to the human gastrointestinal tract [193, 207]. Some species is allochthonous microflora might be transferred from food into small intestine into the large bowel and an altered to microflora, which can collect from human feces [208].

Probiotics and modulation of immune response in *H. pylori* infection

Probiotics are defined by the FAO/WHO "as live organisms which when administered in adequate amounts confer a health benefit to the host." Probiotics have been used as a useful treatment of several gastrointestinal diseases. At the present, most studies showed that the representatives of bacterial probiotics mainly include lactic acid bacteria, particularly *Lacobacillus* spp. and *Bifidobacterium* spp. Species usually used as probiotics such as *L. acidophilus*, *L. plantarum*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L.* gasseri, *L. johnsonii*, *L. delbrueckii*, *L. brevis*, and *L. fermentum*, as well as *Bifidobacterium breve*, *B. infantis*, *B. longum*, *B. thermophillum*, and *B. pseudolongum*.

Numerous reports have shown that various probiotic lactobacilli, or their metabolic products inhibit *H. pylori in vitro*. Strains with this activity include *L. acidophilus* (*johnsonii*) La1 supernatant inhibit *H. pylori* bacterial growth *in vitro* [189]. *L. acidophilus* and *L. casei* subsp. *rhamnosus* have antagonistic effect on *H. pylori* and this inhibition correlated with lactic acid production [209]. The human *L. acidophilus* strain LB secreted an antimicrobial substance (s) diminished the viability of *H. pylori in vitro* independent of pH and the level of lactic acid and showed that the adhesion of *H. pylori* to HT29-MTX cells decreased when lost of *H. pylori* viability [210]. Several studies have shown that *Lactobacillus* can inhibit the adherence of *H. pylori* to mammalian epithelial cells [49, 58, 59] and inhibit *H. pylori*-induced pro-inflammatory cytokines both *in vitro* [36, 49, 60] and *in vivo* (36, 48). *L. gasseri*

OLL2716 (LG21) was effective in both suppressing *H. pylori* colonization and reducing gastric mucosal inflammation in humans [61]. Moreover, viable LG21 can suppress *H. pylori*-induced IL-8 production by MKN45 gastric epithelial cells *in vitro*, and after treatment with LG21 decreases the level of IL-8 concentration in both antrum and body of gastric mucosa of patients infected by *H. pylori* as compared with before treatment [36]. *L. salivarius* was efficiently eradicated *H. pylori* in gnotobiotic murine model [211]. *L. salivarius* WB1004 protects the colonization of *H. pylori* in the stomach of gnotobiotic BALB/c mice [62]. *L. salivarius* UCC118 inhibited *H. pylori*-induced production of IL-8 by AGS gastric epithelial cells *in vitro* by suppression of the *cag* PAI gene expression and intracellular CagA accumulation on *H. pylori* cell [49]. Viable cells and supernatant of *L. bulgaricus* (LBG) attenuated IL-8 production induced with *H. pylori* Sydney strain 1-lipopolysaccharide (LPS) by SGC-7901 cells by inhibition of the Toll-like receptor (TLR) 4 pathway *in vitro* [64]. *L. johnsonii* La1 attenuates *H. pylori*-associated gastritis and reduce the levels of proinflammatory chemokines include macrophage inflammatory protein 2 (MIP-2), and keratinocyte-derived cytokine (KC) in sera and gastric tissues of C57BL/6 mice [63]. More recently, it has been reported that *L. acidophilus* pre-treatment reduced *H. pylori*-induced inflammation in gastric epithelial cells through the inactivation of the Smad7 and NF-κB pathway [65]. Recently, Thiraworawong et al. (2014) showed that conditioned media from *L. plantarum* XB7, *L. salivarius* B101, and *L. rhamnosus* B103 suppressed IL-8 mRNA expression and protein secretion in *H. pylori*-infected AGS cells without inhibiting the growth of *H. pylori* [66]. Moreover, *L. salivarius* strain B37 (LS-B37) and B60 (LS-B60) have been shown to suppress IL-8 production in *H. pylori*-infected AGS gastric epithelial cells [66, 67] and also suppress TNF production from THP-1 monocytoid cell stimulated with LPS [67, 212].

CHAPTER IV
MATERIAL AND METHODS

1. Bacterial strains, cell lines and culture conditions

Bacterial strains used in this study were selected from eight gastric-derived *Lactobacillus* spp. previously shown to suppress *H. pylori*-induced IL-8 production [66] and listed in Table 2. Based on different RAPD and rep-PCR patterns (Figures 7 and 8) and other properties, *L. salivarius* B37 (LS-B37) and B60 (LS-B60) were selected, whereas *L. salivarius* B78 (LS-B78) was used as a same-species negative control. *L. plantarum* XB7 (LP-XB7) previously shown to suppress NF-κB and c-Jun activation and inflammation in rats was selected to characterize IL-8-suppressing substance and other mechanism of IL-8 suppression. All lactobacilli were routinely cultured on deMan Rogosa Sharpe (MRS) agar (Oxoid, Hampshire, UK) anaerobically using AnaeroGen^{TM} sachets (Themo scientific Oxoid) in anaerobe boxes at 37°C for 24-48 h. *Helicobacter pylori* ATCC 43504 (ATCC, Manassas, VA, USA) (*vacA* s1a-m1, *cagA* $^{+}$, *ureC* $^{+}$) was grown on Columbia blood agar (Oxoid, Hampshire, UK) supplemented with 7% (v/v) horse serum (Gibco New Zealand Ltd, Auckland, New Zealand) and 7% (v/v) sheep blood at 37°C for 48 h under microaerophilic conditions using MGC Anaero Pack-Micro Aero sachets (Mitsubishi Gas Chemical Company, Inc, New York, NY). For use in co-culture assays, *H. pylori* were resuspended in antibiotic-free RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA) to a cell density of McFarland 6.

AGS human gastric adenocarcinoma epithelial cells (ATCC CRL-1739) obtained from the American Type Culture Collection (Manassas, VA) were cultured as a monolayer (>80% confluence) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco-Invitrogen) at 37°C and 5% CO_2 atmosphere for 48 h. AGS cells were passaged every 2-3 days. Adherent cells were detached from the flask with 0.25% (v/v) trypsin in 1 mM EDTA (Gibco-Invitrogen) at 37°C for 7-10 min. Detached cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-Invitrogen) and used in the co-culture assay described below.

Table 2: IL-8 inhibitory gastric-derived *Lactobacillus* spp.

Code	Species	IL-8 protein suppre-ssion	IL-8 mRNA suppression at 4 h	Interfere with IL-8 signaling pathway	Interfere with *H. pylori* growth	References
LS-B37*	*L. salivarius*	+	-	-	+	[66, 212]
LS-B60*	*L. salivarius*	+	-	-	-	[66, 67]
LS-B74	*L. salivarius*	+	-	-	-	[66, 212]
LP-B90	*L. plantarum*	+	-	-	-	[66, 212]
LS-B101	*L. salivarius*	+	+	+	-	[66, 212]
LR-B103	*L. rhamnosus*	+	+	+	-	[66, 212]
LC-B106	*L.casei* group	+	-	-	-	[66, 212]
LP-XB7*	*L. plantarum*	+	+	+	-	[66, 67]
LS-B78*	*L. salivarius*	-	-	-	-	[212]

* *Lactobacillus* strains used in this study

2. Preparation of *Lactobacillus*-conditioned media (LCM)

Lactobacillus-conditioned media (LCM) were prepared as previously described [213]. Briefly, lactobacilli were cultured for 24 h, then adjusted to an OD600 of 0.1 in MRS broth and incubated anaerobically for 48 h. Cell-free supernatants were collected by centrifugation at 4000 x g for 10 min at 4°C. Collected supernatants were filtered through a 0.22 µm filter (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany). Filtered supernatant was concentrated by speed vacuum drying at 43°C for 3 h. (Savant instruments, Farmingdale, NY) and resuspended in an equal volume of RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA) for further testing with AGS cells as described below. The conditioned media was stored at -20°C until further analysis.

3. **Bioassay for IL-8 activity in AGS gastric epithelial cells**

AGS gastric epithelial cells were co-cultured with *H. pylori* to induce IL-8 production and treated with LCM to test the effects of *Lactobacillus* spp. produced factors on IL-8 production as previously described [67]. Briefly, AGS cells (2.0×10^4cells/well) were pre-incubated in a 96-well format as described above. After 24 h, the culture supernatant was replaced with fresh RPMI medium containing either 5% (v/v) LCM only or in combination with 3×10^7 colony forming units (CFU)/mL of viable *H. pylori* ATCC 43504 (6.0×10^6 CFU/well) and incubated under 5% CO_2 at 37°C for 24 h. Cell culture supernatants were collected by centrifugation at 125 × g for 7 min at 4°C. IL-8 concentrations in each culture supernatant were measured by using the Quantikine Human IL-8 Immunoassay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. This experiments were performed at least three times independently, each in triplicate.

4. Cell viability assay

In order to determine that the concentrations of IL-8 not mediated with cell death, cell viability test was performed. Cell viability was evaluated by trypan blue dye exclusion assay. Briefly, AGS cell suspensions were mixed with 0.4 (w/v) trypan blue solution (Gibco-Invitrogen) (1:1), and visually examined for the inclusion or exclusion of dye. Trypan blue stains nonviable cells and is excluded by viable cells. The number of total cells and stained cells were counted on a hemocytometer under an inverted microscope within a 1 mm^2 area. The percentage of viable cells was calculated from the ratio of viable cells over total cells.

5. Analysis of *IL-8* gene expression by quantitative real-time PCR

The effects of LCM of LS-B37 and LS-B60 on the transcription of IL-8 in *H. pylori*-treated AGS epithelial cells were performed by quantitative real-time PCR as previously described [66, 214] with the following modifications. Briefly, AGS gastric epithelial cells (5×10^5cells/well) were cultured in a 24-well plate as outlined above. AGS cells were co-incubated with viable *H. pylori* ATCC 43503 (1.5×10^8 CFU/well) with or without LCM (5% v/v) at 37ºC, 5% CO_2 for 2, 4, and 6 h. Cell culture

supernatants were removed by centrifugation and total RNA of treated AGS cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and the purity (A_{260}/A_{280} ratio) were determined by spectrophotometric analysis (ND-1000 spectrophotometer, NanoDrop Technologies, Willigton, USA). Complementary DNA (cDNA) was reverse transcribed from 10 ng total RNA using the SuperScript® VILO™ cDNA Synthesis kit (Invitrogen). qPCR was performed in a LightCycler® 2.0 (Roche, Germany) for 45 cycles of: 10 s at 95°C, 10 s at 65°C, and 25 s at 72°C. The following primers were used to amplify cDNA fragments: IL-8 forward primer (5'-ACACTGCGCCAACACAGAAATTA-3'), IL-8 reverse primer (5'-TTTGCTTGAAGTTTCACTGGCATC-3'); and Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer (5'-GCACCGTCAAGGCTGAGAAC-3'), GAPDH reverse primer (5'-ATGGTGGTGAAGACGCCAGT-3'). The amplified product was detected using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche, Germany) at 530 nm. Melting curve analysis was performed for amplicon specificity and crossing points (Cp) was determined. The fold change of *IL-8* gene expression, relative to GAPDH, was calculated according to the $2^{-\Delta\Delta Cp}$ method [215].

6. Determination of cell signaling pathways by quantitative western blot

The modulation of signaling pathways mediated IL-8 production by LCM of LS-B37 and LS-B60 was examined by western blot as previously described with minor modifications [42, 66]. Briefly, AGS cells (2.0×10^6cells/well) were cultured in a 6-well plate as outlined above. AGS cells were stimulated with *H. pylori* ATCC 43504 (6.0×10^8 CFU/well) in the presence or absence of LCM (5% v/v) for 15, 30 min, 1, 2, and 3 h. Proteins were extracted from whole cell lysates of AGS-treated cells using Mammalian Protein Extraction Reagent (M-PER, Pierce Biotechnology, Rockford, IL, USA) supplemented with Halt protease and phosphatase inhibitors (Pierce Biotechnology) according to the manufacturer's instructions. Protein concentrations from whole cell lysates were determined using the Pierce® BCA protein assay kit (Pierce Biotechnology, Illinois, USA) according to the manufacturer's instructions. Cell extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF)

membranes (Bio-Rad, Philadelphia, PA, USA) at 15 volts for 30 min using Bio Rad Trans-Blot SD Semi Dry Transfer Cell instrument (Bio-Rad, Philadelphia, PA, USA). After blotting transfer, the PVDF membrane was blocked in 10% non-fat milk in Tris-Buffered Saline and Tween 20 (TBST) (50mM Tris, pH 7.5, 150mM NaCl, 0.05% Tween 20). Blocked membranes were incubated with mouse antibodies against NF-κB (p65), phospho-NF-κB (p65), c-Jun, phospho-c-Jun (Santa Cruz Biotechnology, California, USA) and β-actin (Cell signaling Technology, Inc), and then washed with TBST and incubated with horseradish peroxidase-labeled goat anti-mouse secondary antibodies for 1 h. Peroxidase signals were measured and imaged by ChemiDoc™ XRS (Bio-Rad, Philadelphia, USA). Densitometric analyses for protein quantification were carried out using ImageJ 1.45 s software.

7. **Effects of LCM on *cag*A and *cag*E of *H. pylori* in co-culture assay**

To determine whether LS-XB7, LS-B37 and LS-B60 suppress expression of *H. pylori cag*A and *cag*E genes, quantitative PCR assays were performed. Briefly, *H. pylori* 43504 was co-incubated with 5% LCM at 37°C and 5% CO_2 atmosphere for 2, 4 and 6 h. Total RNA was extracted from LCM-treated *H. pylori* pellets using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed from 10 ng RNA using the SuperScript® VILO™ cDNA Synthesis kit (Invitrogen), and qPCR was carried out using a LightCycler® 2.0 (Roche, Germany) for 45 cycles of: 10 s at 95°C, 10 s at 55°C, and 25 s at 72°C. The amplified product was detected using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche, Germany) at 530 nm. Gene targets were amplified using gene specific primers: *cag*A forward primer (5'-GATAACAGGCAAGCTTTTGAGG-3'), *cag*A reverse primer (5'-CTGCAAAAGATTGTTTGCGAGA-3') [216, 217]; *cag*E forward primer (5'-GCGATTGTTATTGTGCTTGTAG-3'), *cag*E reverse primer (5'-GAAGTGGTTAAAAAATCAATGCCCC-3'). [194, 218]; *16S rRNA* forward primer (5'-GCTAAGAGATCAGCCTATGTCC-3'), reverse primer (5'-TGGCAATCAGCGTCAGGTAATG-3') [216, 219]. Gene expression of *cag*A and *cag*E relative to *16S rRNA*, was calculated according to the $2^{-\Delta\Delta Cp}$ method [215]

8. Evaluation of IL-8 suppressing substance(s) in LCM

The properties of IL-8-suppressing substances produced by LS-XB7, LS-B37 and LS-B60 were evaluated for thermostability, size estimation, and protease sensitivity. Heat stability was assessed by heating LCM to 100°C for 15, 30, 60, and 120 min. The molecular size analysis of IL-8 suppressing substances present in LCM were estimated by 3K, 50K, and 100K Amicon® Ultra-4 Centrifugal Filters (Millipore) according to the manufacturer's instruction. Protease sensitivity of LCM was tested by incubation with various enzymes: 1) α-amylase in 20 mM sodium acetate, 7 mM sodium chloride (pH 6.9); 2) lipase and 3) lysozyme each in 50 mM Tris-HCl (pH 7.2); 4) proteinase K and 5) trypsin each in 50 mM Tris-HCl (pH 7.5-7.6). Each enzyme (Sigma, USA) was used at a final concentration of 1 mg/mL and incubated at 37°C for 6 h, with the exception of amylase and lysozyme, which were incubated at 25°C (Table 3). Each enzymatically treated LCM was subsequently subjected to 100°C for 10 min to inactivate these enzymes. All treated (heat-treated, filtered, enzymatically incubated) samples were tested for IL-8 suppressive activity in the co-culture assay outlined above.

