# บทบาทของแลคโตบาซิลลัสสายพันธุ์ไทยในการต้านการอักเสบและการต่อต้านเฮลิโคแบคเตอร์ไพโลไร

นายเธียร ธีระวรวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# ROLE OF *LACTOBACILLUS* THAI STRAINS IN ANTI-INFLAMMATORY AND ANTAGONISTIC ACTIVITY TO *HELICOBACTER PYLORI*

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เธียร ธีระวรวงศ์ : บทบาทของแลคโตบาซิลลัสสายพันธุ์ไทยในการต้านการอักเสบและการต่อต้านเฮลิโคแบคเตอร์ไพโลไร . (ROLE OF *LACTOBACILLUS* THAI STRAINS IN ANTI-INFLAMMATORY AND ANTAGONISTIC ACTIVITY TO *HELICOBACTER PYLORI*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก รศ.ดร.สมหญิง ธัมวาสุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.นพ.ดร.เจมส์ เวอร์ซาโลวิค, รศ.พญ.ดวงพร วีระวัฒนานนท์, ดร.เจนนิเฟอร์ เสพินเลอร์ 177 หน้า.

เฮลิโคแบคเตอร์ไพโลไรเป็นสาเหตุของโรคกระเพาะอาหาร เช่น กระเพาะอาหารอักเสบ แผลเปปติก และมะเร็งกระเพาะอาหาร เพื่อหาโพรไบโอติกแบคทีเรียที่มีศักยภาพ เสริมการรักษาโรคติดเชื้อเฮลิโคแบคเตอร์ไพโลไร ได้นำแลคโตบาซิลลัสที่แยกได้จากกระเพาะอาหารของมนุษย์มาทดสอบกิจกรรมหลัก 2 อย่าง คือ การต้านการอักเสบและการยับยั้งการเจริญของเฮลิโคแบคเตอร์ไพโลไร ในด้านการต้านการอักเสบนั้นพบว่า น้ำเลี้ยงเชื้อของแลคโตบาซิลลัส 8 สายพันธุ์สามารถยับยั้งการสร้าง IL-8 โดยไม่เกี่ยวข้องกับการยับยั้งการเจริญของเฮลิโคแบคเตอร์ไพโลไร น้ำเลี้ยงเชื้อของแลคโตบาซิลลัสซาลิวาเรียส B101 (LS-B101) แลคโตบาซิลลัสแรมโนซัส B103 (LR-B103) และแลคโตบาซิลลัสแพลนทารัม XB7 (LP-XB7) สามารถกดการแสดงออกของ IL-8 mRNA ในเซลล์เยื่อบุผิวกระเพาะอาหาร AGS ที่ถูกกระตุ้นด้วยเฮลิโคแบคเตอร์ไพโลไรผ่านทางการยับยั้ง NF-κB และใน LP-XB7 มีการยับยั้ง c-Jun ด้วย ได้นำ LP-XB7 มาทดสอบการต้านการอักเสบในสัตว์ทดลองโดยใช้หนูแรท Sprague-Dawley พบว่า LP-XB7 สามารถยืดเวลาของการถูกตรวจพบและการตั้งถิ่นฐานในกระเพาะอาหารของหนูที่ติดเชื้อเฮลิโคแบคเตอร์ไพโลไร ลดการอักเสบในกระเพาะและพยาธิสภาพของเนื้อเยื่อกระเพาะอาหาร ยิ่งไปกว่านั้น การให้ LP-XB7 มีความสัมพันธ์กับการยับยั้ง TNF-α และ CINC-1 ในซีรั่ม และการยับยั้ง CINC-1 ในเนื้อเยื่อกระเพาะของหนูแรทที่ติดเชื้อเฮลิโคแบคเตอร์ไพโลไร ผลการทดลองเหล่านี้ชี้นำว่า LP-XB7 หลั่งสารที่สามารถปรับเปลี่ยนการอักเสบระหว่างการติดเชื้อเฮลิโคแบคเตอร์ไพโลไร ในด้านการยับยั้งการเจริญของเฮลิโคแบคเตอร์ไพโลไร จากการตรวจหา 85 สายพันธุ์ของแลคโตบาซิลลัสพบว่า แลคโตบาซิลลัสซาลิวาเรียส B23 (LS-B23) และแลคโตบาซิลลัสซาลิวาเรียส B37 (LS-B37) ยับยั้งการเจริญของเฮลิโคแบคเตอร์ไพโลไรสายพันธุ์มาตรฐานโดยวิธี spot-overlay ได้นำ LS-B37 ซึ่งให้ผลดีที่สุดมาทดสอบต่อพบว่าสารที่หลั่งจาก LS-B37 สามารถทนกรด-ด่าง ทนความร้อน สามารถคงสภาพได้อย่างน้อย 3 เดือน ไม่มีความเกี่ยวข้องกับกรดแลกติก และมีขนาดประมาณ 3-10 kDa LS-B37 ยังสามารถยับยั้งการเจริญของเฮลิโคแบคเตอร์ไพโลไรสายพันธุ์ที่ดื้อยา clarithromycin และ metronidazole ที่แยกได้จากผู้ป่วย ดังนั้น แลคโตบาซิลลัสแพลนทารัม XB7 (LP-XB7) และแลคโตบาซิลลัสซาลิวาเรียส B37 (LS-B37) จึงเป็นสายพันธุ์ที่มีศักยภาพที่จะนำมาใช้ในการเสริมการรักษาโรคติดเชื้อเฮลิโคแบคเตอร์ไพโลไรต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

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THIEN THIRAWORAWONG : ROLE OF *LACTOBACILLUS* THAI STRAINS IN ANTI-INFLAMMATORY AND ANTAGONISTIC ACTIVITY TO *HELICOBACTER PYLORI*. ADVISOR : ASSOC. PROF. SOMYING TUMWASORN, Ph.D., CO-ADVISOR : PROF. JAMES VERSALOVIC, M.D., Ph.D., ASSOC. PROF. DUANGPORN WERAWATGANON, M.D., JENNIFER K. SPINLER, Ph.D., 177 pp.

*Helicobacter pylori* is the causative agent of gastric-associated disease such as gastritis, peptic ulcer, and gastric cancer. To search for probiotic bacteria with the potential adjuncts in the treatment of *H. pylori*-associated diseases, human gastric-derived *Lactobacillus* spp. were tested for the two main activities, anti-inflammatory and antagonistic activity against *H. pylori*. For anti-inflammatory activity, *Lactobacillus* conditioned media (LCM) of eight isolates showed the suppressive effect to IL-8 production which was not related to inhibition of *H. pylori* proliferation. LCM of *L. salivarius* B101 (LS-B101), *L. rhamnosus* B103 (LR-B103), and *L. plantarum* XB7 (LP-XB7) had suppressive effect to IL-8 mRNA expression in *H. pylori*-stimulated AGS cells by contributing to the inhibition of NF-κB and with the addition of c-Jun inactivation for LP-XB7. The anti-inflammatory effect of LP-XB7 was further assessed *in vivo* using a *H. pylori*-infected Sprague-Dawley rat model. Strain LP-XB7 contributed to a delay in the detection and colonization of *H. pylori* in rat stomachs, attenuated gastric inflammation, and ameliorated gastric histopathology. Additionally, the administration of LP-XB7 correlated with the suppression of TNF-α and CINC-1 in sera, and suppression of CINC-1 in the gastric mucosa of *H. pylori*-infected rats. These results suggest that LP- XB7 produces secreted factors capable of modulating inflammation during *H. pylori* infection. For antagonistic activity, screening of eighty-five isolates revealed that *L. salivarius* B23 (LS-B23) and B37 (LS-B37) showed positive result to *H. pylori* reference strains by spot-overlay method. LS-B37 was chosen for further characterization due to its strongest inhibition. Secreted antagonistic substance of LS-B37 was found to be, acid-alkali tolerant, heat-stable, stable over 3 months, not related to lactic acid, and approximately 3-10 kDa. LS-B37 also inhibited the growth of clarithromycin- and metronidazole-resistant *H. pylori* clinical isolates. Therefore, *L. plantarum* (LP-XB7) and *L. salivarius* (LS-B37) show promise as an adjunctive therapy for treating *H. pylori*-associated disease.

Field of Study : Medical Microbiology Student's Signature .....
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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| AP-1 | Activator protein 1 |
| ATCC | American Type Culture Collection |
| BabA | Blood group antigen-binding adhesin |
| BCA | Bicinchoninic acid |
| BHI | Brain heart infusion |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| °C | Degree Celsius |
| Cag | Cytotoxin-associated gene |
| CFU | Colony-forming unit |
| CINC-1 | Cytokine-induced neutrophil chemoattractant-1 |
| cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| DDW | Double-distilled water |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ERK | Extracellular signal-regulated kinase |
| g | Gram |
| G+C content | Guanine+cytosine content |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GERD | Gastroesophageal reflux disease |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| GRAS | Generally recognized as safe |
| GRO | Growth-regulated oncogene |
| h | Hour |
| $H_2$ | Hydrogen |
| H&E | Hematoxylin and eosin |
| HCl | Hydrochloric acid |
| HRP | Horseradish peroxidase |
| *iceA* | Induced-by-contact-with-epithelium gene A |

| | |
|---|---|
| IFN-γ | Interferon-γ |
| IL-1β | Interleukin-1β |
| IL-2 | Interleukin-2 |
| IL-4 | Interleukin-4 |
| IL-5 | Interleukin-5 |
| IL-6 | Interleukin-6 |
| IL-7 | Interleukin-7 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IL-12 (p70) | Interleukin-12 (p70) |
| IL-13 | Interleukin-13 |
| JNK | C-terminal Jun kinase |
| kb | Kilobase |
| KC | Keratinocyte-derived cytokine |
| kDa | kilodalton |
| LCM | *Lactobacillus* conditioned media |
| LDM | *Lactobacillus* defined media |
| LPS | Lipopolysaccharide |
| µg | Microgram |
| µL | Microliter |
| µm | Micrometer |
| M | Molar |
| MALT | Mucosa-associated lymphoid tissue |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocyte chemoattractant protein-1 |
| mg | Milligram |
| min | Minute |
| MIP-2 | Macrophage-inflammatory protein-2 |
| mL | Milliliter |
| mm | Millimeter |
| MRS | deMan Rogosa Sharpe |
| MTT | Thiazolyl blue tetrazolium bromide |
| $N_2$ | Nitrogen |
| NaCl | Sodium chloride |

| | |
|---|---|
| NaOH | Sodium hydroxide |
| NFAT | Nuclear factor of activated T-cells |
| NF-κB | Nuclear factor κB |
| nM | Nanomolar |
| $O_2$ | Oxygen |
| OipA | Outer inflammatory protein |
| OMP | Outer membrane protein |
| PAI | Pathogenicity island |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pI | Isoelectric point |
| PPI | Proton pump inhibitor |
| PVDF | Polyvinylidene difluoride |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute |
| s | Second |
| SabA | Sialic acid-binding adhesin |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SodB | Superoxide dismutase |
| T4SS | Type 4 secretion system |
| Th1 cells | T-helper 1 cells |
| TLR | Toll-like receptor |
| TNF-α | Tumor necrosis factor-α |
| TTC | Triphenyltetrazolium chloride |
| v/v | Volume/volume |
| WHO | World Health Organization |
| w/v | Weight/volume |

# CHAPTER I

## INTRODUCTION

*Helicobacter pylori* is the important pathogen that infects the stomach of humans throughout the world. This pathogen is the main cause of gastritis, peptic ulceration, and gastric adenocarcinoma. Also, gastric adenocarcinoma is the second highest cause of cancer deaths worldwide due to a combination of high incidence rate, aggressive disease course, and lack of effective treatment (1). The colonization of *H. pylori* usually occurs in childhood, but in the absence of appropriate therapy, infection can persist lifelong (2). *H. pylori* persistence is the most important step of pathogenesis. After many years of infection and inflammation, ulcers can take place mainly in mid- or late-adulthood. Eventually, after an even longer period of chronic inflammation and epithelial damage, gastric adenocarcinoma occurs in the late adulthood (3).