Table 3: Condition of enzyme treatment in this study

Enzyme	Buffer	pH	Temperature	Incubation time (h)
α-amylase	20 mM sodium acetate, 7 mM sodium chloride	6.9	25	6
Lipase	50 mM Tris-HCl	7.2	37	6
Lysozyme	50 mM Tris-HCl	6.24	25	6
Proteinase K	50 mM Tris-HCl	7.5	37	6
Trypsin	50 mM Tris-HCl	7.6	25	6

9. **Randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR)**

A single colony of *Lactobacillus* spp. grown on MRS agar was resuspended in 200 µL of double-distilled water (DDW) into density of 10^9cell/mL and DNA was extracted using a High Pure PCR template preparation kit (Roche, USA) according to the manufacturer's protocol. RAPD-PCR with primer M13V was performed as previously described [220, 221] with the following modifications. The primer sequence was 5'-GTT TTC CCA GTC ACG AC-3'. The reaction mixture contained 100 pmol of primer M13V, 5.0 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl), 3.5 mM of $MgCl_2$, 0.4 mM each deoxynucleoside triphosphate (dNTPs; dATP, dCTP, dGTP, dTTP), 1.25 U Taq DNA polymerase (Gibco-Invitrogen), 5.0 µL of the DNA template, and DNAse- and RNAse-free distilled water (Gibco-Invitrogen, UK.) in a volume of 50 µL. RAPD-PCR was carried out by using Eppendorf Master Cycler gradient PCR system (Thermal Master cycler gradient, Germany). The amplification program was 94 °C for 45 s; 3 cycles of 94 °C for 3 min, 40 °C for 5 min, and 72 °C for 5 min; and 32 cycles of 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. Fifteen microlitres of PCR products were electrophoretically separated in a 1.5% agarose gel for 3 h at a constant voltage of 170 V in 1X tris-borate-EDTA (TBE). The similarity among digitized profiles was calculated using Info Quest FP software and UPGMA clustering method.

10. **Repetitive sequence-based PCR fingerprinting (Rep-PCR)**

The rep-PCR fingerprinting using the $(GTG)_5$ primer (5'-GTG GTG GTG GTG GTG-3') was performed according to Gevers *et al*. [222] with a few modifications. PCR mixture containing 100 pmol of $(GTG)_5$ primer, 5.0 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl), 3.5 mM of $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTPs; dATP,dCTP,dGTP,dTTP), 2 U *Taq* DNA polymerase (Gibco-Invitrogen), 5.0 µL of the DNA template, and DNAse- and RNAse-free distilled water (Gibco-Invitrogen, UK.) in a volume of 50 µL. Rep-PCR was carried out by using Eppendorf Master Cycler gradient PCR system (Thermal Master cycler gradient, Germany). Initial denaturation at 94°C for 7 min was followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 40°C for 1 min, and extension at 65°C for 8 min. The last cycle was followed by the final elongation at 65°C for 16 min [223]. Ten microlitres of

PCR products were electrophoretically separated in a 1.5% agarose gel for 3 hours at a constant voltage of 170 V in 1X tris-borate-EDTA (TBE). The similarity among digitized profiles was calculated using Info Quest FP software and UPGMA clustering method.

11. Statistical analyses

All experiments were performed at least three times and the results were reported as mean ± standard deviation. The data were analyzed using the Student's *t*-test with one-tailed distribution and considered statistically significant at a *p*-value ≤ 0.05, unless otherwise stated.

CHAPTER V
RESULTS

1. Random amplified polymorphic DNA (RAPD) and Repetitive-sequence-based PCR (rep-PCR) fingerprinting of IL-8 - inhibitory *Lactobacillus* spp.

Lactobacillus strains were subjected to RAPD fingerprinting using M13V primers. A dendrogram of RAPD patterns of these lactobacilli were analyzed using Info Quest FP software that represents correlation coefficient and clustering of *Lactobacillus* strains as shown in figure 7. In addition, rep-PCR fingerprinting using the (GTG)5 primers was performed and a dendrogram of rep-PCR patterns indicated correlation coefficient and clustering of *Lactobacillus* strains was shown in figure 8. This is one of the rationale used to select *Lactobacillus* strains for further investigation. The results showed that RAPD and rep-PCR patterns of *L. salivarius* B37, B74, and B101 are similar. Since *L. salivarius* B37 (LS-B37) can inhibit *H. pylori* growth and suppress IL-8 production [66, 151], it was selected for this study. The pattern of *L. salivarius* B60 (LS-B60) was different for other *L. salivarius* strain so it was selected for a comparative study in *L. salivarius* strains. *L. plantarum* XB7 (LP-XB7), the strain which was previously shown to suppress NF-κB and c-Jun activation and inflammation in rats [66] was selected for determination of other mechanism of IL-8 suppression and characterization of IL-8-suppressing substance. Therefore, three isolates including LS-B37, LS-B60 and LP-XB7 were selected for further investigation.

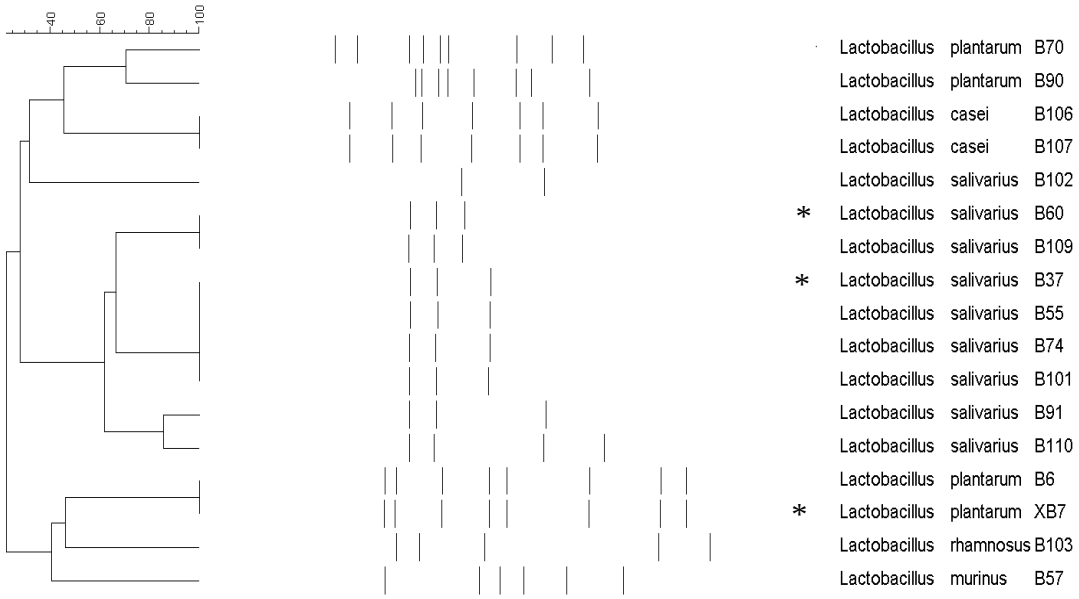


Figure 7: Dendrogram of RAPD patterns of IL-8-inhibitory *Lactobacillus* spp. The similarity of the patterns was calculated using Info Quest FP software and UPGMA clustering method. The strains marked by asterisk were selected for further investigation.

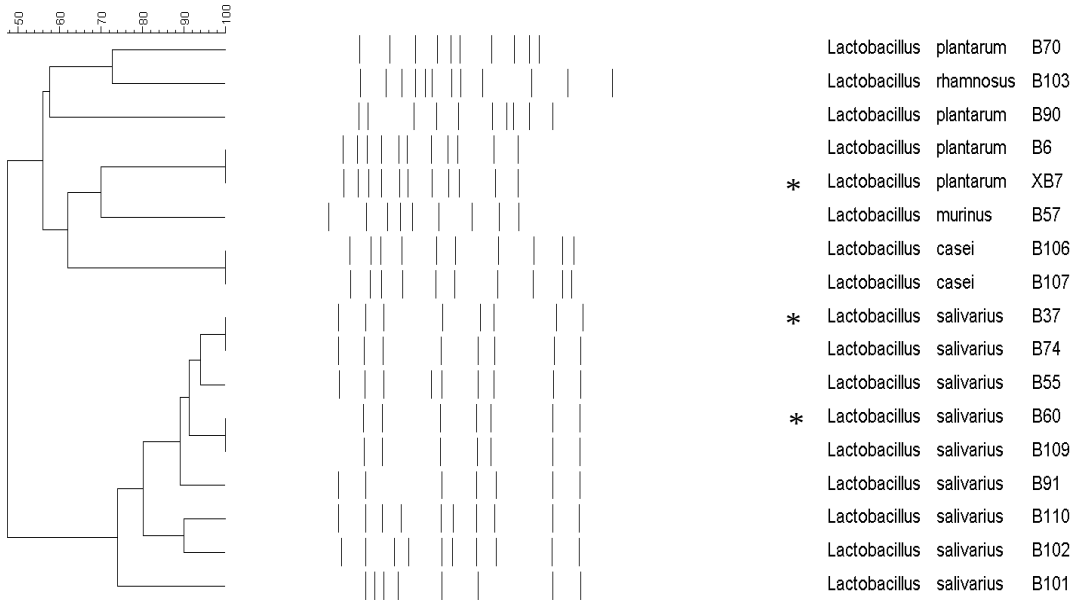


Figure 8: Dendrogram based on cluster analysis of rep-PCR fingerprints obtained from IL-8-inhibitory *Lactobacillus* spp. A dendrogram was calculated using Info Quest FP software and the UPGMA clustering method. The *Lactobacillus* strains marked by triangle were selected for further investigation.

2. Human gastric-derived *Lactobacillus* spp. suppressed IL-8 production by *H. pylori*-induced AGS cells

LP-XB7, LS-B37, and LS-B60 were re-analyzed for their ability to modulate *H. pylori*-mediated IL-8 production *in vitro*. *L. salivarius* strain B78 (LS-B78), a non-inhibitory strain, was used as a negative control. Immunomodulatory activity of produced factors from LP-XB7, LS-B37, and LS-B60 was investigated by adding cell-free conditioned media to AGS cells either alone or in combination with viable *H. pylori* ATCC 43504. LB-XB7, LS-B37, and LS-B60 significantly suppressed IL-8 production in *H. pylori*-stimulated AGS cells ($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively), whereas LS-B78 did not suppressed IL-8 production (Table 4 and Figure 9). The ability to suppress IL-8 production by *Lactobacillus* spp. did not have cytotoxic effects on AGS cells as determined by Trypan Blue dye exclusion assay. Conditioned media of LP-XB7, LS-B37, and LS-B60 did not stimulate IL-8 production when co-cultured with AGS cells in the absence of *H. pylori* (Figure 9).

Table 4: Inhibitory effects of three gastric-derived *Lactobacillus* spp. on IL-8 production in *H. pylori*-induced AGS human gastric epithelium cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation. The experiments were performed three times independent, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to MRS media control.

Sample		**48 h LCM no *H. pylori***		**48 h LCM plus *H. pylori***		**Statistical analysis (T-test)**	
Code	**% TNF suppression**	**IL-8 conc. (pg/ml)**	**SD**	**IL-8 conc. (pg/ml)**	**SD**	**% IL-8 suppression**	***p*-value**
MRS	-	96.61	23.33	1481.43	575.79	-	-
LP-XB7	57.23***	142.41	39.02	684.56	138.88	53.79**	0.0018
MRS	-	95.21	24.77	2003.12	245.48	-	-
LS-B37	25.86	107.9	26.19	1234.96	132.94	38.35***	0.0007
MRS	-	95.58	24.19	2051.18	129.43	-	-
LS-B60	48.03*	107.09	18.87	1196.59	314.5	41.66***	0.0000
MRS	-	100.08	17.97	1777.31	471.11	-	-
LS-B78	-	123.75	29.98	1899.05	157.81	0	0.3509

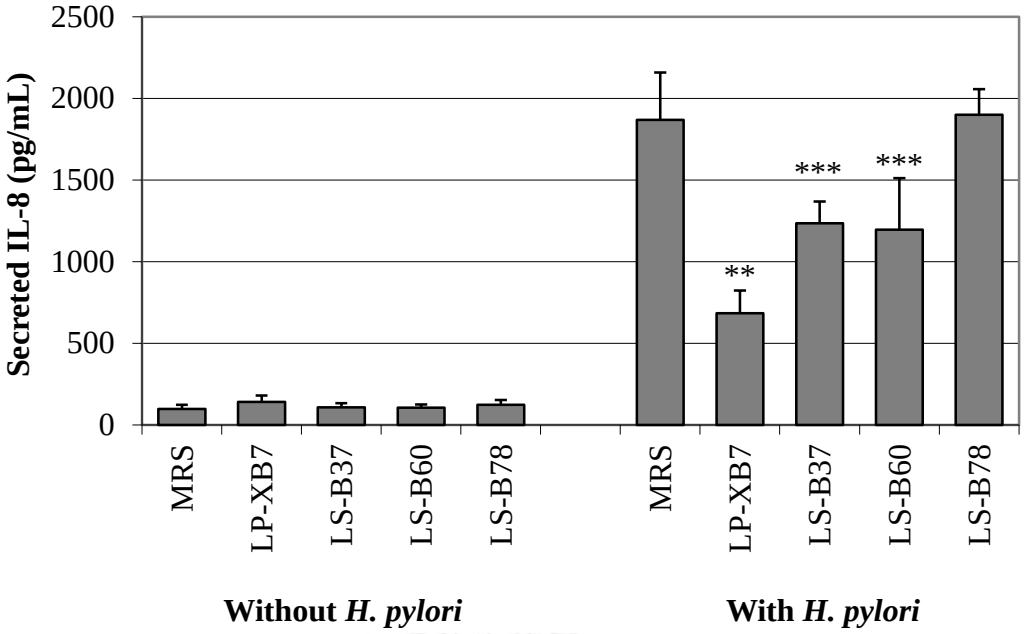


Figure 9: Human gastric-derived *Lactobacillus* spp. were affected on IL-8 production by *H. pylori*-induced AGS cells. LCM from four *Lactobacillus* isolates were tested for the ability to suppress IL-8 production from AGS cells stimulated with *H. pylori*. LCMs were co-incubated with AGS cells either with or without *H. pylori* ATCC43504 for 24 h and IL-8 secretion was measured by ELISA. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control. The experiments were performed three times independently, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to MRS media control.

3. Immunomodulatory effects of 24- and 48 h LCM on IL-8 production in *H. pylori*-induced AGS human gastric epithelial cells

To study the immunomodulatory effect of 24- and 48 h *Lactobacillus* conditioned media, LCM at 24 and 48 hours were prepared and tested the ability to suppress IL-8 production in *H. pylori*-activated AGS cells. Both 24- and 48 h LCM of LS-B37 and LS-B60 significantly suppressed IL-8 production. A 48 h LCM of LP-XB7 suppressed IL-8 production, while 24 h LCM did not suppress IL-8 production as shown in figure 10. Moreover, the 48 h LCM of these strains inhibit IL-8 higher than 24 h LCM. Therefore, we used 48 h of LCM for study in the next experiment.

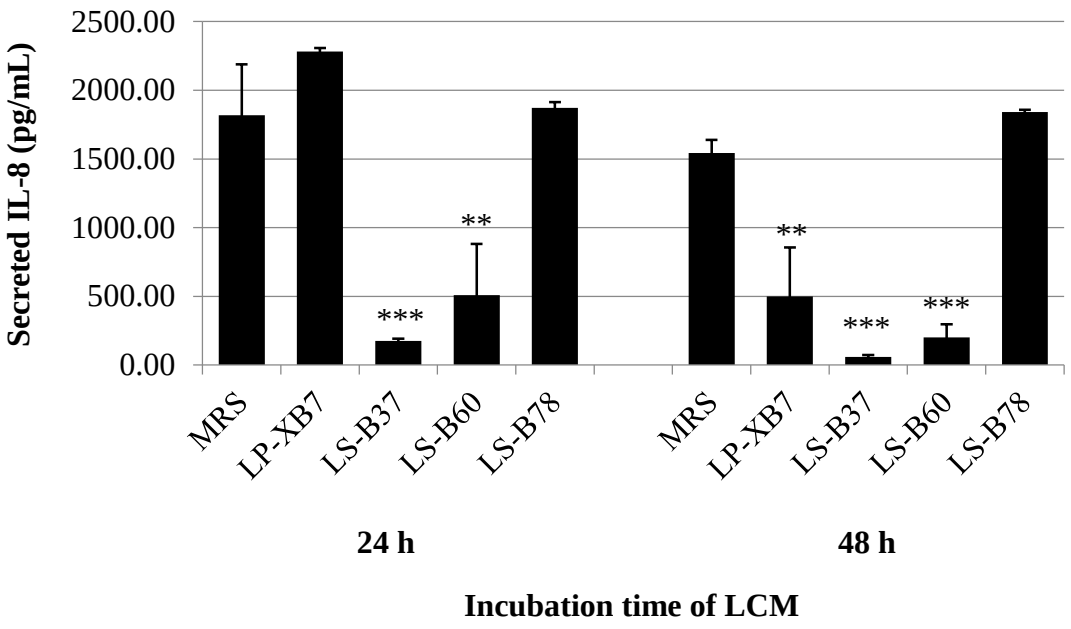


Figure 10: Immunomodulatory effects of 24- and 48 h LCM on IL-8 production in *H. pylori*-induced AGS human gastric epithelial cells. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control. The experiments were performed three times independently, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared to MRS media control.

4. *L. salivarius* B37 and B60 diminished *IL-8* gene expression in *H. pylori*-induced AGS cells

Quantitative RT-PCR was used to determine the effects of IL-8 inhibitory *Lactobacillus* spp. on IL-8 mRNA expression. According to Thiraworawong et al. (2014), LCM of LS-B37 and LS-B60 could not suppress *IL-8* gene expression after incubation with AGS cells for 4 h. Therefore, this study investigated *IL-8* gene suppression effect by LS-B37 and LS-B60 at various time points. AGS cells were co-incubated with MRS control or LCM (5% v/v) of each strain and stimulated with *H. pylori* ATCC 43504 for 2, 4 and 6 h prior to total RNA isolation. Crossing points (Cp) was determined. The Cp represents the number of PCR cycles at which the growth curve enters the log-linear phase. There is an inverse linear relationship between the crossing-point cycle number and the number of template copies present in a PCR reaction. AGS cells co-incubated with RPMI tissue culture medium control and stimulated with *H. pylori* at 2 h, the Cp of *IL-8* gene 17.61 and the Cp of GAPDH about 13.86. After co-incubation 4 h, the Cp of *IL-8* gene 20.05 and the Cp of GAPDH around 13.67, and after co-incubation 6 h, the Cp of *IL-8* gene 22.37 and the Cp of GAPDH around 13.79. As detected by RT-PCR, IL-8 mRNA transcription was distinct in AGS cells within an hour of exposure to *H. pylori* ATCC 43504. Induction was higher after 2 to 4 h of incubation than 6 h. *IL-8* gene expression, relative to *GAPDH* at 2 h, was significantly inhibited approximately 0.5 fold, $p < 0.01$and $p < 0.001$ in the presence of LCM of LS-B37 and LS-B60, respectively. Only LS-B37 suppressed *IL-8* gene expression at 4 h, while LS-B60 did not (Table 5 and Figure 11). In addition, both strains could not suppress *IL-8* gene expression at 6 h incubation (Table 5). The findings show that LCM from LS-B37 and LS-B60 suppress *IL-8* gene expression at earlier time points.

Table 5: Summary the effects of *L. salivarius* B37 and B60 on the suppression of *IL-8* gene expression in AGS cells co-incubated with *H. pylori* for 2 h, 4 h, and 6 h.

***Lactobacillus* strain**	**Incubation time**	**Time I**	**Time II**	**Time III**
L. salivarius (LS-B37)	2 h	+	+	+
	4 h	+	+	+
	6 h	-	-	ND
L. salivarius (LS-B60)	2 h	+	+	+
	4 h	-	-	-
	6 h	-	-	ND

IL-8 mRNA suppression activity: +, positive; -, negative; ND, does not determined.

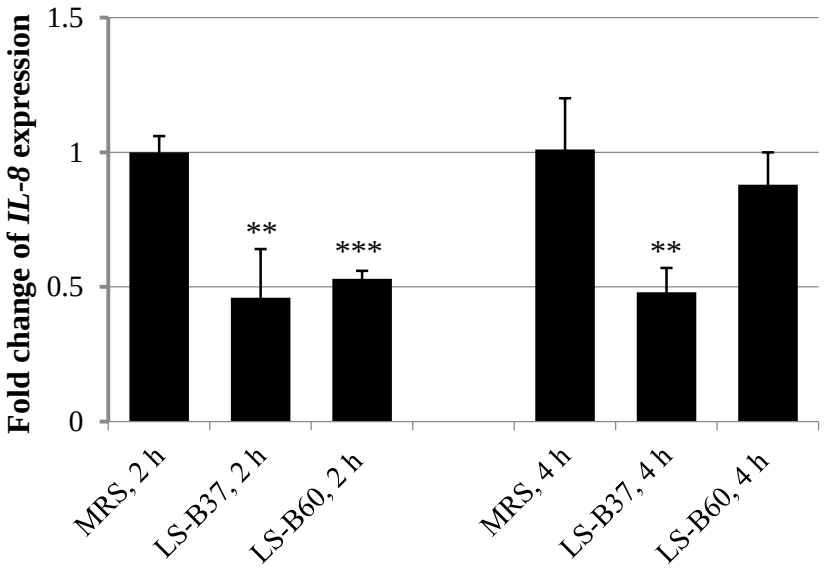
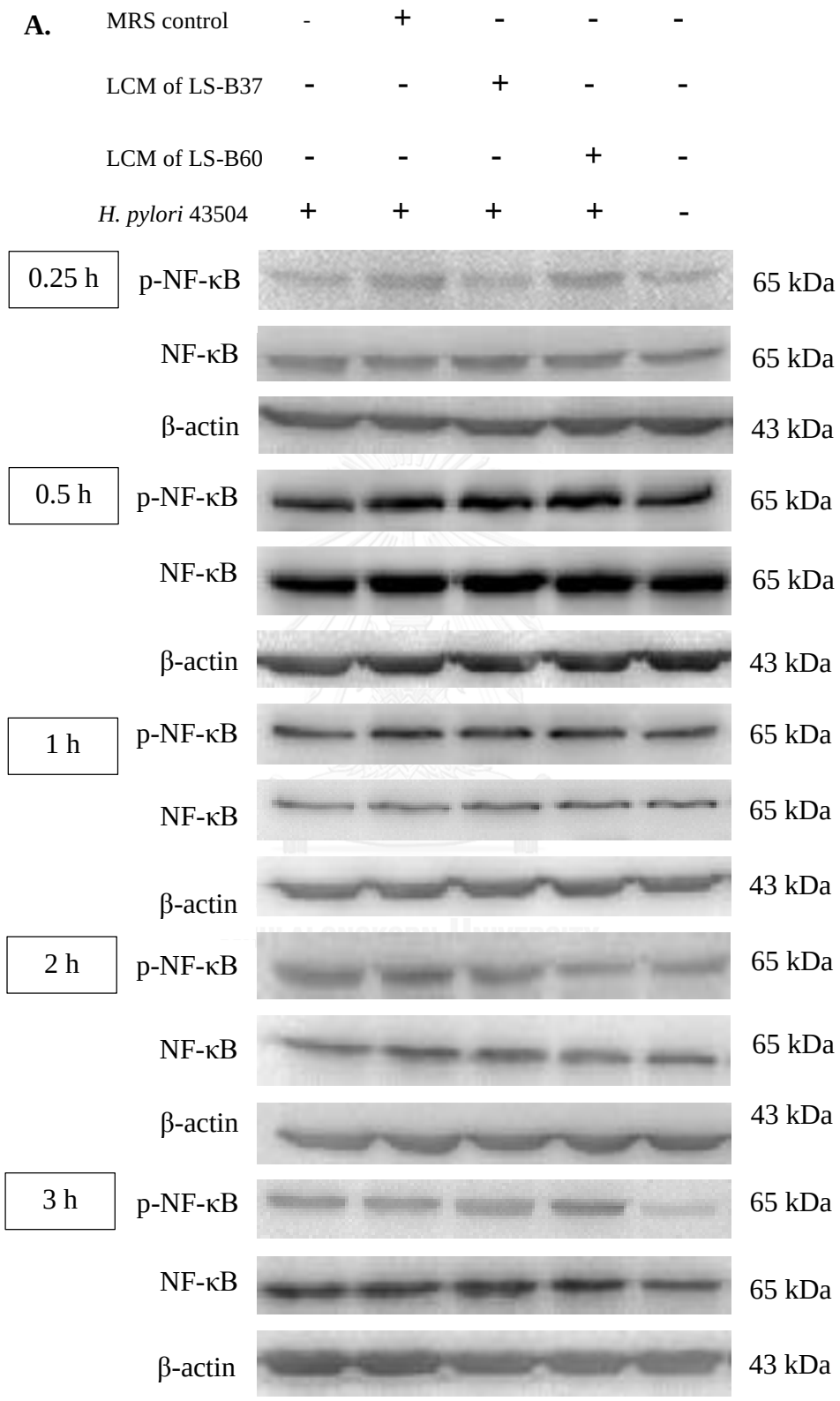


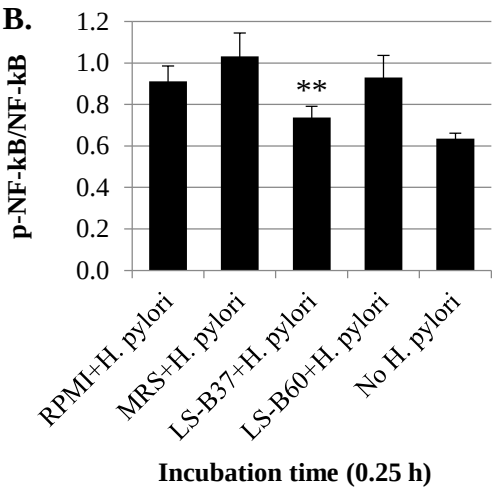
Figure 11: Effects of *Lactobacillus* conditioned media of LS-B37 and LS-B60 on *IL-8* gene expression in *H. pylori*-induced AGS human gastric epithelial cells. After 2- and 4 h of AGS cells stimulated with *H. pylori* and LCM or MRS medium control, *IL-8* gene expression was examined by using quantitative real-time PCR. This experiment was performed with primers specific to *IL-8* and *GAPDH* transcripts. Quantitative gene expression data were normalized to GAPDH as housekeeping gene. Fold change of *IL-8* gene expression was calculated in relative to MRS media control, and results showed the means ± SD, $^{**}p < 0.01$, and $^{***}p < 0.001$ and corresponded to representative experiments. The experiments were performed at least three times independently, each in triplicate.

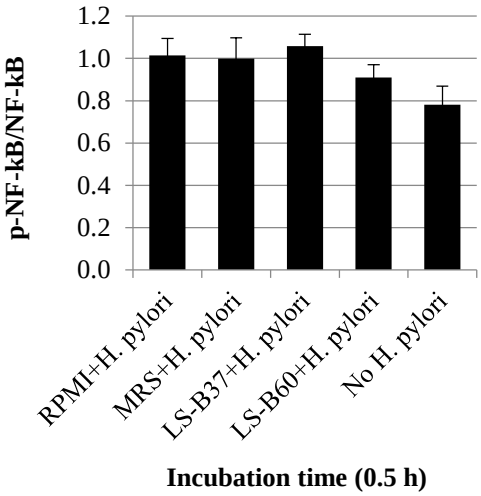
5. *L. salivarius* B37 and B60 inhibited IL-8 production via NF-kB p65 suppression

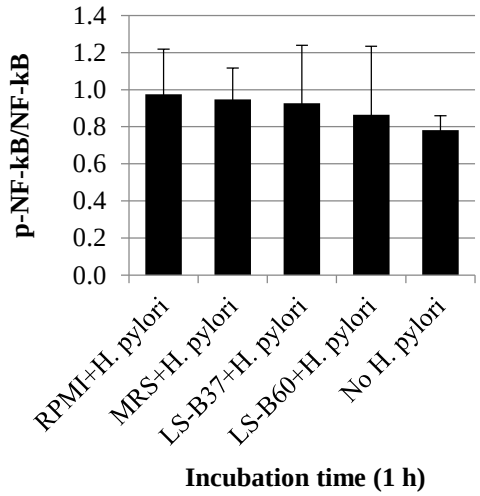
H. pylori infection activates phosphorylation of the NF-κB p65 subunit and AP-1 in gastric epithelial cells resulting in increased *IL-8* gene expression and IL-8 production. To determine whether LS-B37 or LS-B60 affected activation of NF-κB or c-Jun, western blot analysis of phosphorylated NF-κB (p-NF-κB) and phosphorylated c-Jun (p-c-Jun) was conducted. AGS cells were co-incubated with MRS control or LCM (5% v/v) from each strain in the combination of *H. pylori* ATCC43504 for 0.25, 0.5, 1 , 2 and 3 h, then were assayed for effects on p-NF-κB and p-c-Jun concentrations by Western blot. Co-incubation of *H. pylori*-stimulated AGS with LCM of LS-B37 for 0.25 h significantly inhibited p-NF-κB (28.52%) as compared to media control ($p < 0.01$, Figure 12A and 12B). However, the inhibitory effects of p-NF-κB had not occurred after 0.25 h of incubation (Figure 12C). Two hours treatment with LS-B60 LCM resulted in reduced concentrations of p-NF-κB (38.29%, $p < 0.01$, Figure 12B and 12C). In contrast, LCM of both strains did not suppress p-c-Jun (AP-1 subunit) at either time point (Figure 13A, 13B and 13C). These results show that LCM of LS-B37 inhibited the *H. pylori*-stimulated IL-8 production by suppression of NF-κB p65 activation at 0.25 h, while LS-B60 reduced IL-8 production by *H. pylori*-induced AGS cells by suppressing NF-κB p65 activation at 2 h. However, IL-8 suppression of both strains was not associated with the suppression of c-Jun activation.

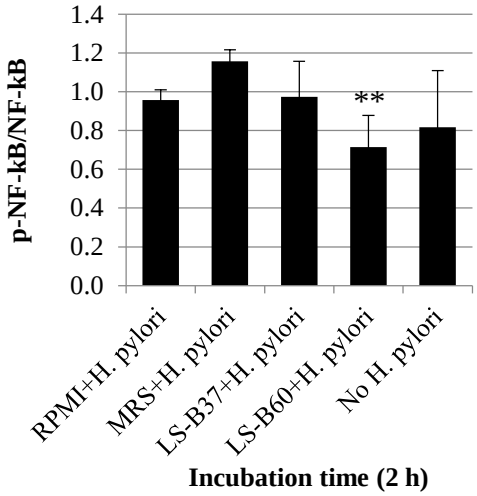

A.
MRS control - + - - -
LCM of LS-B37 - - + - -
LCM of LS-B60 - - - + -
H. pylori 43504 + + + + -
0.25 h
p-NF-κB
65 kDa
NF-κB
65 kDa
β-actin
43 kDa
0.5 h
p-NF-κB
65 kDa
NF-κB
65 kDa
β-actin
43 kDa
1 h
p-NF-κB
65 kDa
NF-κB
65 kDa
β-actin
43 kDa
2 h
p-NF-κB
65 kDa
NF-κB
65 kDa
β-actin
43 kDa
3 h
p-NF-κB
65 kDa
NF-κB
65 kDa
β-actin
43 kDa

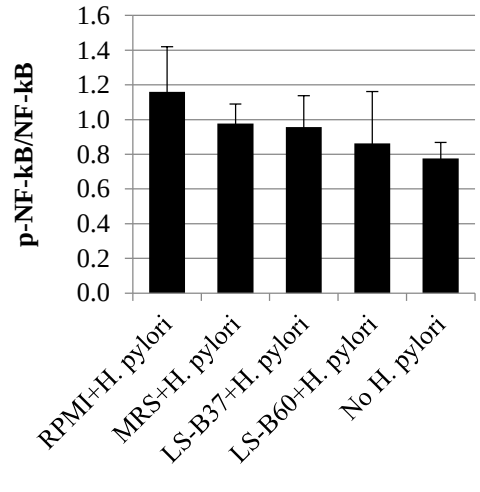
B.


p-NF-kB/NF-kB
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
RPMI+H. pylori
MRS+H. pylori
LS-B37+H. pylori
LS-B60+H. pylori
No H. pylori
Incubation time (0.25 h)


p-NF-kB/NF-kB
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RPMI+H. pylori
MRS+H. pylori
LS-B37+H. pylori
LS-B60+H. pylori
No H. pylori
Incubation time (0.5 h)


p-NF-kB/NF-kB
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RPMI+H. pylori
MRS+H. pylori
LS-B37+H. pylori
LS-B60+H. pylori
No H. pylori
Incubation time (1 h)


p-NF-kB/NF-kB
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
RPMI+H. pylori
MRS+H. pylori
LS-B37+H. pylori
LS-B60+H. pylori
No H. pylori
Incubation time (2 h)


p-NF-kB/NF-kB
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RPMI+H. pylori
MRS+H. pylori
LS-B37+H. pylori
LS-B60+H. pylori
No H. pylori
Incubation time (3 h)