*H. pylori* colonization is known to induce several cytokines regulating accumulation and cellular activation of neutrophils and mononuclear cells (4-10). Among the cytokines caused by *H. pylori* in gastric epithelium, interleukin-8 (IL-8) plays a major role in the mucosal inflammation. The presence of chronic inflammation is associated with gastric cancer that was well described in many studies (11-13). IL-8 is known as a potent neutrophil chemotactic and activating agent (14-18). A variety of cell types such as monocytes, fibroblasts, endothelial and epithelial cells can produce IL-8 (16-18). There is the evidence showing that *H. pylori*-infected and gastric cancer patients have increasing levels of IL-8 in gastric epithelium of both corpus and antrum (19). Some studies demonstrated the stimulation of IL-8 production by *H. pylori* in gastric epithelial cell lines *in vitro* (20, 21). These evidences suggest that IL-8 is an important cytokine induced by *H. pylori*. There are several *H. pylori* factors that induce IL-8 production. Induction of IL-8 production by *H. pylori* strains is associated with the presence of *cag* pathogenicity island (PAI), especially the *cagA* gene (22, 23). Besides *cagA* gene, *cagE* had also been demonstrated in induction of IL-8 secretion of gastric epithelial cell line (24). Although the type 4 secretion system (T4SS) of *H. pylori* can function as molecular syringe to inject CagA into gastric epithelial cells, the soluble components of the bacterial peptidoglycan cell wall known as muramyl dipeptides can also enter the

cells leading to IL-8 production (25). Specific part of *H. pylori* outer membrane protein (OMP) played a role in induction of gastric epithelial cell lines to produce IL-8, and then was named outer inflammatory protein (OipA) (26). Lipopolysaccharide (LPS) of *H. pylori* has been shown to induce IL-8 in a gastric epithelial cell line and it was thought that this IL-8 induction effect might come from a lipophilic portion of LPS, known as lipid A (27). More recently, the well-known virulence factor of *H. pylori*, VacA, has been demonstrated to induce IL-8 production in variety of cell types, including monocyte (28) and eosinophil (29). These findings suggest the networks of immunopathogenesis mediated by IL-8. Several lines of evidence demonstrated that major transcription factors responsible for IL-8 activation by *H. pylori* involved nuclear factor kB (NF-κB) and/or activator protein 1 (AP-1) (28, 30-32). Other signaling factors associated with IL-8 activation are p38 mitogen-activated protein kinase (MAPK) (28), extracellular signal-regulated kinase (ERK) (28, 33), and C-terminal Jun kinase (JNK) (30, 33).

Treatment of choice to eradicate *H. pylori* infection usually consists of 2 antibiotics and a proton pump inhibitor (PPI) and the most successful regimens have achieved eradication rate of 75-90% (34). However, the cure rates following standard therapies are reducing because of antibiotics resistance and poor compliance due to side effects of the medications (35). Probiotics have been proposed as a useful adjunctive therapy to improve either side effect or the rates of eradication (3). A probiotic is defined as a live microorganisms, which when consumed in adequate amounts, confers a health effect on the host (36, 37). The most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* species (38).

Probiotics could modify the immunologic response of the host by interacting with epithelial cells and modulating the secretion of inflammatory cytokines. This interaction would lead to reduction of gastric activity and inflammation (39). In the previous study of probiotic lactobacilli, *L. salivarius* showed the inhibition of the *H. pylori*-stimulated production of IL-8 by gastric epithelial cell *in vitro*. In addition, *H. pylori* could not colonize the stomach of *L. salivarius*-implanted mice *in vivo* (40). Another *in vivo* study demonstrated the ability of *L. johnsonii* La1 to attenuate *H. pylori*-associated gastritis in mice (41). This *L. johnsonii* La1 could also reduce levels of proinflammatory chemokines such as macrophage-inflammatory protein-2 (MIP-2),

chemokine in inflammatory states and a potent murine neutrophil chemoattractants (42). Viable *L. gasseri* OLL2716 showed suppression of *H. pylori*-induced IL-8 production *in vitro*, and also in human gastric mucosa (43). Recently, *L. bulgaricus* has been demonstrated for its ability to inhibit *H pylori* SS1-LPS-induced IL-8 production by inhibition of the Toll-like receptor (TLR) 4 pathway *in vitro* (44). These studies support the possibility of using lactobacilli to modulate *H. pylori*-induced IL-8 production.

On the other hand, probiotics can inhibit the growth of *H. pylori* by secreting antimicrobial substances. Some strains of lactobacilli synthesize antimicrobial compounds related to the bacteriocin family (45, 46). In the previous study of antagonistic effect to *H. pylori*, the antibacterial substance secreted by *L. acidophilus* could probably be lactic acid (47). In the *in vivo* study of gnotobiotic murine model, the antagonistic compound that mediated suppression of *H. pylori* growth is also lactic acid which is secreted from high acid-producing *L. acidophilus* strain (48). However, some authors demonstrated that the antibacterial substance secreted by *L. acidophilus* was independent on pH and lactic acid levels *in vitro* (49). The involvement of lactic acid-independent compounds in inhibitory effect to *H. pylori* has also been reported by several studies (50, 51). However, the exact nature of antimicrobial agents secreted by these strains remains to be clarified. Other known substance that was secreted against *H. pylori* includes hydrogen peroxide (52). The antagonistic effects to *H. pylori* of certain probiotic *L. acidophilus*, garcinol, Protykin and clarithromycin were compared, and the result suggested the possibility of supplementation of probiotic *Lactobacillus* capsules to eradicate *H. pylori* from the stomach (53). However, in this case, the antagonistic substance secreted by *Lactobacillus* was still not identified.

Taken together, these aforementioned findings support the promising of using lactobacilli as probiotic agents against *H. pylori*. Therefore, in the present study, human gastric-derived *Lactobacillus* Thai isolates were tested for probiotic properties that either suppress host inflammatory responses or inhibit the growth of *H. pylori*. The advantage of this study was that gastric-derived *Lactobacillus* has adapted by natural selection to live in the stomach microenvironment. These lactobacilli were thus suitable for using as candidate probiotics in adjunctive therapy of *H. pylori*-associated diseases.

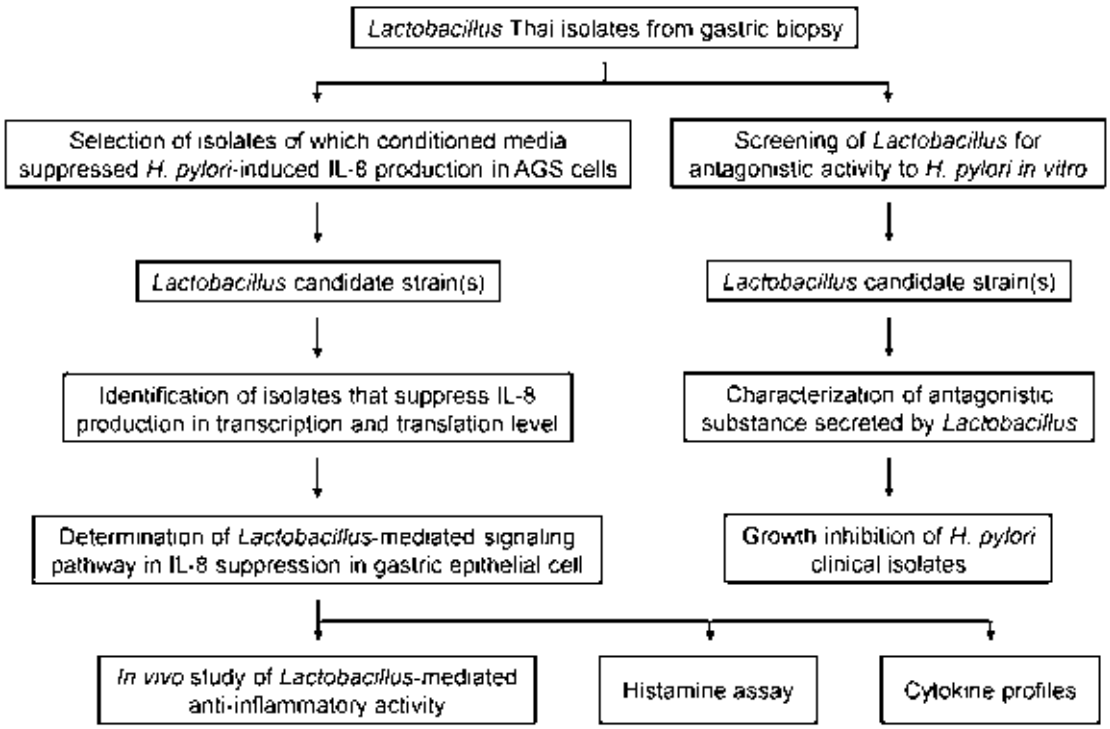
**Hypotheses**

- Specific strains of *Lactobacillus* Thai isolates from gastric biopsy modulate signaling pathway involved in the suppression of IL-8 production by gastric epithelial cells.
- Specific strains of *Lactobacillus* Thai isolates from gastric biopsy exhibit anti-inflammatory activity *in vivo*.
- Specific strains of *Lactobacillus* Thai isolates from gastric biopsy confer antagonistic activity to *H. pylori* growth.

**Objectives**

- Identify *Lactobacillus* Thai isolates that suppress *H. pylori*-induced IL-8 production in gastric epithelial cells
- Explore *Lactobacillus*-mediated signaling pathway involved in IL-8 suppression in gastric epithelial cells
- Determine *Lactobacillus*-mediated anti-inflammatory activity *in vivo*
- Search for *Lactobacillus* Thai isolates from gastric biopsy that inhibit *H. pylori* growth
- Characterize the antagonistic substance secreted by *Lactobacillus* that inhibits *H. pylori* growth

**Conceptual framework**




**Outcomes and applications**

- This study provides scientific evidences for the alternative therapeutic tool by human gastric-derived *Lactobacillus* Thai strains, and offers suitable strains of probiotic for further study in the treatment of *H. pylori* infection.
- The knowledge based on this finding elucidates some parts of the mechanism of *Lactobacillus*-mediated probiosis in *H. pylori*-infected disease.

# CHAPTER II

# LITERATURE REVIEWS

## History of *H. pylori*

During the late of 19th and early of 20th centuries, several lines of evidence demonstrated about the spiral microorganisms presence in the animal stomachs. Shortly after those times, similar spiral bacteria were detected in humans, and some of them showed peptic ulcer disease or gastric cancer. At that time, the etiological role of these bacteria was considered for the development of peptic ulcer disease and gastric cancer (54). The bacteria recognized in the stomach of humans were acknowledged as bacterial overgrowth or food contaminants until the early 1980s. At this moment, Warren and Marshall demonstrated the experiments resulting to the identification with successful culture of a spiral shape bacterium in 58 of 100 patients (55-59). At the beginning, the bacterium was named "*Campylobacter*-like organism", "gastric *Campylobacter*-like organism", "*Campylobacter pyloridis*", and "*Campylobacter pylori*". However, now it was named *Helicobacter pylori* due to the fact that this microorganism is different from the genus *Campylobacter* members (60). In a short time, it is cleared that this bacterium leads to chronic active gastritis, peptic ulcer, gastric adenocarcinomas, and gastric lymphomas (61).

## Genus *Helicobacter* and Phylogeny

The genus *Helicobacter* is a part of the ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. At present, the genus *Helicobacter* contains approximately over 20 recognized species (62). All of the members of the genus *Helicobacter* are microaerophilic bacteria, and most of them are catalase and oxidase positive, while many but not all species are urease positive. *Helicobacter* species can be subdivided into two major ancestries, the gastric *Helicobacter* species and the enterohepatic (nongastric) *Helicobacter* species. Both groups exhibit organ specificity, for instance gastric helicobacters usually cannot colonize in the intestine or liver, and vice versa (63). The summary of *Helicobacter* species that are related with human disease or correlated to animal models of human *Helicobacter* infections is shown in Table 1.

**Table 1.** Characteristics of selected *Helicobacter* species (54)

| Species | Primary mammalian host | Pathology | Animal model |
|---|---|---|---|
| Gastric *Helicobacter* spp. | | | |
| *H. pylori* | Human, primate | Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma | Mouse, Mongolian gerbil, guinea pig, gnotobiotic piglet |
| *H. felis* | Cat, dog, mouse | Gastritis in natural host; may cause peptic ulcers or gastric adenocarcinoma in mouse | Mouse |
| *H. mustelae* | Ferret | Gastritis, peptic ulcer disease, gastric adenocarcinoma, MALT lymphoma | None |
| *H. acinonychis* | Cheetah, tiger, other big cats | Gastritis, peptic ulcer disease | Mouse |
| *H. heilmannii* | Human, dog, cat, monkey, cheetah, rat | Gastritis, dyspeptic symptoms, MALT lymphoma | Mouse |
| Enterohepatic *Helicobacter* spp. | | | |
| *H. hepaticus* | Mouse, other rodents | Proliferative typhlocolitis, hepatitis, hepatocellular carcinoma | None |

## Microbiology of *H. pylori*

### Genome, plasmids, and strain diversity

The genomes size of *H. pylori* is approximately 1.7 Mbp, and G+C content is 35 to 40%. The *H. pylori* 26695 genome consists of 1,587 genes, while the *H. pylori* J99 genome comprises of 1,491 genes (64-66). Two copies of the 16S, 23S, and 5S rRNA genes are included in both genomes. One or more cryptic plasmids are contained in many strains which suggest no holding of antibiotic resistance genes or virulence genes (67). There was a report about *H. pylori*-infecting bacteriophages, however, the details of characterization has not been determined (68). Genetic content of *H. pylori* is considered heterogeneous, this implying the deficiency of clonality, leading to every *H. pylori*-positive isolate containing a different strain (69).

This phenomenon of *H. pylori* in genetic heterogeneity help possible explain the adaptation to the gastric conditions of the host, and also host immune response due to infection of *H. pylori* (70).

**Morphology**

*H. pylori* is gram-negative bacterium with 2 to 4 μm in length and 0.5 to 1 μm in width. Normally, *H. pylori* is spiral-shaped, but it can become rod. Coccoid shapes can occur in culture *in vitro* for a long time or when received antibiotic treatment (71). The coccoid form cannot be *in vitro* cultured and seems to represent dead cells (71). Some report suggested that the coccoids may represent a viable, nonculturable form (72). *H. pylori* has 2 to 6 unipolar, approximately 3 μm in length of sheathed flagella (73). The flagella help *H. pylori* for motility, and allow movement in the mucus layer of gastric epithelial cells (73).

**Growth requirements**

*H. pylori* can live in microaerobic environment with optimal condition at $O_2$ levels of 2 to 5%, as well as 5 to 10%$CO_2$, and high humidity. *H. pylori* does not need $H_2$, although it is not harmful to growth. Normally, *H. pylori* can be cultured at standard microaerobic conditions of 85%$N_2$, 10%$CO_2$, and 5%$O_2$. Optimal temperature to promote growth of *H. pylori* is at 37°C. Natural habitat of *H. pylori* is the acidic microenvironment of gastric mucosa, but *H. pylori* is acknowledged as a neutralophile. *H. pylori* can survive in a brief exposure of pH <4, but growth takes place at the narrow range of pH approximately 5.5 to 8.0 with optimal condition at pH of neutral (74, 75). *H. pylori* is a fastidious microorganism and requires several growth supplementation, commonly with blood or serum. The supplements may help as sources of nutrients (76). Columbia or brucella agar is commonly used as solid media for routine isolation and culture of *H. pylori* with supplementation of horse serum or sheep blood. Primary isolation and routine culture may contain antibiotic mixtures, even if they are not required. Dent supplement comprised of vancomycin, trimethoprim, cefsoludin, and amphotericin B is often used (77). Alternative to Dent supplement is Skirrow supplement that contains vancomycin, trimethoprim,

polymyxin B, and amphotericin B (78). Both mixtures of antibiotics are commercially available. Liquid media normally comprise of brucella, Mueller-Hinton, or brain heart infusion (BHI) broth with supplementation of 2 to 10% calf serum or 0.2 to 1.0% β-cyclodextrins, often with addition of either Dent or Skirrow supplement. There was a report of *H. pylori* growth in chemically defined media (79), but these are not appropriate for isolation and routine growth of *H. pylori*. Most of the commercially available tissue culture media do not promote *H. pylori* growth without the supplementation of serum (80). Cultures of gastric biopsy should be checked during 3-14 days. *H. pylori* forms approximately 1 mm small, smooth, and translucent colonies (81). After many successful subculturing, *H. pylori* strains tend to adapt to the conditions used for growth in the laboratory. For *H. pylori* reference strains, cultures can grow within 1-3 days of incubation. However, after the stationary phase of culture takes place, the rate of *H. pylori* growth is decreased, the morphological change to coccoid form can occur at this point (71). Prolonged culture of *H. pylori* results in a change to the unculturable coccoid form without increasing in size of colony. Triphenyltetrazolium chloride (TTC) can be supplemented in the agar plate with the final concentration of 0.004% in order to facilitate optical detection of *H. pylori* (82). *H. pylori* colonies with the presence of TTC can be seen as dark red and golden shine due to the reduction of TTC to deep red formazan (83). Storage of *H. pylori* for the long term can be done at -80°C in brain heart infusion or brucella broth supplemented with 15-20% glycerol or 10% dimethyl sulfoxide (DMSO). Cultures less than 48 h old are used for optimal viability which spiral-shaped cells are more than 90%.

**Metabolism**

*H. pylori* lives in a narrow host as well as target organ suggesting adaptation to the natural habitat or microenvironment, the mucus layer covering the gastric epithelial cells. As a result, *H. pylori* is deficient in several of the biosynthetic pathways normally found in many enteric bacteria (64, 65, 84-86). *H. pylori* is urease, catalase, and oxidase positive, such these characteristics are often used for identification. *H. pylori* can catabolize glucose. Information from genomic and biochemical data suggests that *H. pylori* cannot catabolize other sugars (84-87).

**Respiration and oxidative stress defense**

*H. pylori* can live in microaerobic conditions. It cannot tolerate high oxygen atmosphere, although at least 2%$O_2$ is required (88). This is due to *H. pylori* utilizes oxygen as a terminal electron acceptor. *H. pylori* cannot use alternative electron acceptors, for instance nitrate or formate. However, there is a single report of *H. pylori* growth in anaerobic condition using fumarate (89). *H. pylori* can be exposed to oxidative stress produced by the active immune system of the human host. *H. pylori* expresses many components of bacterial oxidative stress resistance to defend from oxidative stress including the superoxide stress defense mediated by the iron-cofactored superoxide dismutase (SodB) (90-92), the peroxide stress defense mediated by catalase (KatA) and alkyl hydroperoxide reductase (AhpC) (93, 94).

**Nitrogen metabolism**

Two major sources of nitrogen in the gastric microenvironment are amino acids and urea. Ammonia plays an important role in nitrogen metabolism and acid resistance (75). *H. pylori* can use multiple alternative sources of ammonia (95-100). Large amounts of urease is produced by *H. pylori*, and up to 10% of the total protein content of *H. pylori* has been assessed as urease (101). Urease is a nickel-containing enzyme comprising of 12 UreA and 12 UreB subunits with molecular sizes of 27 kDa and 62 kDa, respectively (102, 103). The UreA and UreB subunits are regulated by an operon containing the *ureA* and *ureB* genes (104). In the urease gene cluster, there is a second operon downstream of the *ureAB* genes that encode the UreIEFGH proteins. The UreEFGH proteins may act as assembly of subunit and nickel incorporation in the urease active sites (105, 106). The function of UreI protein is an acid-activated urea channel (107-110).

**Metal metabolism**

Metabolism of microorganisms is associated with metals. Metals are involved in many aspects such as cofactors of enzymes, catalyzing, electron transport, redox reactions, energy metabolism, and maintaining the osmotic pressure of the cell. Balance of metal load is important otherwise it can cause delay growth and cell death

in case of metal limitation and metal overload. Therefore metal regulation plays a crucial role in living microorganism (54).

*Nickel*

*H. pylori* uses nickel as the cofactor of the essential colonization factors, urease and hydrogenase. In human serum, nickel concentration is very low (2 to 11 nM), and the intaked concentration of nickel has variation depending on the diet and food ingestion (111, 112).

*Iron*

The concentration of free iron is very low in tissues of human or animal hosts. Most iron is composition of hemoglobin or chelated by transferrin in serum or lactoferrin at mucosal layer. In the gastric mucosa, iron sources are lactoferrin. Damaged tissues can release heme compounds as well as iron from degradation of diet. The 11 proteins from *H. pylori* genome are predicted as iron transport and 2 proteins as iron storage proteins (64, 65, 84). In addition, *H. pylori* has multiple ferric iron transport systems (113).

*Copper*

Copper acts as a cofactor of several proteins related in electron transport, oxidases, and hydroxylases, and it may also be associated with the reactive oxygen species formation (114). Several proteins associated with copper transport or copper chaperones are expressed by *H. pylori*. Also, *H. pylori* regulates various proteins contributing to copper export such as CopA (HP1073) and CopA2 (HP1503) P-type ATPases (115-118), and the CrdA (HP1365) copper resistance determinant (119).

*Cobalt*

In *H. pylori*, the cobalt is used as a cofactor of the enzyme arginase that plays the key role in nitrogen metabolism (96, 120). It also modulates the immune response to *H. pylori* (121, 122).

**Cell envelope, outer membrane, and LPS**

The composition of *H. pylori* cell envelope is similar to other Gram-negative bacteria, conprising of an inner (cytoplasmic) membrane, periplasm with peptidoglycan, and an outer membrane. The outer membrane of *H. pylori* contains phospholipids and LPS, and the phospholipid moiety consists of cholesterol glucosides (123-126) that is not common in bacteria. Normally, LPS comprises of lipid A which is a core oligosaccharide and an O side chain. However, the lipid A moiety of *H. pylori* LPS exhibits low biological activity when compared with lipid A from other bacteria (127). *H. pylori* in clinical isolates produce high-molecular- weight (smooth) LPS with presenting of O antigen, while *in vitro* culture, *H. pylori* alters to rough LPS variants that are deficient of the O side chain (128, 129). The *H. pylori* O side chain can be fucosylated, and it mimics Lewis blood group antigens, Lewis x and y. This phenomenon helps contribute to molecular mimicry of host antigens and related with immune evasion (130). In addition, other blood group antigens can be mimicked by the O antigen (131). *H. pylori* exhibits LPS phase variation which is contributing to heterogeneity in the population, and *H. pylori* may use this mechanism to adapt in the gastric mucosa with various conditions (126, 132, 133).

**Prevalence and geographical distribution**

*H. pylori* prevalence reveals large geographical variations. Over 80% of the population including young ages is positive with *H. pylori* infection in developing countries (134). In industrialized countries, the *H. pylori* prevalence is usually under 40%, and shows lower infection rate in children and adolescents than in adults and old persons (135). Among the geographical areas, the *H. pylori* prevalence inversely correlates with socioeconomic conditions, especially related with the living circumstance in the childhood period (136). The *H. pylori* prevalence is frequently

higher between first- and second-generation immigrants from the developing nation into the Western countries (137, 138). In developing countries, *H. pylori* prevalence shows relatively constant, while in the industrialized world, it decreases in rate of *H. pylori* infection (139). This phenomenon can explain due to improvement of hygiene and sanitation which reduced chances of childhood infection, and the elimination of *H. pylori* by antimicrobial treatment. Rate of *H. pylori* infection increases rapidly in the first 5 years of people in developing countries, after that it still remains continuously high suggesting that acquisition of *H. pylori* is occurred early in childhood (140). In contrast, infection rate of *H. pylori* is low in early childhood and slowly rises with increasing age in industrialized countries. Among adults in the Western countries, the incidence of new *H. pylori* infections is under 0.5% per year, the higher *H. pylori* prevalence in the old age indicates a birth cohort effect with higher rate of infection in the past (141, 142). Improvement of hygiene, housing environment, and *H. pylori* elimination from the people results to lower of rate of infection in children (143, 144).

**Transmission and sources of infection**

Host range of *H. pylori* is narrow. *H. pylori* is found specifically in humans and some nonhuman primates. Rarely, *H. pylori* may be isolated from pet animals, therefore, presenting of pets can become a risk factor for infection of *H. pylori* (145-148). However, transmission of *H. pylori* as a zoonosis has not been concluded (149). Infection and transmission of H. pylori is considered as a subsequence of direct contact of human-to-human by oral-oral or fecal-oral route or both ways. Several studies demonstrated detection of *H. pylori* in saliva, vomitus, gastric refluxate, and feces (150-156), however, conclusive evidence of transmission by these courses is still missing. It may become clear for missing the evidences due to most research of *H. pylori* transmission has focused on adults. There was no conclusive data 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 (157). Thus, as a general knowledge, acquisition of *H. pylori* takes place in early childhood which is from members in close family (158-163). An uncertain risk factor for *H. pylori* transmission has been reported for premastication of food by the parent (164). Positive associated with prevalence of *H.*

*pylori* has been informed for crowding of childhood in and outside the family (165, 166), while crowding of adults are less important (163, 167, 168). Several investigators demonstrated the detection of *H. pylori* DNA in sources of environmental water (169-171), but this may be due to contamination of naked DNA or dead *H. pylori*. *H. pylori* has been reported for successful culture from water, but this involved in wastewater and may contain fecal contamination in the source of water (172). Spread of fecal contaminants for *H. pylori* transmission is promoted by infection of *H. pylori* within institutional young members for the period of gastroenteritis (173). Contaminated food is considered for other possible sources of transmission due to *H. pylori* may stay alive temporarily in refrigerated food (174).

### *H. pylori* associated diseases

Gastric colonization of *H. pylori* induces gastritis in all infected persons, but only a small groups progress to obvious clinical signs. *H. pylori* positive patients have been estimated that there is lifetime risk approximately 10-20% to develop ulcer disease, and 1-2% risk of progress to distal gastric cancer (61, 175, 176). The risk of *H. pylori* infection to develop gastric associated diseases depends on a variety of bacterial virulence, host, and environmental factors which may reflect the pattern and severity of gastritis (Figure 1).