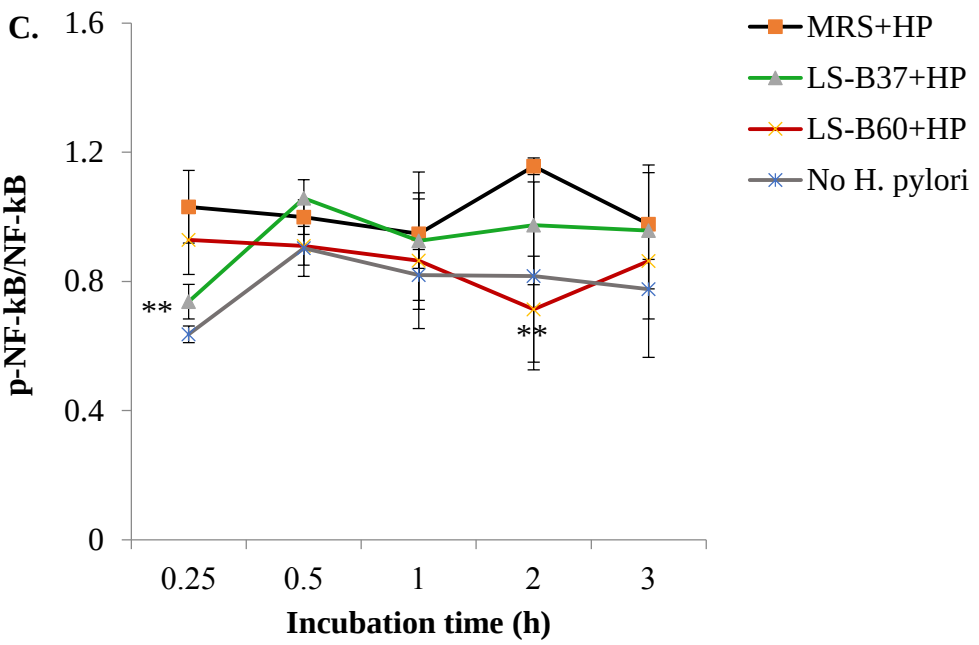
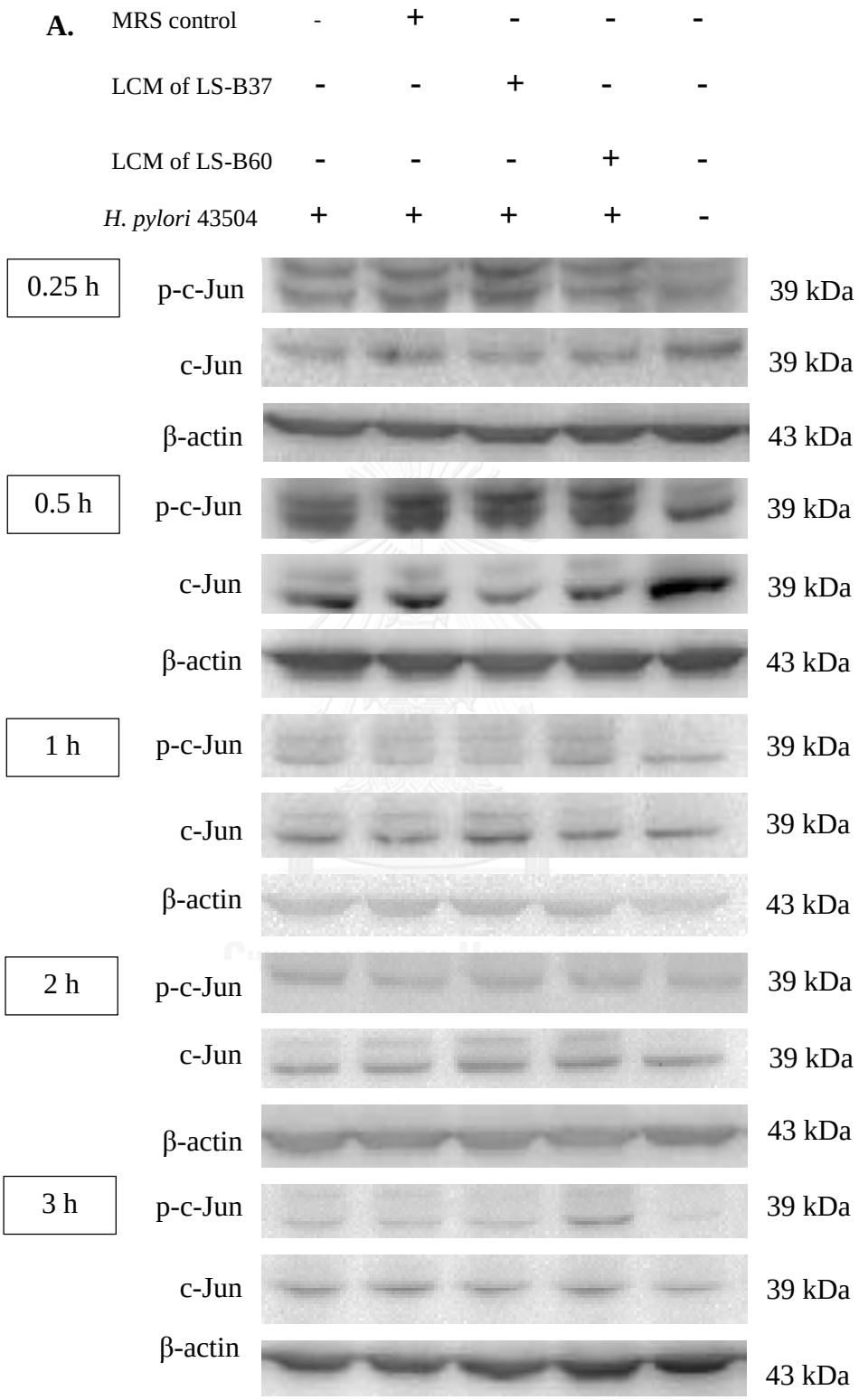
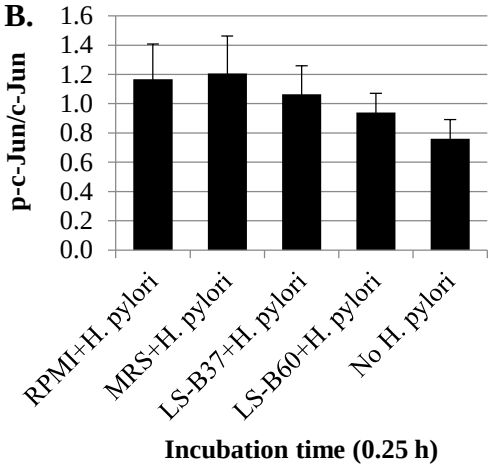


Figure 12: Suppressive effects of LCM of LS-B37 and LS-B60 on *H. pylori*-activated transcription factor (NF-κB) in AGS human gastric epithelial cells. AGS cells were incubated with LCM of LS-B37 and LS-B60 for 0.25, 0.5, 1, 2, and 3 h, and Western blot analysis of p-NF-κB p65 subunit using specific antibodies was performed. Relative protein concentration was quantified by densitometry. The protein level of activated transcription factor (p-NF-κB p65 Ser 536) was normalized to non-activated counterparts (NF-κB p65) and the experiments were performed in three independent experiments. Representative result of Western blot analysis (A). Relative level of p-NF-κB p65 Ser 536 and NF-κB p65 presented as means ± SD (B) and at various time points (C), $^{**}p < 0.01$ as compared to MRS media control.

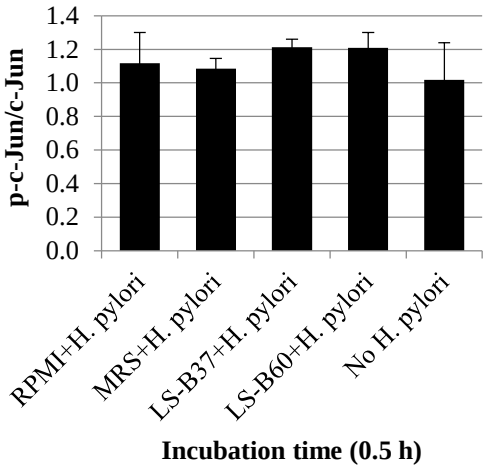
A.


MRS control - + - - -
LCM of LS-B37 - - + - -
LCM of LS-B60 - - - + -
H. pylori 43504 + + + + -
0.25 h
p-c-Jun
39 kDa
c-Jun
39 kDa
β-actin
43 kDa
0.5 h
p-c-Jun
39 kDa
c-Jun
39 kDa
β-actin
43 kDa
1 h
p-c-Jun
39 kDa
c-Jun
39 kDa
β-actin
43 kDa
2 h
p-c-Jun
39 kDa
c-Jun
39 kDa
β-actin
43 kDa
3 h
p-c-Jun
39 kDa
c-Jun
39 kDa
β-actin
43 kDa

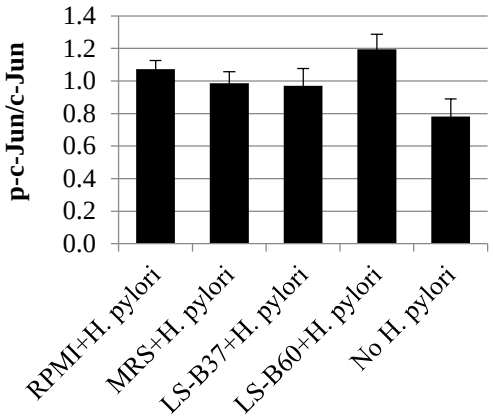
B.



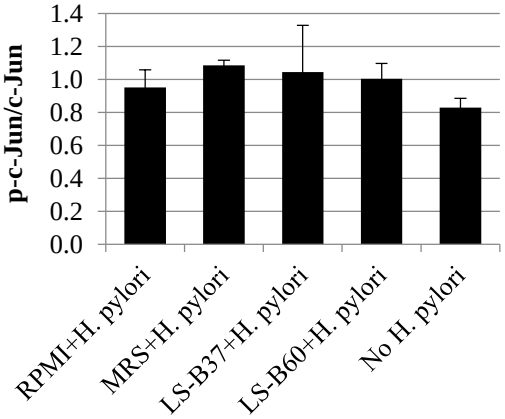
Incubation time (0.25 h)



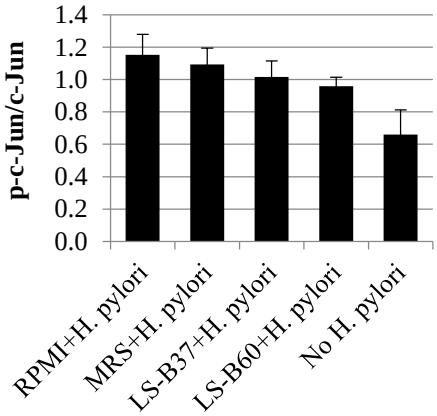
Incubation time (0.5 h)



Incubation time (1 h)



Incubation time (2 h)



Incubation time (3 h)

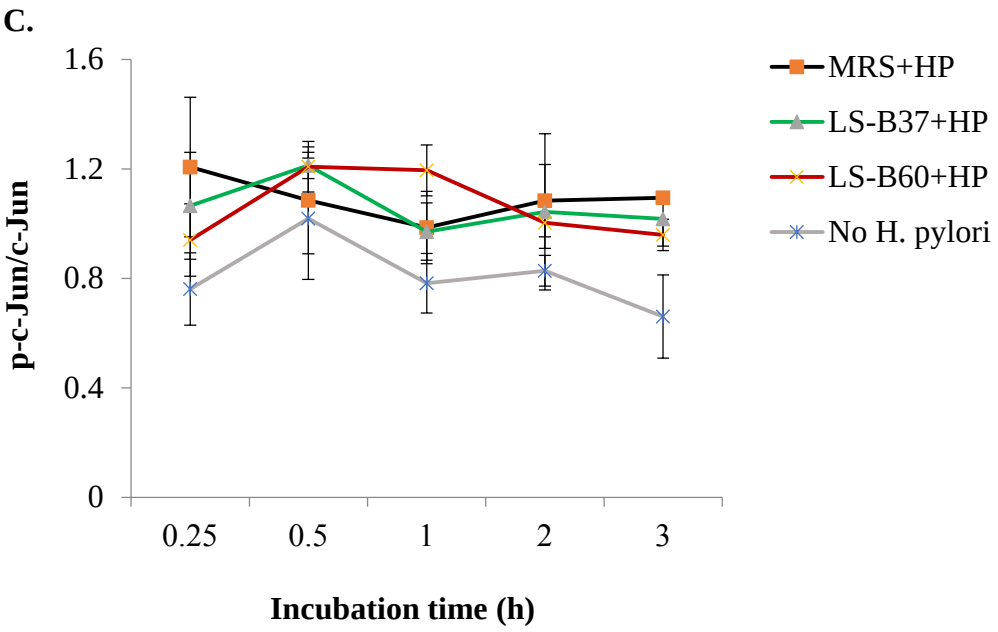
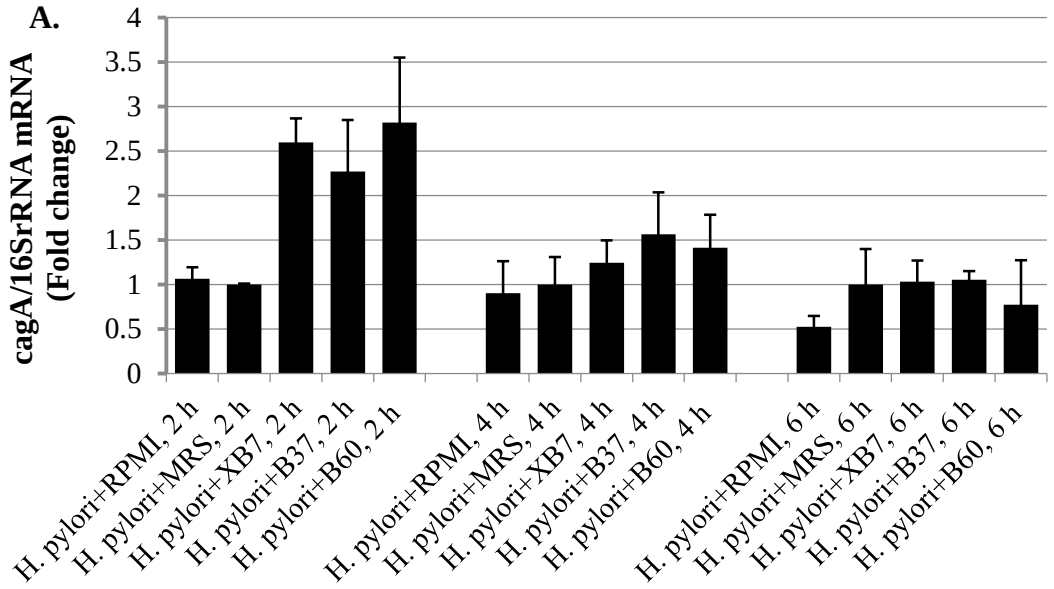


Figure 13: Effects of LCM of LS-B37 and LS-B60 on activation of transcription factor (c-Jun) in *H. pylori*-induced AGS human gastric epithelial cells. AGS cells were incubated with LCM of LS-B37 and LS-B60 for 0.25, 0.5, 1, 2, and 3 h, and Western blot analysis of p-c-Jun subunit using specific antibodies was performed. Relative protein concentration was quantified densitometry. The protein level of activated transcription factor (p-c-Jun) was normalized to non activated counterparts (c-Jun) and the experiments were performed in three independent experiments. Representative result of Western blot analysis (A). Relative level of p-c-Jun and c-Jun presented as means ± SD (B) and at various time points (C).

6. *Lactobacillus*-conditioned media of LP-XB7, LS-B37 and LS-B60 do not affect *cag*A and *cag*E expression

It is well supported that the *H. pylori* virulence factor, CagA, is largely responsible for stimulating IL-8 production by gastric epithelial cells. CagA activates NF-κB, via ERK resulting in the induction of IL-8 secretion [29]. According to Tamura et al. (2006), a mutant *H. pylori* strain constructed by deleting the *cag*E (*cag*E-KO) also could not induce MKN45 gastric epithelium cells to produce IL-8 [36]. Our experiment was aimed at determining whether the inhibitory activity exerted by LP-XB7, LS-B37 and LS-B60 was due to the suppression of *cag*A and/or *cag*E expression. *H. pylori* was co-cultured with MRS control or LCM (5% v/v) from LP-XB7, LS-B37 and LS-B60 for 2, 4 and 6 h and then were assayed for effects on *cag*A and/or *cag*E expression by quantitative real-time PCR relative to 16SrRNA. The results show that, LCM from LP-XB7, LS-B37 and LS-B60 could not suppress both *cag*A and *cag*E at all time points (Figures 14A and 14B). The findings show that the inhibitory activity on *H. pylori*-induced IL-8 production in AGS cells by LP-XB7, LS-B37 and LS-B60 did not mediate with the reduction of *cag*A and *cag*E gene expression.


A.
cagA/16SrRNA mRNA (Fold change)
4
3.5
3
2.5
2
1.5
1
0.5
0
H. pylori+RPMI, 2 h
H. pylori+MRS, 2 h
H. pylori+XB7, 2 h
H. pylori+B37, 2 h
H. pylori+B60, 2 h
H. pylori+RPMI, 4 h
H. pylori+MRS, 4 h
H. pylori+XB7, 4 h
H. pylori+B37, 4 h
H. pylori+B60, 4 h
H. pylori+RPMI, 6 h
H. pylori+MRS, 6 h
H. pylori+XB7, 6 h
H. pylori+B37, 6 h
H. pylori+B60, 6 h
H. pylori co-incubated with LCM

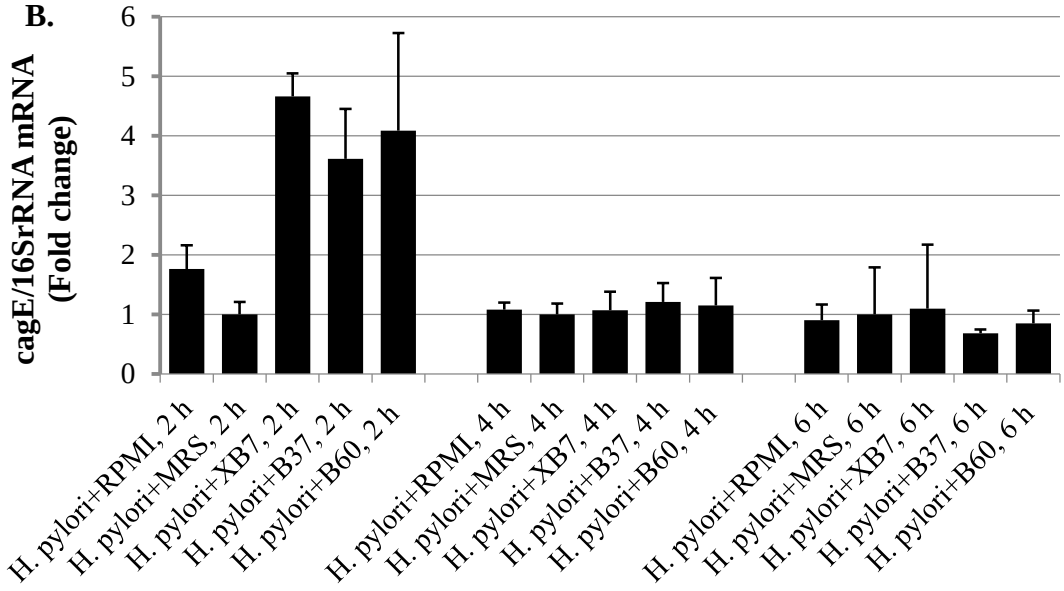

B.
cagE/16SrRNA mRNA (Fold change)
6
5
4
3
2
1
0
H. pylori+RPMI, 2 h
H. pylori+MRS, 2 h
H. pylori+XB7, 2 h
H. pylori+B37, 2 h
H. pylori+B60, 2 h
H. pylori+RPMI, 4 h
H. pylori+MRS, 4 h
H. pylori+XB7, 4 h
H. pylori+B37, 4 h
H. pylori+B60, 4 h
H. pylori+RPMI, 6 h
H. pylori+MRS, 6 h
H. pylori+XB7, 6 h
H. pylori+B37, 6 h
H. pylori+B60, 6 h
H. pylori co-incubated with LCM

Figure 14: *Lactobacillus*-conditioned media of LP-XB7, LS-B37 and LS-B60 do not suppress *cag*A and *cag*E expression. *H. pylori* suspensions were co-incubated with LCMs of LP-XB7, LS-B37 and LS-B60 for 2, 4 and 6 h prior to total RNA isolation. *cag*A and *cag*E expression was measured by using quantitative real-time PCR. This experiment was performed with primers specific to *cag*A, *cag*E and 16SrRNA transcripts. Quantitative gene expression data were normalized to 16SrRNA as housekeeping gene. Fold change of *cag*A (A) and *cag*E (B) gene expression was calculated in relative to MRS media control. The results were illustrated means ± SD, and the experiments were performed at least three times and corresponded to representative experiments.

7. Inhibitory effects of heat-treated LCM of *Lactobacillus* spp. on IL-8 production in *H. pylori*-induced AGS human gastric epithelial cells.

To characterize the nature of IL-8 inhibitory substance (s) in LCM, heat-treated samples were performed. LCM from LP-XB7, LS-B37 and LS-B60 were heated to 100^0C for 15 min, 30 min, 1 h and, 2 h and then tested for the inhibitory activity on *H. pylori*-induced IL-8 production in AGS cells. Heat-treated LCM of LP-XB7, LS-B37 and LS-B60 still inhibited *H. pylori*-induced IL-8 production in AGS cells when compared with MRS control ($p < 0.001$). As illustrated in Figure 15, we found that heat-treated LCMs of LP-XB7, LS-B37 and LS-B60 at all incubation periods (15-120 min) significantly inhibited IL-8 production. These findings show that the inhibitory substance (s) in *Lactobacillus*-conditioned medium of LP-XB7, LS-B37 and LS-B60 are heat-stable for up to 2 h (Table 6 and Figure 15).

Table 6: Inhibitory effects of heat-treated LCM of *Lactobacillus* spp. on IL-8 production in *H. pylori*-induced AGS cells. LCM, *Lactobacillus* conditioned media. The experiments were performed three times independently, each in triplicate. The results were shown as means ± SD, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to MRS media control.

Sample and condition	Time I		Time II		Time III	
	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value
LP-XB7						
normal	41.80*	0.0171	61.73	0.0197	71.76***	0.0007
Heat 15 min.	65.26**	0.0013	51.84	0.0055	59.90*	0.0103
Heat 30 min.	44.18*	0.0301	48.28	0.0202	72.56***	0.000
Heat 1 h.	73.49***	0.0003	56.60	0.0227	67.56***	0.000
Heat 2 h.	61.86***	0.0007	34.83	0.0352	59.77***	0.001
LS-B37						
normal	59.26***	0.0003	73.37***	0.0001	69.62**	0.0040
Heat 15 min.	42.36**	0.0077	47.62**	0.0010	66.44**	0.0081
Heat 30 min.	48.08*	0.0128	73.35***	0.0002	45.97**	0.0072
Heat 1 h.	46.90*	0.0117	82.84***	0.0000	51.54*	0.0353
Heat 2 h.	61.75***	0.0008	51.00***	0.0003	85.11***	0.0008
LS-B60						
normal	49.58**	0.0068	57.96**	0.0058	68.33**	0.0013
Heat 15 min.	53.76*	0.0190	74.69**	0.0023	70.32**	0.0021
Heat 30 min.	23.56**	0.0043	75.88**	0.0021	62.39**	0.0068
Heat 1 h.	82.46***	0.0002	47.74**	0.0092	83.01***	0.0005
Heat 2 h.	74.16**	0.0016	79.37**	0.0019	47.07**	0.0050

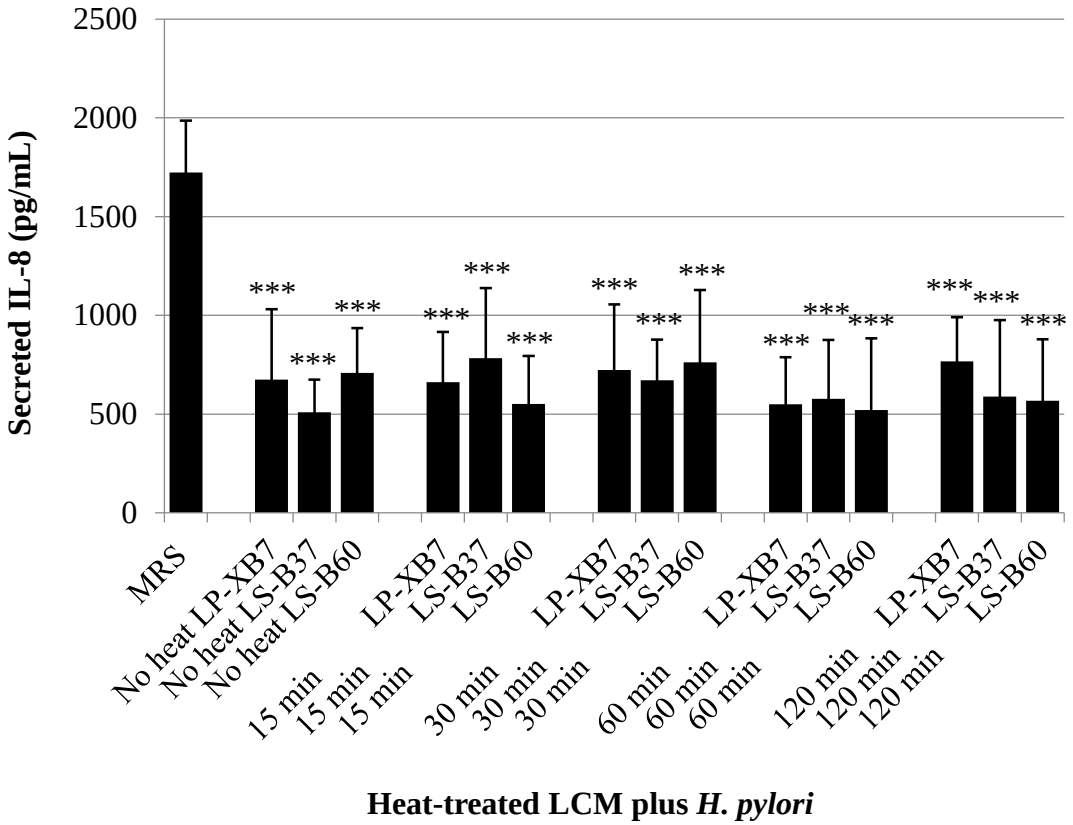


Figure 15: Inhibitory effects of heat-treated LCM of *Lactobacillus* spp. on IL-8 in *H. pylori*-induced AGS cells. AGS cells were incubated with non heat-treated LCM and heat-treated LCM of *L. plantarum* XB7, *L. salivarius* LS-B37 and LS-B60 for 24 h, and IL-8 level in the culture supernatant was determined using a Quantikine Human IL-8 Immunoassay. The experiments were performed three times independently, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference ***$p < 0.001$ as compared to MRS media control.

8. Immunomodulatory effects of fractionated-LCM of *Lactobacillus* spp. on IL-8 productions in *H. pylori*-induced AGS human gastric epithelial cells.

We sought to identify the molecular weight of the IL-8 inhibitory factor (s) present in LCM from LP-XB7, LS-B37 and LS-B60. Size fractionation of LP-XB7, LS-B37 and LS-B60 LCM was performed using 3 kDa, 50 kDa, and 100 kDa Amicon® Centrifugal Filters and subsequently tested for anti-inflammatory capabilities on *H. pylori*-induced IL-8 production in AGS cells. The fractions containing molecules >3 kDa, >50 kDa, and >100 kDa in size of LP-XB7, LS-B37 and LS-B60 significantly suppressed IL-8 production as compared with MRS control ($p < 0.001$), whereas molecules <3 kDa, <50 kDa, and <100 kDa did not suppress *H. pylori*-induced IL-8 production (Table 7-9, Figure 16-18). These data show that the immunomodulatory factor (s) of LP-XB7, LS-B37 and LS-B60 are >100 kDa in size (Figure 19A). Moreover, IL-8 inhibitory activity was increased by using >100 kDa fraction (10% v/v) as compared with >100 kDa fraction (5% v/v) (approximately 0.6 fold) (Figure 19B).

Table 7: Immunomodulatory effects of the fractions of LCM separated by 3K on IL-8 productions in *H. pylori*-induced AGS cells. LCM, *Lactobacillus* conditioned media. The experiments were performed three times independently, each in triplicate. The results were shown as means ± SD, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 as compared to MRS media control.

Sample and fraction	Time I		Time II		Time III	
	IL-8 suppression (%)	***p*-value**	**IL-8 suppression (%)**	***p*-value**	**IL-8 suppression (%)**	***p*-value**
LP-XB7						
normal	77.42***	0.0001	41.80*	0.0171	71.76***	0.0007
<3 kDa	0	-	0	-	0	-
>3 kDa	29.44**	0.0032	30.77**	0.0036	30.55**	0.0073
LS-B37						
normal	59.91***	0.0008	48.60**	0.0042	72.51**	0.0015
<3 kDa	0	-	-	-	0	-
>3 kDa	21.51**	0.0063	57.03**	0.0014	27.78*	0.0200
LS-B60						
normal	62.73***	0.0002	49.58**	0.0068	68.33**	0.0013
<3 kDa	0	-	0	-	0	-
>3 kDa	42.80***	0.0008	28.23**	0.0060	40.67**	0.0064

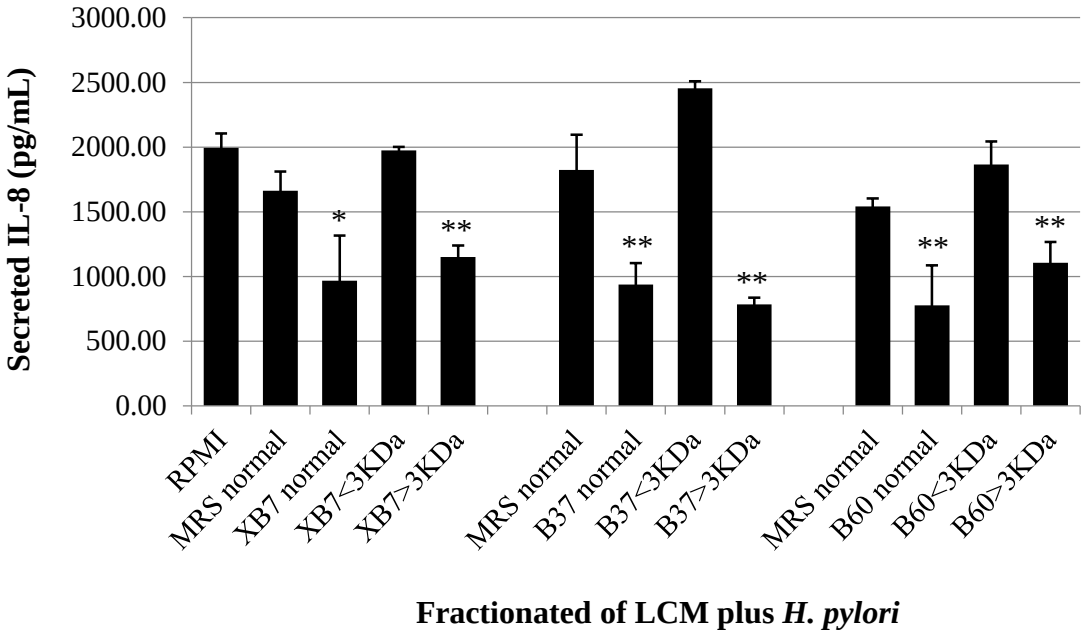


Figure 16: Inhibition of IL-8 secretion by fractionated-LCM >3kDa of *Lactobacillus* spp. LCMs of LP-XB7, LS-B37 and LS-B60 were fractionated by using AmiconUltra-4 100K (Millipore) according to the manufacturer's instruction and then tested for activity on suppression IL-8 production in *H. pylori*-activated AGS cells. The experiments were performed three times independently, each in triplicate. The results were shown as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to MRS media control.

Table 8: Immunomodulatory effects of the fractions of LCM separated by 50K on IL-8 productions in *H. pylori*-induced AGS cells. LCM, *Lactobacillus* conditioned media. The experiments were performed three times independently, each in triplicate. The results were shown as means ± SD, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to MRS media control.