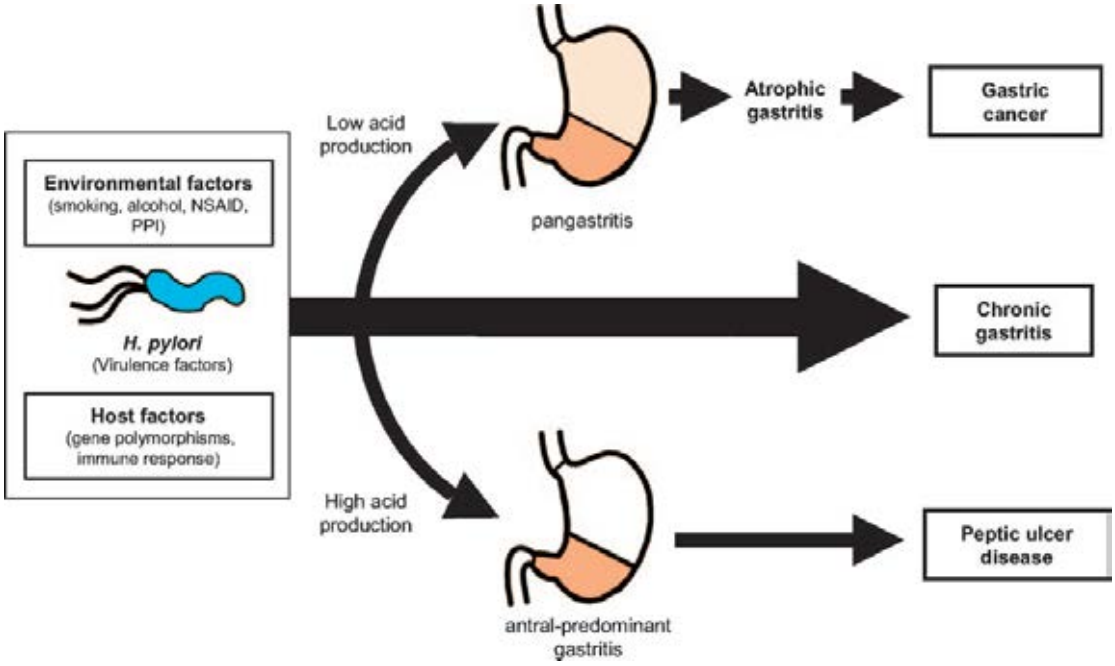



**Figure 1.** Factors associated with gastric pathology and disease outcome in infection of *H. pylori* (54).

### Acute and chronic gastritis

*H. pylori* colonization results to infiltration of neutrophilic and mononuclear cells into gastric mucosa both antrum and corpus. This is the primary step associated with colonization of *H. pylori*, and other *H. pylori* related diseases due to chronic inflammatory course. Several lines of evidence have reported about acute phase of infection as a result of subjects ingested *H. pylori* deliberately or inadvertently (2, 57, 177, 178). *H. pylori* infection was demonstrated in human challenge model which permitted controlled studies of acute phase with intentional *H. pylori* infection in healthy volunteers by well-studied laboratory *H. pylori* strain (179). Also, this report revealed that *H. pylori* colonization with acute phase may be connected with transient nonspecific dyspeptic symptoms, including fullness, nausea, and vomiting, in addition with inflammation of both the proximal and distal gastric mucosa, or pangastritis. This phase is frequently related with hypochlorhydria. It is still missing the evidence for this colonization that can be proceeded with spontaneous clearance or resolving of gastritis. There were a follow up studies of young children using serology or breath tests for detection of infection indicated that

*H. pylori* infection may spontaneously dissolve in some patients among this young age group (180-182). Persistence of *H. pylori* colonization indicated a close correlation between the acid secretion level and the gastritis distribution (Figure 2). The correlation comes from the results of effects of acid on *H. pylori* growth and association of mucosal inflammation with secretion of acid and regulation. The outcomes of *H. pylori* infection can be determined from this interaction. *H. pylori* colonizes the antrum part of stomach in the patients with intact acid secretion, while few parietal cells, acid-secretory, are present. Patients with impairing of acid secretion have a distribution of *H. pylori* in corpus and antrum. In addition, *H. pylori* in the corpus is in closer contact with gastric mucosa resulting to a corpus-predominant pangastritis (183).

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

**Figure 2.** Acid secretion and relation of gastritis pattern in *H. pylori* associated diseases (54).

**Peptic ulcer disease**

Peptic ulcers are usually consisting of gastric or duodenal ulcers which defined as mucosal damages with a diameter of at least 0.5 cm penetrating until the muscularis mucosa. Almost all gastric ulcers take place along with the lesser curvature of the stomach, particularly, between the transition of corpus and antrum

mucosa (184). Duodenal bulb is the gastric acid exposing area where duodenal ulcers mostly occur. *H. pylori* infection is associated with gastric and duodenal ulcer diseases. In the manifestation of *H. pylori* infection, duodenal ulcers occurred approximately 95%, and 85% for gastric ulcers (175). Previous study estimated for lifetime risk of ulcer disease in *H. pylori*-positive patients which is approximately 3-10 times higher than in *H. pylori*-negative patients (185).

**Non-ulcer dyspepsia**

Non-ulcer or functional dyspepsia is defined as the symptoms of upper gastrointestinal suffering without identifiable abnormality throughout diagnostic as well as upper gastrointestinal endoscopy. Symptoms of dyspepsia may exhibit a reflux-like character with heartburn, or may show dysmotility-like with satiety and nausea, or may present ulcer-like with pain and vomiting. Normally, these symptoms occurred approximately 20-40% of adult people in the Western country. Previous report showed that 30-60% of patients with functional dyspepsia associated with *H. pylori*, but the prevalence is not much distinct with the unaffected people (186). In some patients with dyspepsia, early investigation using endoscopy or *H. pylori* examination had advantage, but was not cost-effective (187).

**Atrophic gastritis, intestinal metaplasia, and gastric cancer**

*H. pylori* induced chronic inflammation which results to loss of the normal gastric structure as well as damage of gastric glands, following by substitution of fibrosis and epithelium with intestinal type. That is the process of atrophic gastritis and intestinal metaplasia which arise approximately half of the people colonized by *H. pylori* (188). Distribution and pattern of chronic inflammation causing by *H. pylori* indicates the risk for atrophic gastritis. Therefore, patients with reduction of acid secretion exhibit a more rapid progression directing to atrophy (189) (Figure 2). Loss of gland and intestinal metaplasia increase the risk for gastric cancer approximately 5-90 fold relying on the magnitude and severity of atrophy (190). Previous report revealed that *H. pylori* infection increased the risk of gastric cancer due to consequence of atrophy and metaplasia, which *H. pylori*-positive patients progress to

these phases more often than the uninfected controls (191). The risk of gastric cancer can be increased by *H. pylori* colonization approximately 10-fold, and *H. pylori* was catalog as a class I carcinogen by the World Health Organization (WHO) (192). Previous study of *H. pylori* infection in gastric cancer patients compared with controls demonstrated higher odds ratios for development of distal gastric cancer in case of *H. pylori* infection (193). This is supported by results of *in vivo* studies, *H. pylori* infection induced atrophic gastritis and gastric cancer in Mongolian gerbil model (194-196). *H. pylori* colonization with *cagA*-positive statue increased the risk of development of atrophy and gastric cancer (197, 198). *H. pylori* positive patients have lifetime gastric cancer risk approximately 1-2% in Western countries (176).

**Gastric MALT lymphoma**

Normally, the gastric mucosa does not have lymphoid tissue, but MALT can appear in the mucosa with *H. pylori* colonization. Diagnosis using histological criteria for MALT lymphoma remains controversial. Diagnosis methods include histological appearance by routine microscopy, immunohistochemistry, and PCR. Almost, MALT lymphoma patients relate with *H. pylori* positive status (199). *H. pylori* positive patients also had a significantly increasing risk for progress of gastric MALT lymphoma (200). However, due to diagnostic controversies and rarity of this illness, the exact occurrence in *H. pylori*-positive patients is still missing. In *H. pylori*-positive patients, MALT lymphomas arise approximately less than 1% (201).

**Gastroesophageal reflux disease**

*H. pylori* infection might protect from development of gastroesophageal reflux disease (GERD) which benefit to the host. This concept is slowly acknowledged due to repeated observations in GERD patients with low prevalence of *H. pylori* (202). Increasing incidence of GERD after *H. pylori* eradication has been reported in previous study (203). *H. pylori* induced corpus gastritis which reduced acid output. The hypothesis for relation of *H. pylori* and GERD arose due to acid suppression from inflammation of the gastric corpus causing by *H. pylori*, therefore, protecting the

patients from development of GERD. However, there is still missing the conclusive evidence for the incidence of *H. pylori* infection and GERD.

### Extragastric diseases

Relationship between *H. pylori* and extragastric diseases has been discovered. This topic continues as an interesting issue in the field of *H. pylori* related diseases, particularly, the role of *H. pylori* for the development of cardiovascular and hematologic diseases (204).

## Histopathology

*H. pylori* infection eventually leads to the development of gastric diseases. *H. pylori* colonization of the gastric mucosa induces an inflammatory response, particularly, Th1 (T-helper 1) predominance. The event initially starts by acute gastritis, is followed by active chronic gastritis, and it can last for life in this condition in case of lacking of treatment (188). However, persons with *H. pylori* positive status are mostly unaware of the inflammation because of no clinical signs. The inflammatory response is considered as appearing of neutrophils, mononuclear cells, and Th1 cells which function for clearance of intracellular infections. *H. pylori* is not an intracellular bacteria, therefore, response of Th1 leads to damage of epithelial cell rather than the elimination of *H. pylori*. The inflammation can promote production of reactive oxygen species which may affect to DNA damage reflecting the induction of mutation, thus, gastric cancer may occur initially in this phase (54).

## *H. pylori* virulence factors

### Cag PAI

The PAI is encoded in *cag* PAI. The *cag* PAI consists of approximately 30 genes. *H. pylori* acquired *cag* PAI from an unknown source, and it inserted into glutamate racemase gene (24). *H. pylori* strains may contain all parts of PAI (*cag*+), be deficient in whole piece (*cag*-), or lack in some elements, but the latter status is

typically similar to phenotype of *cag*- type (205). *H. pylori* strains with *cag*+, *cag*−, and intermediate status differ in many countries, but mostly *cag*+ strains are predominance, for instance *cag*+ type can be found ubiquitously in Japan and China. One of the PAI genes called *cagA* and serological response to the CagA protein has been applied for using as the *cag* PAI marker. *H. pylori* strains with possession of *cagA*+ are more usually related with peptic ulcer and gastric cancer than strains with *cagA*- type (22, 206-208). Another gene in *cag* PAI of *H. pylori* called *cagE* showed the effect for stimulation of IL-8 production which led to inflammation of gastric epithelium (24). T4SS is builded from genes encoding proteins in the *cag* PAI. Bacteria use T4SS for translocation of bacterial products directly into the cytoplasm of host cell (24). The T4SS is visualized as a sheathed rigid needle which connects *H. pylori* and epithelial cell together (209, 210). The CagA protein is injected through this needle into gastric epithelium (211-215). Consequently, phosphorylation of CagA on tyrosine residues is occurred by Src family kinases in the host cell which is stimulating phase of cell signaling pathways (216, 217). The summarization of CagA signaling and effects in epithelium is shown in Figure 3. The most obvious *in vitro* effect causing by CagA is called hummingbird phenotype which is stimulation of cell-shape change as well as elongation of the cell (211, 218). When epithelial cells are contacted by T4SS, the soluble components of the bacterial peptidoglycan cell wall called muramyl dipeptides can enter into the cell. The peptides are detected by the cell recognition system for intracellular bacteria, termed as Nod1 (25).

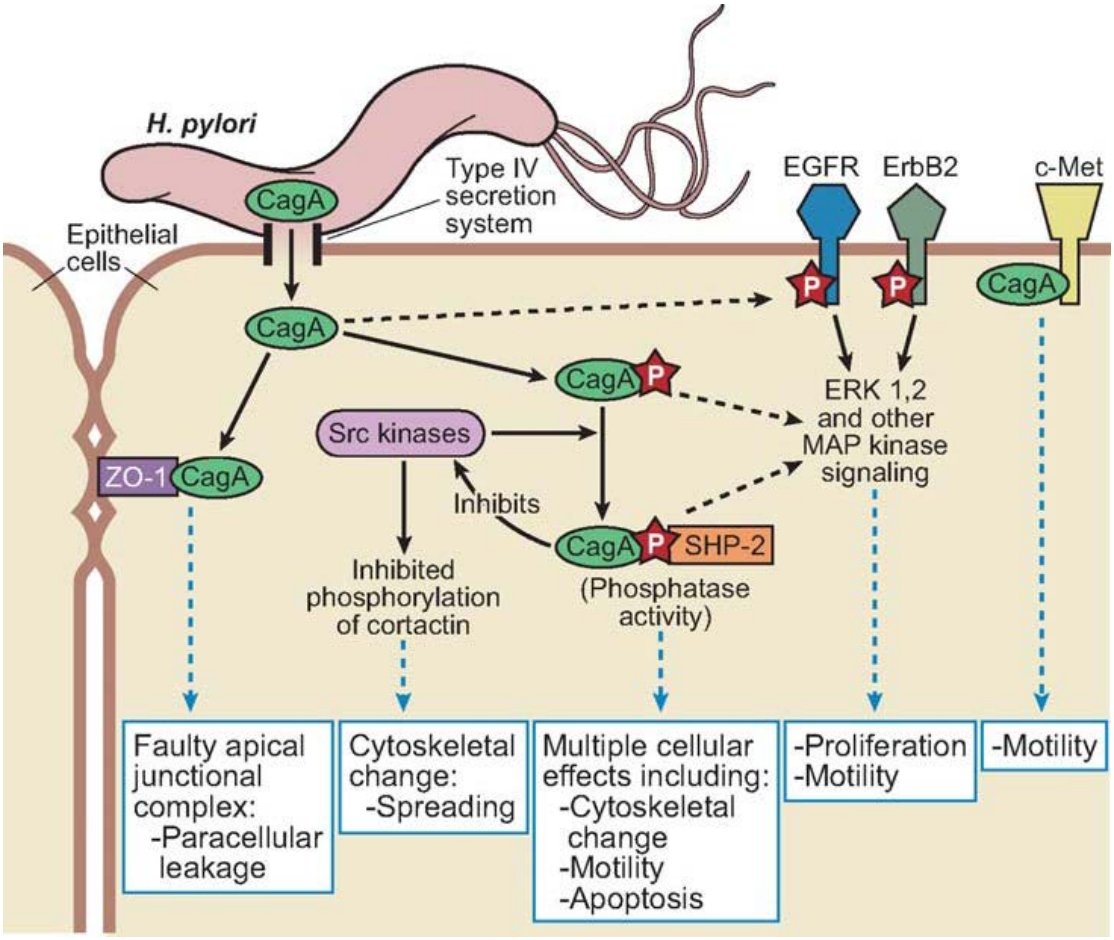



**Figure 3.** Effects and CagA signaling in epithelial cells (1). The green oval indicates the CagA protein. Red P is tyrosine phosphorylation. Dashed black line is activation where precise pathways are doubtful. Dashed blue lines and blue boxes show the effects of CagA in the epithelial cell.