Sample and fraction	Time I		Time II		Time III	
	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value
LP-XB7						
normal	31.76*	0.0342	50.30**	0.0068	77.42***	0.0001
<50 kDa	0	-	0	-	0	-
>50 kDa	19.33*	0.0103	45.42***	0.0006	24.62**	0.0076
LS-B37						
normal	69.11***	0.0004	71.54***	0.0000	68.55***	0.0003
<50 kDa	0	-	0	-	0	-
>50 kDa	18.04***	0.0008	44.33**	0.0021	50.95**	0.0098
LS-B60						
normal	33.31**	0.0036	60.42**	0.0047	62.73***	0.0002
<50 kDa	0	-	0	-	0	-
>50 kDa	31.74***	0.0000	42.10**	0.0013	41.37***	0.0009

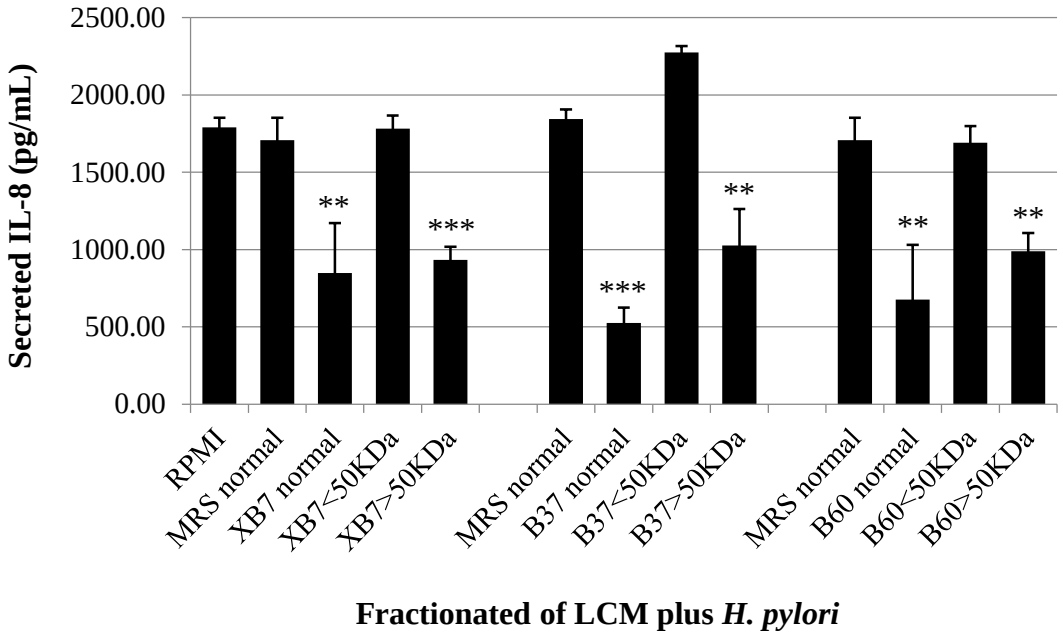


Figure 17: Inhibition of IL-8 secretion by fractioned-LCM >50kDa of *Lactobacillus* spp. LCMs of LP-XB7, LS-B37 and LS-B60 were fractionated by using AmiconUltra-4 100K (Millipore) according to the manufacturer's instruction and then tested for activity on suppression IL-8 production in *H. pylori*-activated AGS cells. The experiments were performed three times independently, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to MRS media control.

Table 9: Immunomodulatory effects of the fractions of LCM separated by 100K on IL-8 productions in *H. pylori*-induced AGS cells. LCM, *Lactobacillus* conditioned media. The experiments were performed three times independently, each in triplicate. The results were shown as means ± SD, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 as compared to MRS media control.

Sample	Time I		Time II		Time III	
	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value	IL-8 suppression (%)	*p*-value
LP-XB7						
normal	60.62***	0.0002	89.39***	0.0005	65.69**	0.0027
<100kDa	0	-	0	-	0	-
>100kDa	64.41***	0.0001	57.23***	0.0008	64.66***	0.0005
LS-B37						
normal	86.48***	0.0001	84.87***	0.0002	91.34***	0.0000
<100kDa	0	-	0	-	0	-
>100kDa	67.33***	0.0001	54.34**	0.0048	65.52***	0.0003
LS-B60						
normal	62.78**	0.0022	56.51**	0.0047	87.90***	0.0001
<100kDa	0	-	0	-	0	-
>100kDa	68.07***	0.0002	47.21*	0.0135	65.52***	0.0003

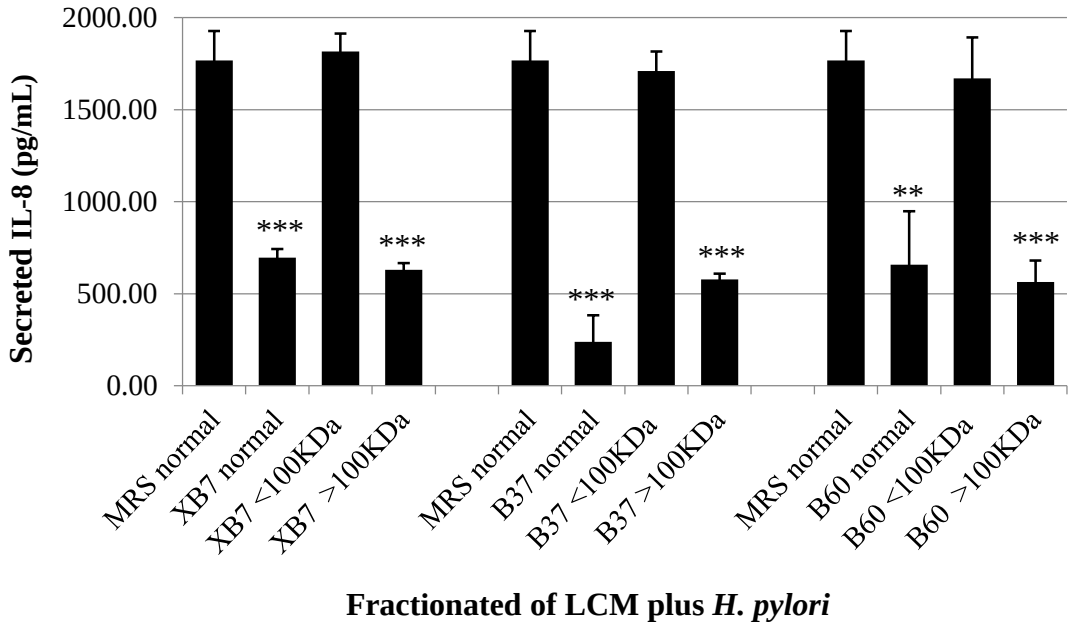
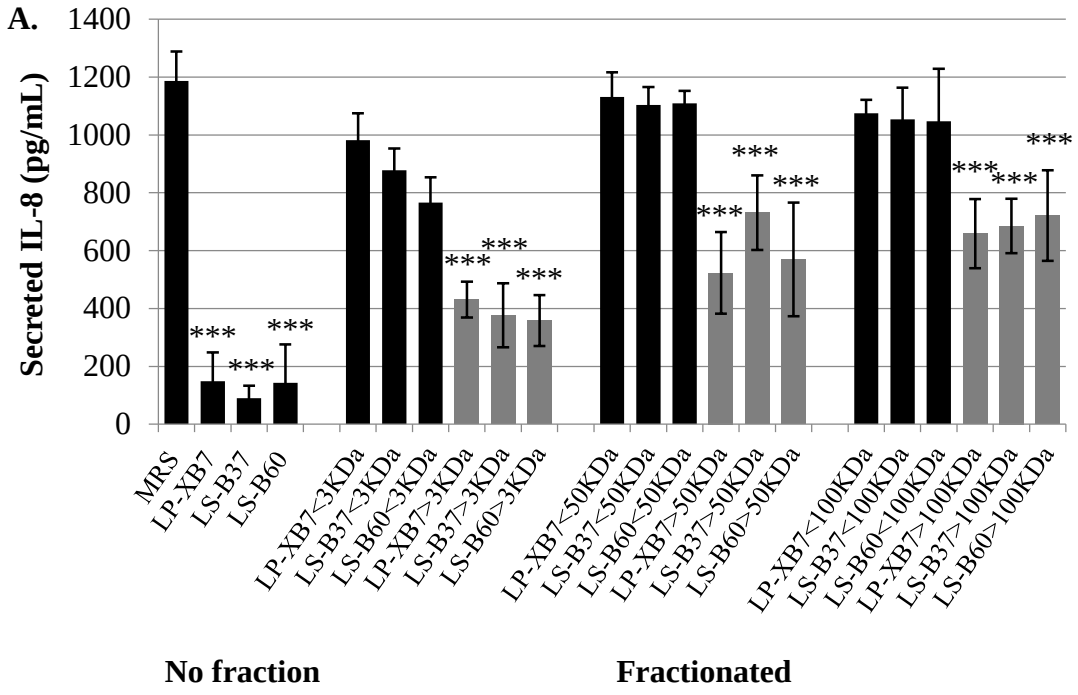


Figure 18: Inhibition of IL-8 secretion by fractionated-LCM >100kDa of *Lactobacillus* spp. LCMs of LP-XB7, LS-B37 and LS-B60 were fractionated by using AmiconUltra-4 100K (Millipore) according to the manufacturer's instruction and then tested for activity on suppression IL-8 production in *H. pylori*-activated AGS cells. The experiments were performed three times independently, each in triplicate. The results were expressed as the mean ± SD of triplicate determinations, and an asterisk indicated a statistically significant difference $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to MRS media control.


A.
Secreted IL-8 (pg/mL)
1400
1200
1000
800
600
400
200
0
***
MRS
LP-XB7
LS-B37
LS-B60
LP-XB7<3KDa
LS-B37<3KDa
LS-B60<3KDa
LP-XB7>3KDa
LS-B37>3KDa
LS-B60>3KDa
LP-XB7<50KDa
LS-B37<50KDa
LS-B60<50KDa
LP-XB7>50KDa
LS-B37>50KDa
LS-B60>50KDa
LP-XB7<100KDa
LS-B37<100KDa
LS-B60<100KDa
LP-XB7>100KDa
LS-B37>100KDa
LS-B60>100KDa
No fraction
Fractionated

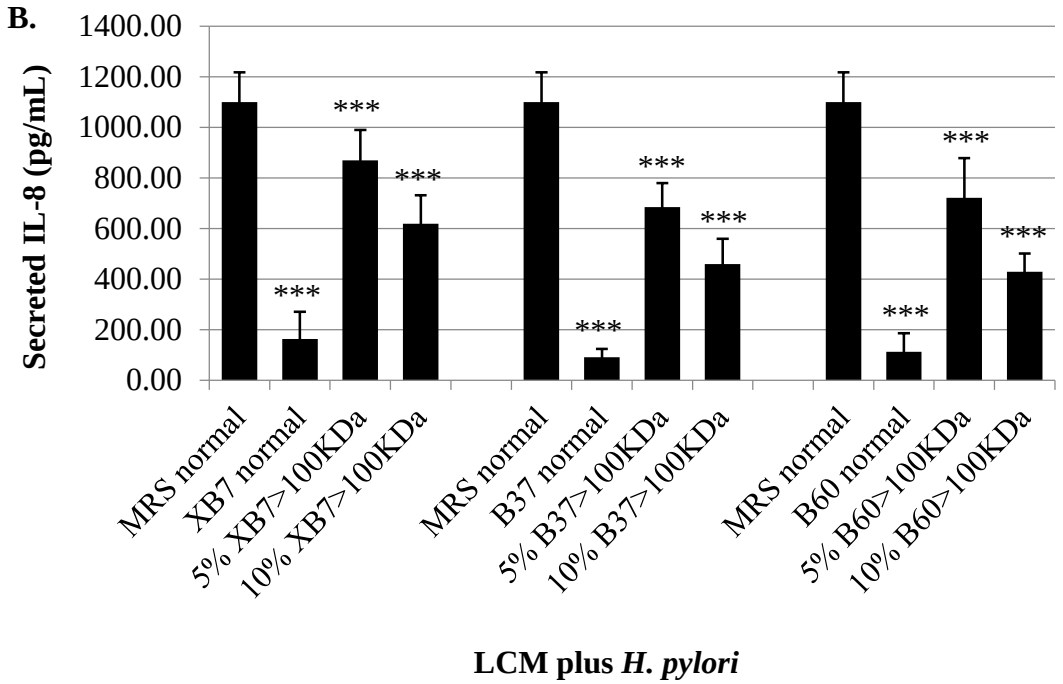
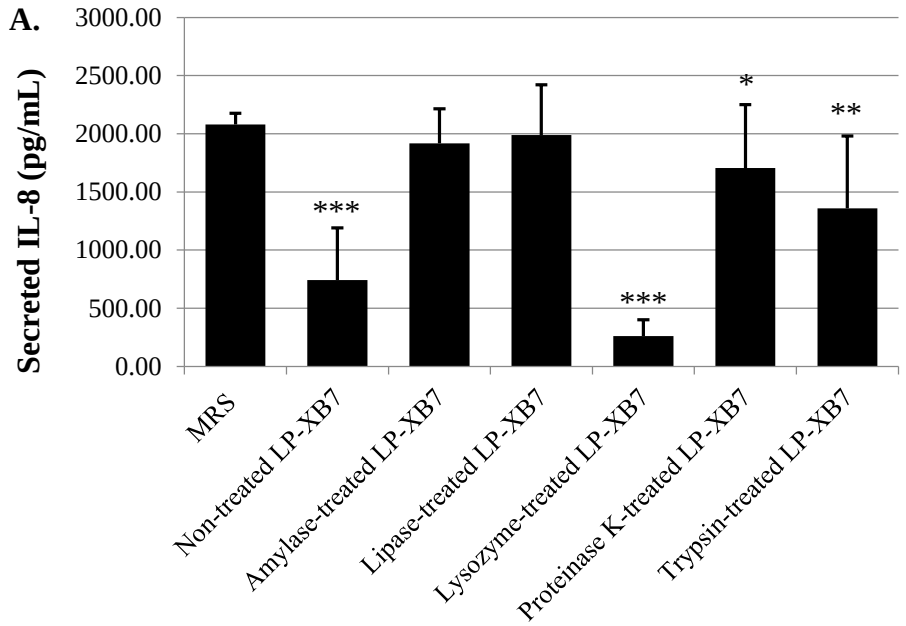

B.
Secreted IL-8 (pg/mL)
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
***
MRS normal
XB7 normal
5% XB7>100KDa
10% XB7>100KDa
MRS normal
B37 normal
5% B37>100KDa
10% B37>100KDa
MRS normal
B60 normal
5% B60>100KDa
10% B60>100KDa
LCM plus H. pylori

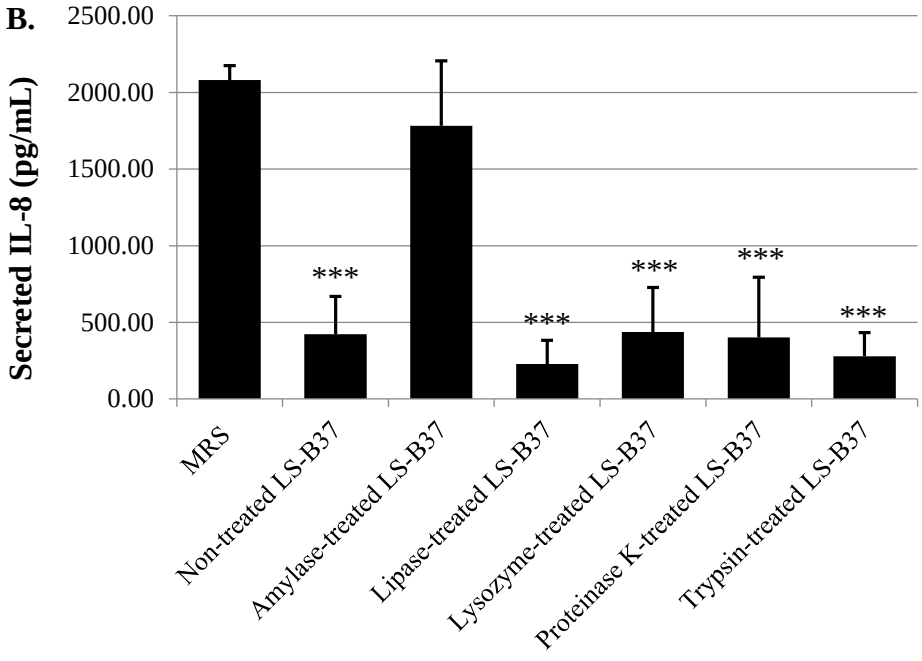
Figure 19: Summary of Inhibition of IL-8 secretion by fractionated-LCM >3kDa, >50kDa, and >100kDa of *Lactobacillus* spp. LCM of LP-XB7, LS-B37 and LS-B60 were fractionated by using AmiconUltra-4 3K, 50K, and 100K (Millipore) according to the manufacturer's instruction and then tested for activity on suppression IL-8 production in *H. pylori*-activated AGS cells (A). The experiments were performed three times independently, each in triplicate and the results showed as means ± SD, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ as compared to media control.

9. Enzyme sensitivity of *Lactobacillus*-conditioned media on *H. pylori*-induced IL-8 production in AGS human gastric epithelial cells

To determine whether the IL-8 inhibitory factor (s) consists of carbohydrates, lipids, proteins, and/or the components of cell wall, treatment LCMs with α-amylase, lipase, proteinase K, trypsin, and lysozyme were performed. LCM from LP-XB7, LS-B37 and LS-B60 were incubated with each of these enzymes for 6 hours and then tested for the anti-inflammatory ability on *H. pylori*-induced IL-8 production in AGS cells. Treatment of LS-B37 LCM with α-amylase decreased IL-8 suppression as compared to non-treated LCM (>4 fold change) (Figure 20B). Treatment of LS-B37 LCM with lipase, proteinase K, trypsin, and lysozyme had no effect on IL-8 suppression. Treatment of LP-XB7 LCM with α-amylase and lipase also diminished the inhibition of *H. pylori*-induced IL-8 production (>4 fold change), whereas the treatment with proteinase K and trypsin slightly affected IL-8 suppression (Figure 20A). Treatment of LS-B60 LCM with α-amylase, and proteinase K diminished the inhibition of *H. pylori*-induced IL-8 production (>4 fold change), whereas the treatment with lipase and trypsin slightly affected IL-8 suppression (Figure 20C). No effect on IL-8 suppression was detected when LS-XB7 and LS-B60- LCM was treated with lysozyme (Figure 20A and C). These data suggest that IL-8 suppression by LS-B37 LCM is due to a produced polysaccharide, while that of LP-XB7 and LS-B60 LCM is more complex consisting of components of polysaccharides, lipids and, proteins. Summary of the inhibitory effects of these enzyme treatments was shown in Figure 21. The effect of various treatments of LCM on the ability of IL-8 suppression was summarized in Table 10.



Enzyme-treated LCM plus *H. pylori*



Enzyme-treated LCM plus *H. pylori*

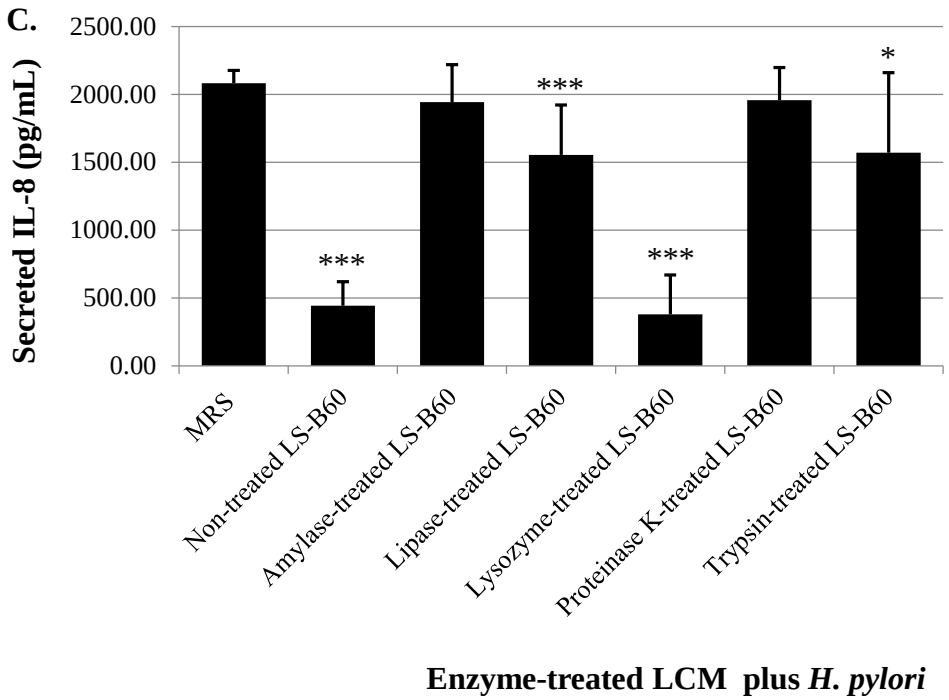


Figure 20: Inhibitory effect of enzyme-treated LCM of *L. plantarum* LP-XB7, *L. salivarius* LS-B37 and LS-B60 on IL-8 production in *H. pylori*-induced AGS cells. The IL-8 suppression effect of enzyme-treated LCM of LP-XB7 (A), LS-B37 (B) and LS-B60 were investigated. LCMs of each isolate was treated with enzymes, α-amylase, lipase, lysozyme, proteinase K, and trypsin and then incubated with AGS cells in combination with *H. pylori* for 24 h. IL-8 level in the culture supernatant was determined using a Quantikine Human IL-8 Immunoassay. The experiments were performed three times independently, each in triplicate and the results were shown as means ± SD, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to media control.

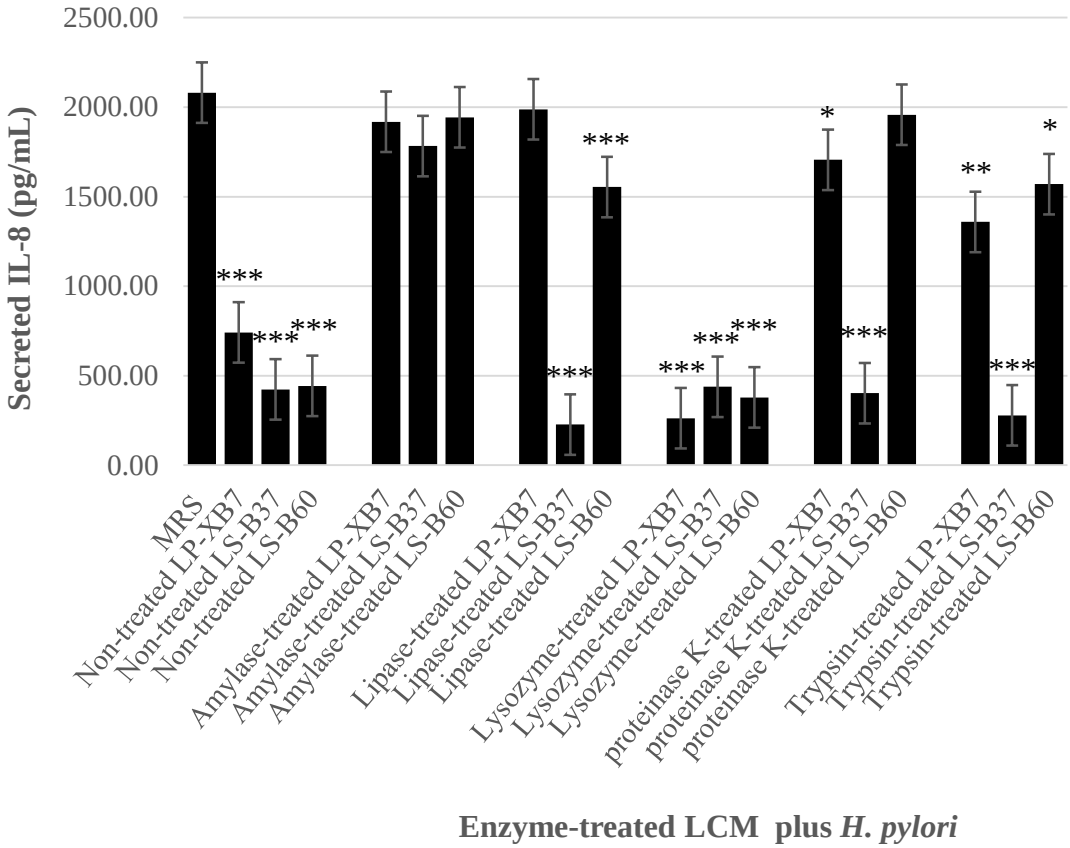


Figure 21: Summary of the inhibitory effect of *L. plantarum* LP-XB7, *L. salivarius* LS-B37 and LS-B60 on IL-8 production in *H. pylori*-induced AGS cells. The IL-8 suppression effect of enzyme-treated LCM of LP-XB7, LS-B37 and LS-B60 were investigated. LCMs of each isolate was treated with enzymes, α-amylase, lipase, lysozyme, proteinase K, and trypsin and then incubated with AGS cells in combination with *H. pylori* for 24 h. IL-8 level in the culture supernatant was determined using a Quantikine Human IL-8 Immunoassay. The experiments were performed three times independently, each in triplicate and the results were shown as means ± SD, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ as compared to media control.

Table 10: Summary of effect of various treatments of conditioned media from *Lactobacillus* spp. on the ability of IL-8 suppression.

Treatment		**Activity**		
Temperature 100°C	Time (minutes)	*L. plantarum* (LP-XB7)	*L. salivarius* (LS-B37)	*L. salivarius* (LS-B60)
	15	+	+	+
	30	+	+	+
	60	+	+	+
	120	+	+	+
Amicon® Ultra4 Centrifugal Filter	Molecular weight fraction size			
	> 3 kDa	+	+	+
	>50 kDa	+	+	+
	>100 kDa	+	+	+
	<3 kDa	-	-	-
	<50 kDa	-	-	-
	<100kDa	-	-	-
Enzymes	Type of enzyme			
	α-Amylase	-	-	-
	Lipase	-	+	+[a]
	Lysozyme	+	+	+
	Proteinase K	+[a]	+	-
	Trypsin	+[a]	+	+[a]

IL-8 suppression activity: +, positive; -, negative

[a], slightly positive

CHAPTER VI
DISCUSSION

H. pylori infection induces a host inflammatory response with the production of proinflammatory cytokines and chemokines which lead to infiltration of inflammatory cells including neutrophils, lymphocytes, and macrophages at the site of infection and initiating inflammatory damage of gastric mucosa reference. IL-8 is a potent neutrophil chemotactic and activating agent [10-14] and associated with disease activity. A variety of cell types such as monocytes, fibroblasts, endothelial, and epithelial cells can produce IL-8 [15]. There is evidence demonstrated that *H. pylori*-infected and gastric cancer patients have increased levels of IL-8 in gastric epithelium [16]. Some studies demonstrated the stimulation of IL-8 production by *H. pylori* in gastric epithelial cell lines *in vitro* [17, 18]. *H. pylori* infection caused an increased induction of IL-8 mRNA and protein expression in gastric epithelial cells *in vitro* and *in vivo* [17, 19-21]. These evidences suggest that IL-8 is an important chemokine induced by *H. pylori* colonization which induces the activation of NF-κB in gastric epithelium cells and resulting in up-regulation of *IL-8* gene transcription.

Some probiotics, including lactobacilli, have been shown to diminish inflammatory cytokine in *H. pylori* infection models both *in vitro* [36, 49, 60] and *in vivo* [36]. The present study demonstrates that conditioned media of *L. salivarius* (LS-B37 and LS-B60) can diminish IL-8 production by *H. pylori*-induced gastric epithelial cells at transcriptional level by reducing *IL-8* gene expression at 2 hours. Our result is supported by recent experiment that *L. salivarius* strain UCC118 decreased IL-8 production of *H. pylori*- infected gastric epithelium cell [49]. Furthermore, *H. pylori*-activated p-NF-κB expression was reduced at 15 minutes in the presence of LS-B37, but LS-B60 did not suppress at the same time. LS-B60 has ability to inhibit NF-κB activation at 2 hours after incubation with conditioned media and *H. pylori* in AGS gastric epithelial cells. The findings suggest that the suppression of p-NF-κB activation is time specific for these strains due to LS-B37 suppresses NF-κB activation at 15 min, while LS-B60 suppresses the activation of NF-κB at 2 h. However, this study found that the suppression of IL-8 production is not mediated by the suppression of

p-c-Jun activation. Our findings are supported by the recent evidence that gastric-derived *L. salivarius* B101 has ability to suppress IL-8 production by inactivation of NF-κB at 1 h and continued to 4 h. Moreover, *L. plantarum* XB7 modulated IL-8 production by suppression of NF-κB activation at 2 h and also suppressed c-Jun phosphorylation at 4 h [66]. Yang et al. (2012) reported that pretreatment *L. acidophilus* with human gastric epithelial cancer cell lines (MKN45) for 8 h followed by *H. pylori* co-incubation for 1 h ameliorated *H. pylori*-stimulated gastric inflammation by modulation of NF-κB activation [65]. The result is compatible with the finding from Thiraworawong et al. (2014) showing that anti-inflammatory properties of *L. salivarius* B101 was mediated through the inhibition of NF-κB activation at 2 h [66].

Previous study showed that protein of *cag*A is largely responsible for stimulating IL-8 production by gastric epithelial cells both *in vitro* and *in vivo*. CagA can activate NF-κB via ERK resulting in induction of IL-8 secretion [29] and knock-out *cag*E mutant of *H. pylori* could not induce MKN45 gastric epithelium cells to produce IL-8 production [36]. This study aimed to determine whether the IL-8 inhibitory activity exerted by *L. plantarum* (LP-XB7), *L. salivarius* (LS-B37), and (LS-B60) was due to the suppression of *cag*A and/or *cag*E expression. The finding demonstrates that lactobacilli in this study could not decrease the activation of *cag*A and *cag*E of *H. pylori* after co-culture of their conditioned media with *H. pylori*. The results suggest that IL-8 suppression ability of these lactobacilli was not contributed from the inhibition of *cag*A and *cag*E gene expression. This result agreed with the previous evidence by Ryan et al. (2009) that the expression of *cag*A gene (HP0547) was not altered in the presence of *L. salivarius* UCC118 [49].

The difference of the activity, molecular size, and stability of IL-8 suppressing factors were reported to be varied among microorganisms. Frick. et al. (2007) demonstrated that *Lactobacillus fermentum* can suppress *Yersinia enterocolitica*-induced IL-8 production by a possibly secreted soluble factor phospholipid of <10kDa molecular weight in size [224]. Thomas et al. (2012) showed that *L. reuteri* ATCC PTA 6475 is able to produce histamine that suppresses pro-inflammatory TNF production in THP-1 monocytoid cells by the modulation of PKA and ERK signaling [225]. Sougioultzis et al. (2006) reported that *Saccharomyces boulardii* (Sb) a probiotic yeast, produces a small water-soluble, and heat-stable anti-inflammatory factor (<1kDa) that

inhibits NF-κB-mediated *IL-8* gene expression in monocytes and intestinal epithelial cells [226]. In this study, we characterized the substance(s) in conditioned media from LP-XB7, LS-B37, and LS-B60 that suppress IL-8 production in *H. pylori*-stimulated AGS human gastric epithelial cells by testing the heat stability, molecular size and the resistance to various enzymes. The findings indicate that the immunomodulatory substance (s) of these *Lactobacillus* strains are heat stable and >100kDa molecular size. The result agreed with the finding from Schillde et al. (2012) that the active secreted component of *Lactobacillus paracasei* that suppress IP-10 secretion in small intestinal epithelial cell line Mode-K cells stimulated with TNF, is a protein or protein complex with a molecular mass of >100kDa [227]. Additionally, the substance of *L. salivarius*-B37 (LS-B37) is sensitive to amylase but stable to various degrading enzymes such as lipase, lysozyme, proteinase K, and trypsin while the substance of either *L. plantarum*-XB7 (LS-XB7) or *L. salivarius*-B60 (LS-B60) is sensitive to all of these enzymes albeit with different magnitudes (Table 10). The possibility of the findings is that LS-B37 may produce the factor consisting polysaccharides that suppress IL-8 production. Previous report of Yasuda et al (2008) demonstrated that the high molecular mass polysaccharide moiety of cell well is the relevant immune modulator that may function to suppress excessive immune response during the activation of macrophage by *L. casei* Shirota [228]. LP-XB7 and LS-B60 may produce the molecules (s) comprised of components of polysaccharide, lipid, and protein which inhibit *H. pylori*-induced IL-8 production. Schillde et al. (2012) reported that Lactocepin secreted by *L. paracasei* is a protease that selectively degrades secreted, cell-associated, and tissue-distributed IP-10, resulting in reduced lymphocyte recruitment after intraperitoneal injection in an ileitis model [227]. It is probable that immunomodulatory substance(s) in LCM of LP-XB7 and LS-B60 is complex consisting of components of polysaccharide, lipid and protein, and the components are different as manifested by their susceptibility to various enzymes. In conclusion, the preliminary identification of immunomodulatory substance (s) of these lactobacilli shows that they are water-soluble, heat-stable, and >100kDa molecular weight in size. Further characterization and identification of the immunomodulatory substances of these lactobacilli may provide us useful pharmacologic agents for treatment of *H. pylori*-associated diseases.