### VacA

VacA is a toxin which forms a pore (219), and the action involves in vacuolation (220). Hexameric pores are formed by VacA, and it selects an anions and small neutral molecules as well as urea. This activity of urea permease may be help in acid survival by offer a substrate for urease (221). *vacA* appears in all *H. pylori* strains. *vacA* has polymorphic and variation in two regions called the mid- and signal regions (222, 223) (Figure 4). The signal region types are s1 and s2, and the mid-region types are m1 and m2. Normally, the *vacA* gene consists of any

combination of signal and mid-region types, however, the combination of s2/m1 is rare (223, 224). The *vacA* with s2 type is nontoxic in function (223, 225). The s1/m1 type of *vacA* is considered as toxic in wider range of cells than s1/m2 (225, 226). The *H. pylori* strains with s2-type of *vacA* are rarely related with disease in human studies (223, 227-229). The *H. pylori* strains both s1/m1- and s1/m2- type of *vacA* may relate to disease, but, gastric cancer patients typically have *vacA* with s1/m1-type (230-232).

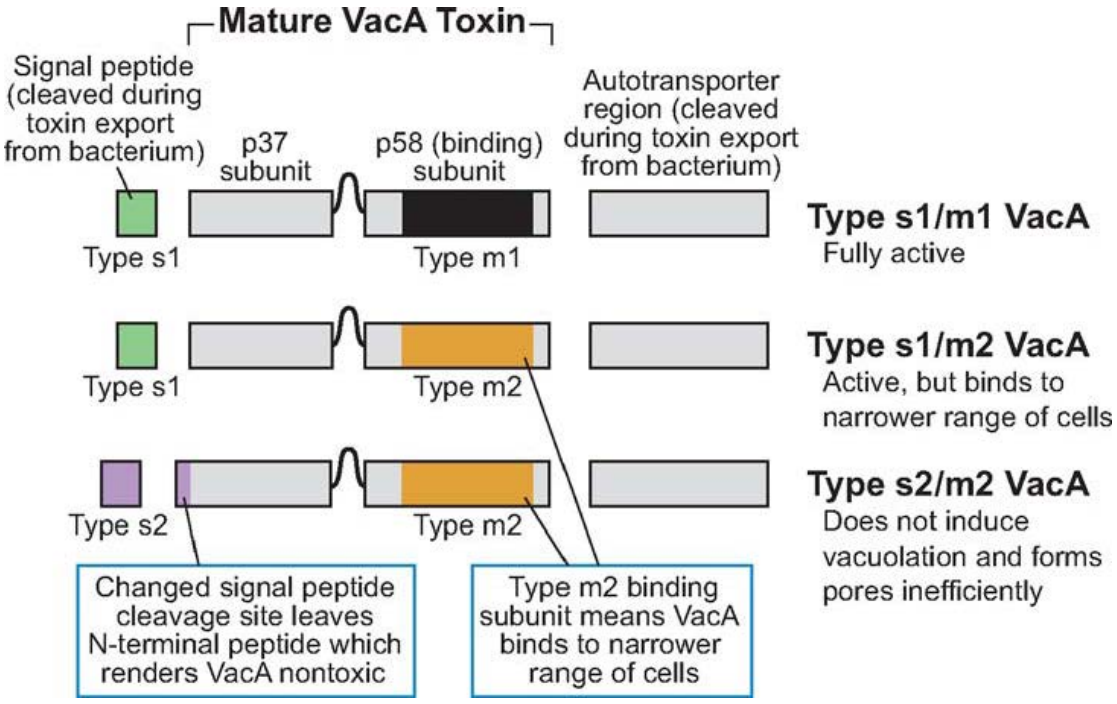



**Figure 4.** Polymorphism of VacA protein (1).

### Adhesion of *H. pylori* and blood group antigen-binding adhesin A

*H. pylori* is free-living in mucus of stomach, but a small number of *H. pylori* can adhere to gastric epithelial cells. Adhesion can be occurred by binding to blood group antigens which was expressed on the gastric epithelium. In this case, The Lewis b is the most important antigen (233). Expression of Lewis b in the stomach is related with higher density of *H. pylori*. In stomach where expression of Lewis b is not occurred, expression of other blood group antigens for instance Lewis x and

Lewis a increase density of *H. pylori* as well, but in a lesser degree (234). The transgenic mice with expression of human Lewis b result to gastritis and gastric ulcer while the wild-type mice did not, suggesting the confirmation for the importance of Lewis b and disease caused by adhesion of *H. pylori* in epithelium (235). *H. pylori* sialic-acid-binding adhesin, SabA, is used to bind with Lewis x (236). However, most study has focused on binding of Lewis b in the blood group antigen-binding adhesin (BabA) (237). *H. pylori* strains with expression of *babA2* gene, encoding of active BabA protein, are related with increasing of proliferation and inflammation in gastric epithelial cells as well as increasing of the risk for duodenal ulcer, gastric atrophy, intestinal metaplasia, and gastric cancer (238-240).

**OipA**

OipA, 34 kDa, is *H. pylori* outer membrane protein which plays a role in induction of IL-8 production of gastric epithelial cells (26). The OipA encoding gene is present in all strains of *H. pylori*. The *oipA* gene expression is regulated by phase variation through a variable number of CT dinucleotide repeating in the 5' region of the gene (26). OipA expression is strongly related with increasing of IL-8 production *in vitro* and *in vivo* (26, 241, 242).

**Induced-by-contact-with-epithelium gene A**

The induced-by-contact-with epithelium gene (*iceA1*) transcription is induced when *H. pylori* contacts with gastric epithelial cells. The presence of *iceA1* rather than a homologue called *iceA2* has been correlated with duodenal ulcer and gastric cancer (243, 244).

**Immune response**

Infection of *H. pylori* leads to induction of a Th1-polarized response which is not clearing the infection (245-247). The response to the cellular rather than the humoral immunity has been demonstrated for the role of sterilizing immunity (248-251). The development of *H. pylori* inducing gastritis or pathology is normally

acknowledged for depending to Th1 cells as well as Th1 cytokines (245, 247, 252-255). *H. pylori* showed down-regulation of inflammation and control the immune response of the host by a wide variety of virulence factors which are associated with inducing and maintaining of immune response to proinflammation. In *H. pylori*-infected mice, transfer of unfractionated splenocytes showed induction to gastritis, delayed-type hypersensitivity, and metaplasia (256).

**Interleukin-8**

In 1989, IL-8 was identified as a novel neutrophil-activating cytokine (16). IL-8 is produced by a precursor of 99 amino acids, and secreted after cleavage of 20 residues signal sequence. N-terminal extracellular processing generates several biologically active variants of the mature form. The predominant variant contains 72 amino acids, and it has a molecular weight of 8,383. It is a basic protein which has pI of 8.3 and comprises of four cysteines forming two disulfide bridges. It can torelate with plasma peptidases, heat, extreme pH, as well as other denaturing treatments. However, when reduction of disulfide bonds are occurred, it is rapidly inactivated (16). Study from nuclear magnetic resonance spectroscopy and X-ray crystallography showed the forming of IL-8 in a dimer (257). The monomer has a short, conformational flexible in N-terminal domain which is anchored by the two disulfide bonds toward a core component comprising of three antiparallel b-strands followed by a terminal a-helix. Originally, IL-8 is considered as a neutrophil-activating protein due to the basis of two *in vitro* effects which are chemotaxis and granule enzymes release (16). In the rabbit studies, intradermal injection of human IL-8 demonstrated great amount of neutrophil accumulation and plasma exudation with effect of rapid and long lasting. The infiltration of the cells was significantly after 15 min, and up to 10 h, the neutrophil immigration was still detectable (258, 259). The IL-8 mRNA expression and the biological active cytokine production were detected in endothelial cells, fibroblasts, keratinocytes, synovial cells, chondrocytes, and epithelial cells (16, 260). IL-8 gene transcription required the combination of NF-κB and AP-1 activation or NF-κB and NF-IL6 activation which depended on the cell type (261). *H. pylori* induced cell signaling of the host by activation of transcription factors resulting to IL-8 production and gastric inflammation (262). The schematic of host cell signaling stimulated by *H. pylori* is shown in Figure 5.

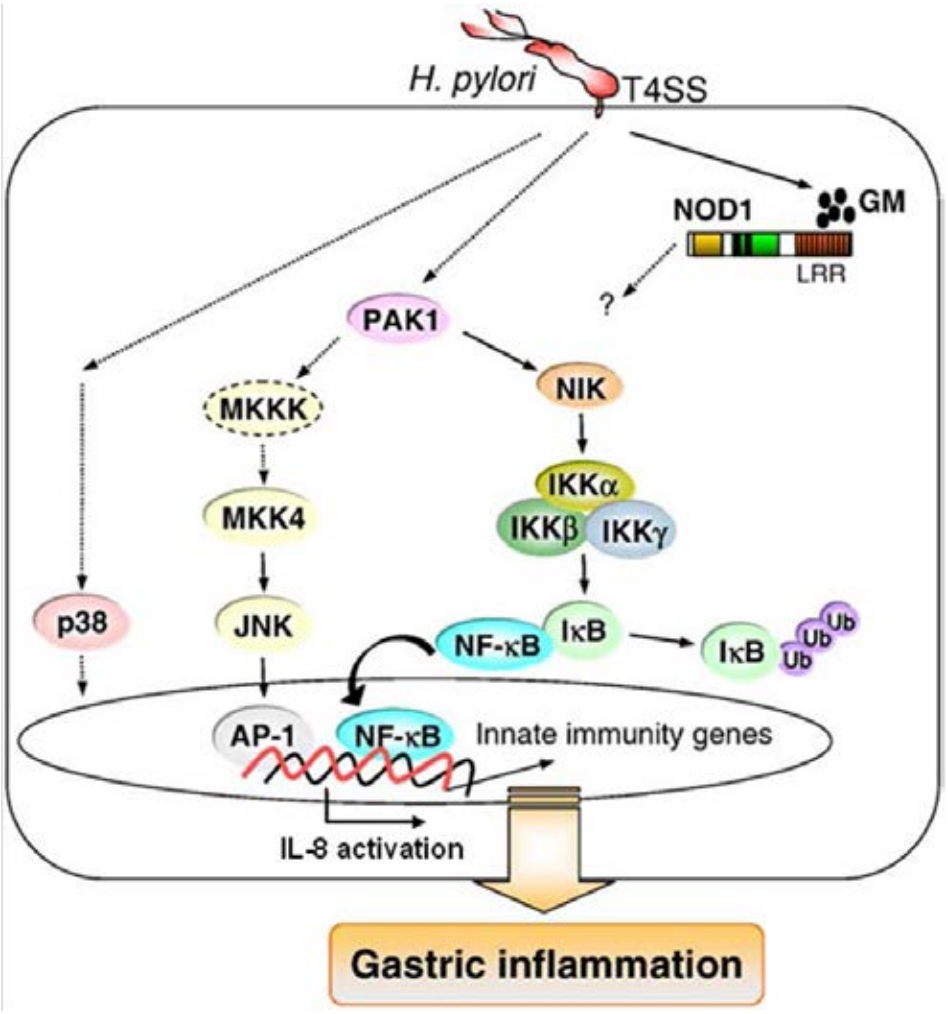



**Figure 5.** *H. pylori* induced host cell signaling resulting to gastric inflammation [modified from (262)].

**Growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1**

The growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), known as rat IL-8, is the C-X-C chemokines which is homologue in structure and function to human IL-8. It plays a crucial role for neutrophil activating and chemoattractive to inflammatory site. In the rats, GRO/CINC-1 can be used as an indication for detection of inflammation similar to IL-8 production in humans (263-265).

**Tumor necrosis factor-α**

In the patients with *H. pylori* associated gastritis, tumor necrosis factor-α (TNF-α) is secreted for responding to inflammation (266). TNF-α is a main mediator for response of the host to Gram-negative bacteria, as well as in the septic shock syndrome stimulated by LPS or bacterial superantigens (267). Interferon-gamma (IFN-γ) can enhance the TNF-α secretion in LPS-activated mononuclear phagocytes or antigen-induced T cells. TNF-α has multiple biological roles such as stimulation of adhesion molecules expression which assists the neutrophil extravasation into the lamina propria of mucosal tissue, leukocytes and T-lymphocytes activation, introduction of cytokine production by macrophages and monocytes (268, 269), and apoptosis induction (270).