CHAPTER VII
CONCLUSION

Lactobacillus conditioned media (LCM) from human gastric-derived *L. salivarius* strains B37 and B60 (LS-B37 and LS-B60) inhibited IL-8 production from *H. pylori*-induced AGS cells, and also attenuated IL-8 mRNA transcription like that of *L. plantarum*-XB7 (LP-XB7). However, LS-B37 and LS-B60 interfered with IL-8 mRNA expression via the suppression of NF-kB activation, while LP-XB7did via NF-κB and c-Jun.

Expression of *H. pylori* virulence genes *cag*A and *cag*E was not affected by LP-XB7, LS-B37 and LS-B60 conditioned media. It is possible that the suppressive effects of LP-XB7, LS-B37 and LS-B60 were not associated with the inhibition of CagA and CagE which are crucial *H. pylori* virulence factors.

The IL-8 inhibitory substance(s) of LP-XB7, LS-B37 and LS-B60 is heat-stable and >100 kDa. The substance of LS-B37 is sensitive to amylase but stable to various degrading enzymes including lipase, lysozyme, proteinase K, and trypsin while the substance of either LP-XB7 or LS-B60 is sensitive to all of these enzymes albeit in various magnitudes. It was thus assumed that the polysaccharide in LCM of LS-B37 is responsible for the inhibition of NF-κB activation in AGS cells stimulated with *H. pylori*. The IL-8 inhibitory effect of LP-XB7 and LS-B60 on *H. pylori*-induced AGS cells is probably due to different complex components of polysaccharides, lipids and, proteins.

These results showed that human gastric derived-*Lactobacillus* produce multiple immunomodulatory factors capable of suppressing *H. pylori*-induced IL-8 production, making them viable probiotic candidates for the use as adjunctive therapy for treatment of *H. pylori* infection.

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APPENDIX

APPENDIX A

MATERIALS AND REAGENTS

Materials

- 0.22 µm Surfactant-free cellulose acetate membrane filters (Minisart, Sartorius Stedim Biotech GmbH, Germany)
- 0.25% Trypsin in 1 mM EDTA (Gibco-Invitrogen, USA)
- 0.4% Trypan blue solution (Gibco-Invitrogen, USA)
- 10% Ammonium persulphate (APS) (Thermo Scientific)
- 40% Acrylamide/Bis solution (Bio-Rad Laboratories)
- 96-Well Flat-bottom tissue culture plates (Nunclon D, Denmark)
- 100% Methanol (Merck, Germany)
- Absolute alcohol (Merck, Germany)
- AGS human gastric adenocarcinoma epithelial cells, CRL-1739 (ATCC, USA)
- Amicon Ultra-4 3K, 50K, and 100K (Millipore Ireland B.V., Ireland)
- Anaerobic box (Oxoid, UK)
- Anaerobic indicator (Oxoid, UK)
- AnaeroPack-Anaero (Mitsubishi Gas Chemical Company, Inc, Japan)
- AnaeroPack-MicroAero (Mitsubishi Gas Chemical Company, Inc, Japan)
- Barrier tips; 20, 100, 200, and 1,000 µl (Neptune, Mexigo)
- Bovine serum albumin (BSA) (Sigma-Aldrich, USA)
- Brain heart infusion (BHI) agar (BBL™, BD, USA)
- Brain heart infusion (BHI) broth (BBL™, BD, USA)
- Chloroform (Merck, Germany)
- Columbia blood agar (Oxoid, Hampshire, UK)
- Conical centrifuge tube; 15, 50 mL (Nunc, USA)
- Counter (Thailand)
- Cryovial (Nunc, Denmark)
- deMan Rogosa Sharpe (MRS) agar (Oxoid, UK)
- deMan Rogosa Sharpe (MRS) broth (Oxoid, UK)

- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA)
- Disodium hydrogen phosphate (Na_2HPO_4) (Sigma-Aldrich, Germany)
- Enzymes; α-amylase, lipase, lysozyme, proteinase K, and trypsin (Sigma-Aldrich, USA)
- Fetal bovine serum (Gibco-Invitrogen, New Zealand)
- Glycerol (Merck, Germany)
- Glycine (Sigma, USA)
- Halt phosphatase inhibitor single-use cocktail (Pierce Biotechnology, USA)
- Halt protease inhibitor single-use cocktail EDTA-free (Pierce Biotechnology, USA)
- Hemacytometer (Bright line) (BOECO, Germany)
- Horse serum (Gibco-Invitrogen, New Zealand)
- Human IL-8 ELISA kit (DuoSet, R&D Systems, USA)
- Hydrochloric acid (HCL) (Merck, Germany)
- Isopropanol (2-Propanol) (Merck, Germany)
- LightCycler Capillary (Roche, Germany)
- LightCycler® FastStart DNA MasterPLUS SYBR Green (Roche, Germany)
- McFarland 6 (Thailand)
- Methanol (Merck, Germany)
- Microcentrifuge tube (Eppendorf, USA)
- M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, USA)
- PageRuler Prestained Protein Ladder (Pierce Biotechnology, USA)
- Pierce ® BCA Protein Assay Kit (Pierce Biotechnology, USA)
- Potassium chloride (KCL) (Sigma-Aldrich, UK)
- Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich, Germany)
- Primary antibodies, p-NF-kB p65 (Ser 536), NF-kB p65, β-actin, p-c-Jun, and c-Jun (Santo Cruz Biotechnology, Inc, USA)
- Polyvinylidene difluoride (PVDF) membrane (Bio-Rad, PA, USA)
- RPMI 1640 medium (Gibco-Invitrogen, USA)
- Secondary antibody, goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Inc, USA)

- Serological pipettes; 5, 10, and 25 mL (Corning, USA)
- Sheep blood (Faculty of Veterinary Medicine, Chulalongkorn University, Thailand)
- Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Germany)
- Sodium chloride (NaCl) (Sigma-Aldrich, Germany)
- Sodium dihydrogen phosphate (NaH_2PO_4)(Sigma-Aldrich, Germany)
- Sodium hydroxide (NaOH) (MERCK, Germany)
- Substrate Reagent Pack (DY999) (R&D, USA)
- Sulfuric acid (H_2SO_4) (MERCK,Germany)
- SuperScript®VILO cDNA synthesis kit (Invitrogen, USA)
- SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, USA)
- Syringe (Nipro, Thailand)
- Tetramethyl-ethylenediamine (TEMED) (Thermo scientific)
- Tissue culture flask; 25, 75 cm^2 (Nunc, Denmark)
- Tissue culture plates; 6, 12, 24 and 96 wells (Nunc, Denmark)
- Tris base (Sigma-Andrich, USA)
- Trizol® reagent (Invitrogen, USA)
- Tween20 (Merck, Germany)
- Yeast extract (Difco, USA)

Equipments

- -20°C Freezer (Sanyo, Japan)
- -80 °C Freezer (Sanyo, Japan)
- Anaerobic chamber (Concept Plus, Ruskinn Technology Ltd,UK)
- Autoclave (Hirayama, Japan)
- Auto pipette: P-20, P-100, P-200, P-1000 (Gilson, France)
- Auto pipette: P-10, P-1000 (Socorex, Switzerland)
- Biological safety cabinet (Astec-Microflow, Bioquell UK Ltd, UK)
- Bio Rad Trans-Blot SD Semi Dry Transfer (Bio-Rad, PA, USA)
- ChemiDoc™ XRS (Bio-Rad Laboratories, Inc, USA)
- CO_2 incubator (BINDER GmbH, Germany)
- Electrophoresis (Wealtec, Taiwan)
- Eppendorf Master Cycler Gradient Thermal Cycler (Germany)
- Equipment gel electrophoresis apparatus (Bio-Rad Laboratories)
- Fireboy (IBS, Switzerland)
- Hemocytometer (Boeco, Germany)
- Heat block (Scientific Industries, Inc, USA)
- Hotplate (Tekstir® Hot plate)
- Incubator (Memmert GmbH, Germany)
- Inverted microscope (Olympus, Japan)
- LightCycler® 2.0 Instrument (Roche, Germany)
- Micropipettes (Gilson, France)
- Mini-PROTEAN® 3 Cell (Bio-Rid Laboratories, Inc, USA)
- Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc, USA)
- Multi-channel pipette (Socorex, Switzerland)
- NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc, USA)
- pH meter (Thermo Fisher Scientific, Inc, USA)
- PowerPac™ Universal Power Supply (Bio-Rad Laboratories, Inc. USA)
- Power supply (ELITE 300 plus, USA)
- Rectangular jar (Mitsubishi Gas Chemical Company, Inc, Japan)

- Refrigerated centrifuge (Sanyo, Japan)
- Safety cabinet (Augustin, Thailand)
- Spectrophotometer (Bio-Rad Smart Spec^{TM} Plus, Bio-Rad Laboratories, Inc, USA)
- Speed-vacuum drying (Savant instruments, USA)
- Vernier caliper (Mitutoya, Japan)
- Vertical Laminar Flow workstation (Microflow, UK)
- Ultrasonic water bath (GEN-PROBE, Germany)
- Water bath (MemMert GmbH, Germany)
- UV Transilluminator (Bio-Rad, USA)

Software and programs

- Multalin program (http://bioinfo.genotoul.fr/multalin/multalin.html)
- GenBank DNA database search (www.ncbi.nlm.nih.gov/BLAST)
- ImageJ 1.45 s software
- EndNote X4

APPENDIX B

MEDIA FORMULA

Media for *H. pylori*

1. Columbia agar with 7% horse serum and 7% sheep blood

Columbia blood agar (oxoid)	39	g
Hose serum (Gibco)	70	mL
Sheep blood	70	mL
Distilled water	860	mL
Total	1,000	mL

2. Brain heart infusion broth with 10% horse serum

Brain heart infusion broth (BBL™)	37	g
Hose serum (Gibco)	100	mL
Distilled water	900	mL
Total	1,000	mL

3. Stock media (brain heart infusion broth with 30% glycerol and 5% horse serum plus yeast extract)

Brain heart infusion broth (BBL™)	3.7	g
Yeast extract	0.15	g
Hose serum (Gibco)	5	mL
Glycerol	30	mL
Distilled water	65	mL
Total	100	mL

Media for *Lactobacillus*

1. MRS agar

MRS agar (Oxoid)	62	g
Distilled water	1,000	mL
Total	1,000	mL

2. MRS broth

MRS broth (Oxoid)	52	g
Distilled water	1,000	mL
Total	1,000	mL

3. Stock media (MRS broth plus 20% glycerol)

MRS broth (Oxoid)	5.2	g
Glycerol	20	mL
Distilled water	80	mL
Total	100	mL

4. 0.85% Normal saline solution (NSS)

Sodium chloride (NaCl)	8.5	g
Distilled water	1,000	mL
Total	1,000	mL

APPENDIX C

MEDIA AND SOLUTION

FOR TISSUE CULTURE, BIOASSAY, AND ELISA

1. RPMI 1640 medium

RPMI 1640	1	wrap
$NaHCO_3$	2	g
DDW	1,000	mL

RPMI 1640 1 wrap and 2 g of $NaHCO_3$ were dissolved in 1,000 of DDW, then filtered by using 0.22 µm filter, and aliquoted into 250 ml bottle and stored in 4°C. The medium was added with 10% of fetal bovine serum when desired.

2. 5X Phosphate buffer saline (PBS) pH 7.2

NaCl	40.03	g
KCl	1.006	g
Na_2HPO_4	5.750	g
KH_2PO_4	1.021	g
DW	1,000	mL

The components were dissolved in 1,000 ml of DW, then adjusted to pH 7.2, and autoclaved at 121ºC for 15 min. The solution was filtered with 0.2 µm and stored at room temperature.

3. Reagent Diluent (RD)

Bovine serum albumin (BSA)	1	g
PBS (pH 7.2-7.4)	100	mL

One gram of BSA was dissolved in 100 ml of PBS (pH 7.2 - 7.4), filtered by using 0.22 µm filter paper, and stored at 4°C.

4. Stop solution (2N H_2SO_4)

20 N H_2SO_4	10	mL
Sterile DW	90	mL

Ten ml of 20N H_2SO_4 was slowly added into 90 ml DW in lamina flow hood and stored in glass bottle.

FOR WESTERN BLOT ANALYSIS

1. 10% Ammonium persulphate (APS)

APS	1	g
DDW	10	mL

One gram of APS was dissolved in 10 ml of DDW, then aliquoted into 1.5 ml eppendorf tube and stored at -20°C or prepared fresh for each run.

2. 10% Sodium dodecyl sulfate (SDS)

SDS	10	g
DDW	100	mL

Ten grams of SDS was dissolved in 100 ml of DDW and stored at room temperature.

3. 1.5 M Tris-HCl (pH 8.8)

Tris base	18.2	g
HCL (conc.)	adjust	pH
DDW	100	mL

Tris base (18.2 g) was dissolved in DDW, adjusted to pH 8.8 with HCL and adjusted to a total volume 100 ml, and stored at room temperature.

4. 0.5 M Tris-HCl (pH 6.8)

Tris base	6.05	g
HCL (conc.)	adjust	mL
DDW	100	mL

Tris base (6.05 g) was dissolved in DDW, adjusted to pH 6.8 with HCL, adjusted to a volume of 100 ml with DDW, and stored in room temperature.

5. 10X Running buffer

Tris base	30.3	g
Glycine	144	g
SDS	10	g
DDW	1,000	mL

The components above were dissolved in 1,000 ml of DDW and stored at room temperature.

6. 10X Blotting buffer: 1L

Tris base (25mM)	30.3	g
Glycine (192 mM)	144	g
DDW	1,000	mL

The components above were dissolved in 1,000 ml of DDW and stored at room temperature.

7. 1X Blotting buffer: 1L

10X blotting buffer	100	mL
Methanol	200	mL
DDW	700	mL

10X Blotting buffer was mixed with methanol, adjusted volume to1,000 ml with DDW, and stored at room temperature.

8. 5X Sample loading buffer: 10 mL

0.5 mM Tris-HCL (pH 6.8)	6.5	mL
Glycerol	3	mL
SDS (10% SDS)	1	g
Bromophenol blue	0.002	g
5% β-Mercaptoethanol	0.5	mL

The components above were mixed and aliquoted into 1.5 ml eppendorf tube. The solution was stored -20°C.

9. 10X Tris-Buffered Saline (TBS): 1L

Tris base (50mM)	60.5	g
NaCl (150mM)	88.06	mL
DDW	1,000	mL

Tris base (60.5 g) and NaCl (88.06 g) were dissolved in DDW, adjusted to pH 7.5, adjusted volume to 1,000 ml with DDW, and stored at room temperature.

10. Tris-Buffered Saline and Tween 20 (0.1% Tween 20) (TBST)

1X TBS	1,000	mL
Tween 20	1.0	mL

One ml of Tween 20 was dissolved in 1X TBS 1,000 ml, and stored at room temperature.

11. 10% Skim milk

Skim milk	10	g
TBST	100	mL

Ten grams of skim milk was dissolved in TBST 10 ml, and stored at room temperature.

VITA

Miss Wimonrat Panpetch was born on August 10, 1982 in Krabi, Thailand. She graduated with Bachelor' s degree of Science in Applied Biology from the Faculty of Science at Suan Dusit Rajabhat University in 2004 and finished her Master's degree of Science in Medical microbiology from Interdisciplinary Program of Medical Microbiology, Graduate School, Chulalongkorn University in 2009. During her study in Ph.D. program, she received the scholarship from the Royal Golden Jubilee Ph.D. Program, Thailand Research Fund (TRF).

FIELDS OF INTEREST

Molecular Bacteriology, Immunology, Cell Biology, Inflammation, Helicobacter pylori, Probiotic

PUBLICATION

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POSTER PRESENTATION

Panpetch, W., Thiraworawong, T., Tumwasorn, S. Human gastric biopsy-derived lactobacilli suppress Helicobacter pylori-induced interleukin-8 production from gastric epithelial cells in vitro. Joint Conference in Medical Science 2011: Chula-Rama-Siriraj (JCMS 2011). June 15-17, 2011. IMPACT Muang Thong Thani, Thailand.

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