**Interleukin-1β**

In human study, *H. pylori* infection can enhance significantly higher mucosal interleukin-1β (IL-1β) level in the infected patients leading to mucosal inflammation than uninfected controls (271). It also increases the level of serum *H. pylori* antibodies which is correlated with level of mucosal IL-1β. In addition, infection of *H. pylori* with CagA positive status showed increasing level of IL-1β in the patients (271). IL-1β, encoded by *IL1B* gene, is a cytokine and a member in the IL-1 family (272). IL-1 is produced by activated macrophages which respond to infection. It stimulates proliferation of thymocyte, induces synthesis of acute phase protein by hepatocytes, and introduces the fever. The gene encoded IL-1β has a region of 7.5 kb, and the coding element consists of seven exons (272).

**NF-κB**

Originally, NF-κB was considered as a transcriptional regulator of immunoglobulin kappa chain genes (273). The role of NF-κB is regulating a wide variety of cytokine genes including IL-8. NF-κB activation can take place in the cytosol of resting cells. Cytosolic NF-κB is regulated by a family of inhibitor proteins, known as IκB, which block the sequence of nuclear translocation of NF-κB family members. In the cells with proper stimulation, phosphorylation of IκBα can occur

leading to its degradation, which frees the NF-κB for translocation into the nucleus as well as activating transcription of genes consisting NF-κB enhancer components. The IκBα transcription is upregulated by NF-κB resulting to generate feedback loop. The functional NF-κB consists of a homo- or heterodimer, similar to AP-1. These dimers are composed of rel family members of proto-oncogenes, c-Rel, RelA, RelB, p50, and p52, which share a region of 300 amino acid termed the Rel-homology domain (273).

**AP-1**

AP-1 is a dimeric complex comprising of Jun polypeptides, c-Jun, JunB, and JunD, and Fos polypeptides, c-Fos, FosB, Fra-1, and Fra-2 (273). The complex can be any type of Jun homodimers, Jun heterodimers, or Jun-Fos heterodimers. The crucial role in regulating AP-1 activity is post-translational modifications of the component polypeptides. For instance, nagative regulation of DNA binding is phosphorylation of two or three C-terminal residues in the c-Jun DNA binding domain (274), while phosphorylation of two N-terminal serine residues induces transactivating potential of c-Jun (275). A possible regulatory role for the stimulus-induced expression or activation of different AP-1 component polypeptides is suggested due to the ability to express specific combinations of dimers holding different DNA binding affinities or transactivating properties. AP-1 is related with other transcription factors such as nuclear factor of activated T-cells (NFAT), and can change their ability of transactivation. The transcription factor of AP-1 has been demonstrated for regulation of *c-jun* and *c-fos* genes (276, 277).

**Animal models**

**Mouse**

The mouse is used in many studies for determination of *H. pylori* pathogenesis. Due to the immune response of mouse has been well studied, and the variety and availability of knockout mouse which is deficient in some specific parts of the immune system. Also, housing of the mouse is not expensive (278-280). Nevertheless, using of the mouse model has limitation due to infection of *H. pylori* in

mouse leads to lymphocytic gastritis without development to *H. pylori* related pathology including peptic ulcers or gastric adenocarcinoma. The stomach condition of the mouse is different from the stomach of human, thus it may lack some parts for progression of gastric pathogenesis. The stomach of the mouse is not sterile which other bacteria may have an influence on the result of *H. pylori* infection (278-280). Thus, the use of mouse may limit to determination of *H. pylori* mutant colonization properties (54).

**Rat**

Rat model of *H. pylori* infection has been developed successfully which demonstrated a mild to moderate chronic inflammation in mucosa. Healing of gastric ulcer was also delayed in the rat infected by *H. pylori* (281). The Wistar rats have been shown to develop chronic active gastritis by *H. pylori* SS1. This study of Wistar rat suggests the possibility of using in vaccine development, testing of novel therapeutic agents, and determination of pathogenesis mechanisms (282). Sprague-Dawley rat model of chronic *H. pylori* infection has been reported for development of mild to moderate mucosal inflammation in infected rat, and this study suggested the simple model of *H. pylori* infection in the rat (283). Recently, gastritis model of Wistar rats infected with *H. pylori* has been introduced for applying in the study of chemotherapeutic intervention (284).

**Mongolian gerbil**

Mongolian gerbil has been reported for colonization of *H. pylori* in its gastric mucosa (285). This model has an advantage resembling to the mouse in case of animal husbandry and size, while the disadvantage is due to outbred, deficiency of the immune system knowledge, and lacking of the tool for investigation of genetic mutation. Long-term colonization of *H. pylori* in Mongolian gerbil led to development of gastric pathology resembling to human patients. It also develops of peptic ulcers, intestinal metaplasia (286-288), and gastric cancer (194, 195, 289-292).

### Guinea pig

The advantage of the guinea pig is alike the mouse for husbandry and size of animal. The stomach of the guinea pig has common characteristic with the stomach of human including IL-8 production (293-295). However, limitation of this model is due to long-term colonization of *H. pylori* results to gastritis without development of peptic ulcer, lymphoma, and gastric cancer. Thus, the guinea pig model is limited to use in the study of immunization and vaccination of *H. pylori* (293-295), supplementation of vitamin (296), and testing of *H. pylori* adhesin mutation (297).

### Gnotobiotic piglet

Gnotobiotic piglet has a stomach similar to human in dietary habit as well as the anatomical and physiological traits (298). *H. pylori* colonization in gnotobiotic piglet develops gastritis (299), gastric ulcers and MALT lymphoma (300-302). Gnotobiotic piglet has been applied to assess antimicrobial therapies and vaccination (303, 304).

### Nonhuman primate

Nonhuman primate is most genetically related to humans. The limitation is the expensive cost of housing, and animal availability. The rhesus monkey was developed for using as animal model for *H. pylori* infection (305-308). *H. pylori* colonization in rhesus monkeys showed gastritis and a Th1 predominant immune response (309, 310). It has been reported that infection of *H. pylori* in this model leading to atrophic gastritis (311, 312).

## Diagnosis

Several methods have been developed for diagnosis of *H. pylori* infection. The methods are commonly consisting of two groups which are invasive and noninvasive tests. The invasive tests comprise of gastric biopsies for histology, culture, or other tests. The noninvasive tests comprise of peripheral specimens

including blood, breath specimens, stools, urine, or saliva for determination of antibodies, bacterial antigens, or urease activity. The usage of each method for testing in the patients bases on the basis of local experience and the clinical condition (313-315). The summary of diagnostic tests is shown in Table 2.

**Table 2.** Diagnostic test for *H. pylori* infection (54)

| Diagnostic method | Sensitivity and specificity [a] | Typical application | Remarks |
|---|---|---|---|
| Invasive methods | | | |
| Histology | >95% | "Gold standard" in routine hospital diagnostics | Requires expert pathologist; also provides histological data on inflammation and atrophy |
| Culture biopsy | >95% | Alternative gold standard | Allows for testing of antimicrobial sensitivity; requires specific microbiological expertise |
| Rapid urease (CLO) test | >90% | Cost-effective and rapid test | Requires an additional test for confirmation of *H. pylori* infection |
| Noninvasive methods | | | |
| Urea breath test | >95% | Alternative gold standard | Very useful, reliable test to evaluate success of eradication treatment of *H. pylori*; limited availability due to requirement of expensive equipment |
| Fecal antigen test | >90% | Not widely used yet | Simple test but may not be reliable for evaluation of success of eradication treatment of *H. pylori* |
| Serology | 80-90% | Mainly used for epidemiological studies | Insufficient reliability for routine screening; cannot prove ongoing infection due to immunological memory |

[a] Global range, depending on regional variations and subjects.

**Treatment**

Resistance rates of *H. pylori* to antibiotics are different in each country as well as in the same country but in different areas (316). Treatment of choice is mainly based on the resistance rates to antibiotics of *H. pylori*. Triple therapy of clarithromycin and amoxicillin with PPI is still the best choice to treat *H. pylori* infection in some countries. However, in countries with clarithromycin resistance >20% of *H. pylori*, the eradication rate may decrease. The use of bismuth-based quadruple therapy, or nonbismuth sequential or concomitant therapies may help in this resistant case. In developing countries, vaccination may be the promising tool, but it still awaits for discovery (316).

**Adjunctive therapy**

Several studies have determined the possible role of using probiotics as adjunctive therapy to gain benefit for patient of *H. pylori* infection. Recently, evaluation of probiotics in detail has been carried out (316). For instance, pretreatment for 4 weeks of *L. gasseri* demonstrated improvement of eradication rates from 69.3 to 82.6% along with standard triple therapy (317).

**Probiotic**

The main group of probiotic microorganisms belongs to *Lactobacillus* and *Bifidobacterium*. However, other bacteria and yeasts can be used as probiotics (318) (Table 3). Lactobacilli and Bifidobacteria are Gram-positive lactic acid producing bacteria which are a normal microflora in animals and humans. *Lactobacillus* is non-spore forming and rod-shaped bacteria. *Lactobacillus* requires complex nutrition as well as strictly fermentative, aerotolerant or anaerobic, and aciduric or acidophilic. *Lactobacillus* can be found in a several types of habitat, for instance human and animal mucosal membranes, on plants or material of plant origin, sewage, fermented milk products, and fermented food (318).

**Table 3.** Microorganisms used as probiotics [modified from (318)]

| Lactobacilli [a] | Bifidobacteria | Others |
|---|---|---|
| *L. acidophilus* (LA-5) | *B. longum* (BB536) | *Enterococcus faecalis* [b] |
| *L. crispatus* (*L. acidophilus* Gilliland) | *B. longum* (SP 07/3) | *Enterococcus faecium* [c] |
| *L. johnsonii* (LA1) | *B. bifidum* (MF 20/5) | *Lactococcus lactis* |
| *L. gasseri* (PA 16/8) | *B. infantis* | *Streptococcus thermophilus* |
| *L. (para)casei*, *L. casei* shirota, *L. casei* defensis | *B. animalis* (*B. animalis* subsp. *lactis* BB-12) | *Propionibacteria* |
| *L. rhamnosus* (LGG) | *B. adolescentis* | *E. coli* [c] (*E. coli* Nissle 1917) |
| *L. reuteri* (17938) | *B. breve* | *Sporolactobacillus inulinus* [c] |
| *L. plantarum* (299 and 299v) | | *Spores of Bacillus cereus* toyoi |
| | | *Saccharomyces boulardii* [d] |

[a] Commercial names of specific strains are given in brackets.

[b] Mainly used in pharmaceutical preparations.

[c] Mainly used in animal husbandry.

[d] Re-classified as a strain of *S. cerevisiae*.

### Selection criteria of probiotic

Probiotics have been selected from a wide variety of lactic acid bacteria and other microorganisms for health promoting properties to the hosts. Therefore, a number of selection criteria were set up as follow [modified from (318)].

- Safe for humans which is free from pathogenic and toxic effects
- Origin from healthy persons due to microorganisms are best adapted to the microenvironments of the gut, and known as generally recognized as safe (GRAS)

- Tolerance to gastric and bile acid plus resistance to digestive enzymes which allow the survival through the passage of stomach and intestinal tract
- Health promoting effects in the gut and systemically
- Grant a positive influence to the gut flora such as production of antimicrobial substances, vitamins, amino acids, and anti-inflammatory compounds

**Mechanisms of action of probiotic**

Probiotic bacteria have several roles of action and various influences to the host (319) (Table 4). Various microorganisms have an effect in the gut microenvironment, epithelial and mucosal barrier function, and immune system of mucosa. The immunomodulatory effects are associated with several cell types of innate and adaptive immune responses, for instance epithelial cells, dendritic cells, monocytes/macrophages, B and T lymphocytes, and NK cells.

**Table 4.** Mechanisms of action of probiotic [modified from (319)]

| |
|---|
| **Antimicrobial activity** |
| Decrease local pH |
| Secrete antimicrobial compounds |
| Inhibit bacterial invasion |
| Block bacterial adhesion to epithelial cells |
| **Enhancement of Barrier Function** |
| Increase mucus production |
| Enhance barrier integrity |
| **Immunomodulation** |
| Effects on epithelial cells |
| Effects on dendritic cells |
| Effects on monocytes/macrophage |
| Effects on lymphocytes |
| - B lymphocytes |
| - NK cells |
| - T cells |
| - T cell redistribution |

**Immunomodulation of probiotics to *H. pylori***

The inflammation from *H. pylori* infection is characterized by various chemokines and cytokines release responding for gastric inflammation. The response of cytokine is initially displayed by release of IL-8 leading to recruit of neutrophils and monocytes to the gastric mucosa (320). TNF-α, IL-1, and IL-6 were produced by activated monocytes and dendritic cells in the lamina propria (7). IL-1 and IL-6 stimulated $CD4^+$ T cells produce several cytokines as well as IL-4, IL-5, IL-6, and IFN-γ (320). However, the immune response is unable to clear the *H. pylori* infection and continues inflammation. Probiotics could modulate the immune response of the host through interaction of epithelial cells as well as anti-inflammatory cytokines secretion leading to reduction of gastric inflammation (39). In animal studies, probiotic properties of lactic acid bacteria may be played by modulation of immune regulation which controlled the balance of proinflammatory and anti-inflammatory cytokines resulting in decline of gastric inflammation (321,

322). In several animal studies, intake of probiotic demonstrated reduction of specific IgG antibodies to *H. pylori* infection (40, 48, 323). Also, intake of probiotic showed enhancement of mucosal barrier by inducing local IgA responses resulting to a stability of mucosa (324). The probiotic effects to the immune response have a wide variety of outcomes. Different probiotic strains may lead to different immune responses which rely on the immune status of the host (325).

**Antagonistic activity of probiotics to *H. pylori***

Probiotics can inhibit growth of *H. pylori* by secretion of antimicrobial substances. *Lactobacillus* can produce antagonistic substances called bacteriocin (45, 46). Other antimicrobial substances include lactic acid, acetic acids, and hydrogen peroxide, which are end products of lactic acid fermentation (52). The lactic acid produced by lactobacilli showed the inhibitory effects against *H. pylori* (48, 323, 326). Lactic acid could inhibit urease of *H. pylori*. Nevertheless, the antagonistic properties of lactobacilli against *H. pylori* vary in each strain. For instance, even if *L. johnsonii* La10 produced lactic acid as *L. johnsonii* La1, but *L. johnsonii* La10 could not inhibit *H. pylori* (50). In contrast, several studies reported that *L. acidophilus* LB, *L. casei*, *L. johnsonii* La1, and *L. lactis* demonstrated inhibitory properties against *H. pylori* by using other mechanisms which were not related with lactic acid and pH (49, 327, 328). The inhibition of *H. pylori* by proteinaceous substances has been reported in several studies (49-51, 327, 328). The antagonistic substances produced by *Lactobacillus* against *H. pylori* are summarized in Table 5.

**Table 5.** Antagonistic substances produced by *Lactobacillus* against *H. pylori*

| Antagonistic substance | Produced strain | Source | Tested pathogen | Reference |
|---|---|---|---|---|
| Lactic acid | *L. salivarius* WB1004 | Wakamoto Pharmaceutical Co., Tokyo, Japan | *H. pylori* No. 130 (*cagA*$^+$, *vac*$^+$) | (48) |
| Heat-labile substance | *L. casei* Shirota | Yakult Honsha Co., Ltd, Tokyo, Japan | *H. pylori* NCTC 11637 | (328) |
| Heat-stable substance | *L. acidophilus* LB | Human stool (Lacteol Laboratory, Houdan, France) | *H. pylori* 1011 | (49) |
| Autolysin | *L. acidophilus* CRL 639 | Centro de Referencia para Lactobacilos (CERELA) | *H. pylori* CCUG 17874 | (51) |
| Heat-stable substance | *L. johnsonii* La1 | Nestlé Research Center, Lausanne, Switzerland | *H. pylori* 69A (Cag-negative strain), *H. pylori* 1101 (Cag-positive strain) | (50) |
| Lactic acid | *L. casei* Shirota | Yakult Honsha Co., Ltd, Tokyo, Japan | *H. pylori* SS1, *H. pylori* clinical isolates | (323) |
| Extracellular secretory substance, probably lactic acid | *L. acidophilus* | Lyophilized cultures (Lactisyn; Franco Indian Pharmaceuticals, Bombay, India) | *H. pylori* clinical isolates | (47) |
| Unidentified substance | *L. acidophilus* DDS-1J | Department of Food Science and Technology, University of Nebraska, Lincoln, USA | *H. pylori* ATCC 49503 | (53) |
| Non-proteinaceous substance | *L. salivarius* UCC119 | Chicken intestine | *H. pylori* CCUG 17874 | (329) |
| Unidentified substance | *L. delbrueckii* subsp. *bulgaricus* | 'Genesis Laboratories' Ltd, Sofia, Bulgaria | *H. pylori* clinical isolates | (330) |
| Bacteriocin (bulgaricin BB18) | *L. delbrueckii* subsp. *bulgaricus* (BB18) | Bulgarian dairy products | *H. pylori* HPW26, HPP112, HPK78, HPKK160 | (331) |

# CHAPTER III

# MATERIALS AND METHODS

## Bacterial strains and culture conditions

*Lactobacillus* Thai isolates from gastric biopsies of dyspeptic patients (332) were cultured on deMan Rogosa Sharpe (MRS) agar (Oxoid, Hampshire, UK) under anaerobic conditions (10%$CO_2$, 10%$H_2$, and 80%$N_2$) at 37°C for 24 h. Eleven isolates of gastric-derived *Lactobacillus* were used for anti-inflammatory assay (Table 6). Phenotypic identification in the species level of 11 isolates was performed by using API 50 CHL (BioMérieux, France) according to the manufacturer's instructions with database version 5.1 (https://apiweb.biomerieux.com/servlet/Identify) in addition to the results of previous genotypic identification by 16S rDNA analysis (332). Eighty-five isolates of gastric-derived *Lactobacillus* spp. were used for antagonistic activity assay.

**Table 6.** Eleven isolates of gastric-derived *Lactobacillus* spp. used for anti-inflammatory assay

| Isolate | Species | % Identity [a] | Patient sex | Patient age (y) |
|---|---|---|---|---|
| B6 | *L. plantarum* | 100.0 | Female | 45 |
| B37 | *L. salivarius* | 99.5 | Male | 44 |
| B57 | *L. murinus* | 96.8 | Male | 53 |
| B60 | *L. salivarius* | 99.0 | Female | 48 |
| B70 | *L. plantarum* | 100.0 | Male | 34 |
| B74 | *L. salivarius* | 99.5 | Female | 59 |
| B90 | *L. plantarum* | 100.0 | Female | 58 |
| B101 | *L. salivarius* | 100.0 | Female | 74 |
| B103 | *L. rhamnosus* | 99.5 | Male | 67 |
| B106 | *L. casei* group | 99.0 | Male | 72 |
| XB7 | *L. plantarum* | 99.3 | Male | 60 |

[a] Nucleotide sequences were analyzed using the Ribosomal Database Project II (RDP-II; http://rdp.cme.msu.edu).

*H. pylori* reference strains ATCC 43504, ATCC 51110, ATCC 51932 (ATCC, Manassas, VA), and 13 clinical isolates with known susceptibility to clarithromycin and metronidazole were cultured on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco New Zealand Ltd, Auckland, New Zealand) and 7% (v/v) whole sheep blood (Faculty of Veterinary Medicine, Chulalongkorn University, Bangkok, Thailand) under microaerobic conditions (5%$O_2$, 10%$CO_2$, and 85%$N_2$) at 37°C for 48-72 h. *H. pylori* clinical isolates were obtained from National Gastric Cancer and *Helicobacter pylori* Research Center, Pathumthani, Thailand.

**_Lactobacillus_ conditioned media**

For anti-inflammatory assay, *Lactobacillus* conditioned media (LCM) were prepared as previously described (333). Briefly, overnight cultures of *Lactobacillus* in MRS broth was measured the optical density at 600 nm ($OD_{600}$) by using spectrophotometer (Bio-Rad Smart Spec$^{TM}$ Plus, Bio-Rad Laboratories Inc, Hercules, CA), diluted to an $OD_{600}$ of 0.1 in MRS broth, and incubated under anaerobic conditions at 37°C for 48 h. The culture supernatant was collected by centrifugation at 4000 x *g*, 4°C for 10 min, and filter sterilized through 0.22 µm surfactant-free cellulose acetate membrane filters (Minisart, Sartorius Stedim Biotech GmbH, Goettingen, Germany). The filtered conditioned media were concentrated by speed vacuum drying (Savant instruments, Farmingdale, NY), and resuspended in an equal volume of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco-Invitrogen, Carlsbad, CA) for further using in co-culture assay. The LCM were stored at -20°C until use in the experiments.

For antagonistic activity assay, LCM were prepared as above with the exception that appropriate amount of MRS broth was used to resuspend the dried conditioned media in the last step.

**Gastric epithelial cells**

The AGS human gastric adenocarcinoma epithelial cells, CRL-1739 (ATCC), were culture in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-Invitrogen) at 37°C in a humidified 5%$CO_2$ atmosphere for 48 h. The

cultured monolayer cells were detached using 0.25% (v/v) trypsin in 1 mM EDTA (Gibco-Invitrogen) at 37°C for 7-10 min. Detached cells were resuspended in fresh media. The resuspended cells were used in the co-culture assay.

**Co-culture for IL-8 production assay**

The experiment was performed as previously described (334). Briefly, The 2 x $10^4$ AGS cells suspended in 200 µL of RPMI 1640 medium were seeded in each well of 96-well flat-bottomed tissue culture plate (Nunclon D, Roskilde, Denmark), and pre-incubated at 37°C in a humidified 5%$CO_2$ atmosphere for 24 h. After pre-incubation, the culture supernatant was removed and replaced by fresh RPMI medium. The 5% (v/v) LCM were added to the cells alone or in combination with 6.0 x $10^6$ CFU/well of viable *H. pylori* ATCC 43504 (multiplicity of infection of 300). The co-culture plate was incubated at 37°C in a humidified 5%$CO_2$ atmosphere for 24 h. The culture supernatants were collected by centrifugation at 125 x *g*, 4°C for 7 min, and stored at -20°C until further use. The IL-8 level in the culture supernatant was measured by using human CXCL8/IL-8 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The experiments were performed in three independent experiments each in triplicate.

**Cell viability assay**

Cell viability test was performed in order to determine that the IL-8 level was not associated with cell death. Two methods using trypan blue dye exclusion and thiazolyl blue tetrazolium bromide (MTT) assay were done to confirm viability of cells.

For trypan blue dye exclusion assay, 10 µL of AGS cell suspensions were mixed with equal volume of 0.4% (w/v) trypan blue solution (Gibco-Invitrogen), ratio of cell:dye = 1:1, and loaded into hemocytometer. The trypan blue could not stain viable cells due to exclusion of dye while the death cells were stained. The number of total, viable, and death cells were counted under an inverted microscope. The viable cell percentage was calculated from ratio of viable cells and total cells.

For MTT assay, MTT was purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA), and the solution of 5 mg/mL of MTT in phosphate buffered saline (PBS) was prepared. After collection of culture supernatant by centrifugation as described above, 100 µL of RPMI 1640 medium was added into the wells, followed by adding of 10 µL of 5 mg/mL of MTT solution. After incubation at 37°C in 5%$CO_2$ atmosphere for 30 min, the MTT solution was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added in order to dissolve the insoluble purple formazan. The optical density was measured at a wavelength of 570 nm ($OD_{570}$) by using a microplate reader. The cell viability was calculated from constructed standard curve with known cell number.

**Inhibition of *H. pylori* proliferation**

A disc diffusion assay was performed to ensure that IL-8 suppressive effect of LCM from co-culture assay was not associated with the inhibition of *H. pylori* proliferation. The concentration of 5% (v/v) LCM used in co-culture assay and various concentrations of LCM for comparative purpose were prepared and tested by disc assay. *H. pylori* ATCC 43504 was streaked for a lawn growth on Columbia agar containing 7% (v/v) horse serum and 7% (v/v) sheep blood. Sterile 6 mm membrane disc (Whatman, Maidstone, UK) soaked in each concentration of LCM at room temperature for at least 1 h was subsequently placed on the agar, and incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo, Japan). A disc soaked with MRS broth was served as a control. The experiments were performed in three independent experiments each in duplicate.

**IL-8 mRNA expression assay**

The 5 x $10^5$ AGS cells in 500 µL of RPMI 1640 medium were seeded into each well of 24-well flat-bottomed tissue culture plate (Nunclon D), and pre-incubated at 37°C in a humidified 5%$CO_2$ atmosphere for 24 h. After pre-incubation, the culture supernatant was removed and replaced by fresh RPMI medium. The 5% (v/v) LCM were added to the cells alone or in combination with 1.5 x $10^8$ CFU/well of viable *H. pylori* ATCC 43504 (multiplicity of infection of 300). The co-culture plate

was incubated under atmosphere air containing 5%$CO_2$ at 37°C for 4 h. The culture supernatants were removed by centrifugation at 125 x *g*, 4°C for 7 min. The treated AGS cells were RNA extracted by using TRIzol® reagent (Invitrogen), and converted to cDNA with SuperScript® VILO cDNA synthesis kit (Invitrogen) (335). The RNA concentration was measured by using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). The Real-Time RT-PCR was done by using LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche, Germany) with a LightCycler® 2.0 instrument (Roche) according to the manufacturer's instructions. The forward and reverse primers consisted of IL-8 forward primer (5'-ACA CTG CGC CAA CAC AGA AAT TA-3'), IL-8 reverse primer (5'-TTT GCT TGA AGT TTC ACT GGC ATC-3'), GAPDH forward primer (5'-GCA CCG TCA AGG CTG AGA AC-3'), and GAPDH reverse primer (5'-ATG GTG GTG AAG ACG CCA GT-3') (335). The programs used for LightCycler® Carousel-Based System instrument were set according to the manufacturer's instructions with minor modification of amplification steps as following settings, analysis mode: quantification, cycles: 45, denaturation at 95°C for 10 s, annealing at 65°C for 10 s, extension at 72°C for 25 s, with acquisition mode: single. The analysis was performed by calculation of fold change of IL-8 gene expression relative to GAPDH gene expression according to the $2^{-\Delta\Delta CT}$ method (336, 337). The experiments were performed in three independent experiments each in triplicate.

**Cell signaling**

The 2 x $10^6$ AGS cells in 2 mL of RPMI 1640 medium were seeded into each well of 6-well flat-bottomed tissue culture plate (Nunclon D), and pre-incubated at 37°C in a humidified 5%$CO_2$ atmosphere for 24 h. After pre-incubation, the culture supernatant was removed and replaced by fresh RPMI medium. The 5% (v/v) LCM were added to the cells alone or in combination with 6.0 x $10^8$ CFU/well of viable *H. pylori* ATCC 43504 (multiplicity of infection of 300), and incubated under atmosphere air containing 5%$CO_2$ at 37°C for 0.5, 1, 2, or 4 h. After culture supernatant was removed by centrifugation at 125 x *g*, 4°C for 7 min, the treated cells remaining in 6-well plate were used for whole cell protein extraction using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Illinois, USA) supplemented with Halt protease inhibitor single-use cocktail EDTA-free (Pierce) and Halt phosphatase inhibitor single-use cocktail (Pierce) according to the manufacturer's instructions.

Protein concentration was determined using bicinchoninic acid (BCA) method by Pierce® BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. The mixture of 15 µg of protein sample and 6X sample buffer were heated at 95°C for 5 min before loading into the gel wells. The proteins were separated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Mini-PROTEAN® 3 Cell (Bio-Rad, Philadelphia, USA). The gel was 0.75 mm thickness, 10% resolving and 4% stacking gel. The marker was PageRuler Prestained Protein Ladder (Pierce). The gel electrophoresis was run at 100 constant volts with PowerPac™ Universal Power Supply (Bio-Rad).

After electrophoresis was completed, the protein samples in the gel were transferred onto polyvinylidene difluoride (PVDF) membrane by western blotting with Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) at constant current 400 mA for 3 h. After blotting transfer, the PVDF membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TTBS (0.1% (v/v) Tween 20, 50mM Tris, pH 7.5, 0.15 M NaCl), followed by probing of primary antibodies corresponding to phospho (p)-NF-κB p65 (Ser 536), NF-κB p65, β-actin, phospho (p)-c-Jun, and c-Jun for 1 h, washed with TTBS, and treated with secondary antibody goat anti-mouse IgG-horseradish peroxidase (HRP) for 1h. The primary antibodies for p-NF-κB p65 (Ser 536), p-c-Jun, and c-Jun were diluted at 1:400 while NF-κB p65, and β-actin were diluted at 1:800. The secondary antibody was diluted at 1:5,000. All of antibodies were purchased from Santa Cruz Biotechnology (California, USA). The chemiluminescence was carried out by using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The signal of image was detected by ChemiDoc™ XRS (Bio-Rad) and Quantity One software (Bio-Rad). The densitometric analysis was completed by ImageJ software version 1.45s. The experiments were performed in three independent experiments.

**Cytokine profiles by Luminex assay**

In order to further determine whether *Lactobacillus* secreted factors modulate cytokines other than IL-8, the culture supernatants from the same IL-8 production co-culture assay described above were screened by MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 14 Plex (Millipore, Billerica, MA) according to

manufacturer's instructions in Luminex 100 platform (Luminex Corporation, Austin, TX) with the following settings, 50 events/bead, sample size 100 µL, gate settings 8,000 to 15,000, and time out 60 s. Analytes at concentrations exceeding the minimum detectable dose were assessed, raw data were acquired by MasterPlex CT version 1.2.0.7, and analyzed by MasterPlex QT version 5.0.0.73 (Hitachi MiraiBio, San Francisco, CA). The experiments were performed in three independent experiments each in duplicate.

**Histamine assay**

*Lactobacillus* was subjected to histamine assay as previously described (338) in order to determine whether the anti-inflammatory substance secreted by gastric-derived *Lactobacillus* is histamine. Briefly, overnight cultures of *Lactobacillus* in MRS broth was measured the optical density at 600 nm ($OD_{600}$) by using spectrophotometer, diluted to an $OD_{600}$ of 0.1 in *Lactobacillus* defined media (LDM), and incubated under anaerobic conditions at 37°C for 24 h. Twenty-four-hour cultures of *Lactobacillus* in LDM were then measured the optical density at 600 nm ($OD_{600}$) by using spectrophotometer before collection of supernatants. The culture supernatant was collected by centrifugation at 4000 x *g*, 4°C for 10 min, and filter sterilized through 0.22 µm PVDF membrane filters (Millipore). The filtrated supernatants were diluted in PBS before testing of histamine. The level of histamine concentrations in the supernatant was determined by using Histamine ELISA kit (Neogen, Lexington, KY) according to the manufacturer's instructions. *L. reuteri* ATCC PTA 6475, histamine producing strain, was used as positive control. The non-IL-8 suppressing strains were also included in the experiments.

**Animal model**

The male Sprague-Dawley rats were used as previously described (283). The experimental procedures in this study were approved by the Ethical Committee for animal care and use of Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (certificate number: 03/54, research project number: 13/53). Twenty-four male Sprague-Dawley rats, five-week old, weight 150-200 g (National Laboratory

Animal Center, Mahidol University, Bangkok, Thailand) were divided into three groups, 8 rats in each group, consisting of control, *H. pylori*-infected, and *Lactobacillus*-treated groups. The rats in *H. pylori*-infected and *Lactobacillus*-treated groups were pre-treated with 5 mg/mL of streptomycin suspended in water for 3 days before inoculation of *H. pylori*. Then $10^8$-$10^{10}$ CFU/mL of viable *H. pylori* ATCC 43504 suspended in PBS was given to the rats by gavage feeding 1 mL/rat twice daily at the time interval of 4 h for 3 consecutive days. After *H. pylori* inoculation, the rats were given access to food and water *ad libitum* for 1 week. In the *Lactobacillus*-treated groups, the rats were gavaged feeding with $10^6$ CFU/mL of *Lactobacillus* candidate strain suspended in PBS in the volume of 1 mL/rat once daily for 7 days. The rats were food restricted overnight before sacrifice. All rats in each group were sacrificed for collecting the sample by intraperitoneal injection of overdose of sodium thiopental.

The rats were opened the chest cavity for cardiac puncture to collect the blood. The serum was prepared for detection of serum cytokines by allowing the blood to clot at room temperature for 2 h, centrifuged at 1000 x *g*, 4°C for 20 min. The serum was collected and stored at -20°C until cytokine assay. The stomach was removed, opened at the side of greater curvature, and washed with PBS to clean the remnant of a food. The gastric tissue (2 $mm^2$) of antrum part was used in urease test for detection of *H. pylori*. *H. pylori* produces urease to degrade urea into ammonia and $CO_2$ resulting in a conversion of color from yellow to pink within 48 h if *H. pylori* infection occurred. Another 2 $mm^2$ of gastric tissue from antrum area were grinded with micropestle in 500 µL of PBS for detection of cytokines in the tissue. The homogenous of grinded tissue was centrifuged at 1800 x *g*, 4°C for 10 min, collected supernatant, and stored at -20°C until experiments of cytokine assay. The cytokines in the sample both gastric tissue and serum were measured for the levels of CINC-1, IL-1β, and TNF-α by using Quantikine ELISA kit (R&D Systems) corresponding to rat CXCL1/CINC-1, IL-1β/IL-1F2, and TNF-α immunoassay, respectively, according to the manufacturer's instructions.

The remaining of stomach was fixed with 10% formaldehyde in 0.2 M sodium phosphate buffer pH 7.4 at room temperature, and processed with standard methods for histopathology to determine of gastric inflammation and *H. pylori* colonization. Briefly, the stomach was embedded in paraffin, sectioned, and stained with

hematoxylin and eosin (H&E). For unclear results of *H. pylori* presence, the Giemsa staining would be used. The assessment of gastric inflammation and *H. pylori* colonization were scored by a blind pathologist. The gastric inflammation was measured by using the updated Sydney System (339). Infiltration of mononuclear and polymorphonuclear leukocyte in the gastric mucosa was scored from 0 to 3 for normal, mild, moderate, and marked inflammatory changes, respectively. The *H. pylori* colonization was scored as follow; score 0, no bacteria detected; score 1, mild colonization in some gastric crypts; score 2, mild colonization in most gastric crypts; score 3, moderate colonization in all gastric crypts.

**Antagonistic assay with *H. pylori* ATCC strains**

*Lactobacillus* was tested for growth inhibition of *H. pylori* reference strains from the American Type Culture Collection (ATCC) by the two following methods.

*Spot-overlay*

Eighty-five isolates of *Lactobacillus* were screened for antagonistic activity to *H. pylori* ATCC 43504, ATCC 51110, and ATCC 51932 by spot-overlay method as previously described (340) with modification. Twenty-four hour culture of *Lactobacillus* in brain heart infusion (BHI) broth in 96-well plate was spotted on BHI agar in 140 mm petri plate by using a frogger (DAN-KAR CCRP, MA, USA), and incubated under anaerobic conditions at 37°C for 48 h. *Lactobacillus* was grown as spots and overlaid with BHI soft agar (0.75% agar) containing viable *H. pylori* supplemented with 10% (v/v) horse serum (Gibco) at the final concentration of *H. pylori* $1x10^8$ CFU/mL, and incubated under microaerobic conditions at 37°C for 72 h. The clear zone around each spot of *Lactobacillus* which had the size greater than or equal to 1 mm was interpreted as positive results. The experiments were done at least in three independent experiments each in duplicate. The *Lactobacillus* strains with positive result were confirmed for their inhibitory effects against *H. pylori* by disc diffusion method.

*Disc diffusion (LCM-disc)*

This method focused on the secreted antagonistic substance of *Lactobacillus* in the broth. The advantage of this method was the ease for further characterization of antagonistic substance. *Lactobacillus* isolates with positive result by spot-overlay method were tested for their secreted antagonistic activity against *H. pylori* ATCC 43504. Sterile 6 mm membrane disc (Whatman) was soaked in 1- and 5-fold concentrated LCM at room temperature for at least 1 h. *H. pylori* ATCC 43504 was streaked for a lawn growth on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood, then the disc was placed on the agar. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). A disc soaked with MRS broth was served as a control, including controls from *Lactobacillus* isolates with negative results by spot-overlay method. The experiments were performed in three independent experiments each in duplicate.

**Antagonistic assay with *H. pylori* clinical isolates**

After characterization of antagonistic substance (details below), *Lactobacillus* with antagonistic activity was tested with *H. pylori* clinical isolates with known susceptibility patterns of clarithromycin and metronidazole by LCM-disc method using 10-fold concentrated LCM. *H. pylori* ATCC strains were also included in this experiment, especially, *H. pylori* ATCC 43504 was served as experimental control.

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* clinical isolates or ATCC strains for a lawn growth. The 10-fold concentrated 48-h LCM of *Lactobacillus* with antagonistic activity were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). The experiments were performed in three independent experiments each in duplicate.

**Characterization of antagonistic substance**

The properties of antagonistic substance produced by *Lactobacillus* that inhibited *H. pylori* growth was tested against *H. pylori* ATCC 43504 by disc diffusion method as following aspects.

**Dose-dependent manner of inhibition**

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 48-h LCM were concentrated 10 folds and dropped in a volume of 10, 15, and 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If the antagonistic substance inhibited *H. pylori* growth in a dose-dependent manner, the zone of inhibition would increase in relation to the used volume. The experiments were performed in three independent experiments each in duplicate.

**pH sensitivity**

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 10-fold concentrated 48-h LCM with adjusted the pH of 2-11 by using HCl or NaOH were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If antagonistic substance was sensitive to the tested pH, the zone of inhibition would decrease or disappear. LCM adjusted with double-distilled water (DDW) in the same volume as HCl or NaOH were used as control. The experiments were performed in three independent experiments each in duplicate.

### Thermostability

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 10-fold concentrated 48-h LCM with heat-treated at 100°C for 15, 30, and 60 min were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If antagonistic substance was heat-labile, the zone of inhibition would decrease or disappear with increasing heat treatment. Non-heat-treated LCM were used as control. The experiments were performed in three independent experiments each in duplicate.

### Enzymatic sensitivity

The 48-h LCM were incubated with each enzyme at the final concentration of 1 mg/mL in the conditions shown in Table 7. All enzymes were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA).

**Table 7.** Enzymes and conditions used for enzymatic treatment

| Enzyme | Buffer | pH | Temperature (°C) | Incubation time (h) |
|---|---|---|---|---|
| Pepsin | 10 mM sodium citrate | 2.0 | 37 | 6 |
| Proteinase K | 50 mM Tris-HCl | 7.5 | 37 | 6 |
| Trypsin | 50 mM Tris-HCl | 7.6 | 25 | 6 |
| 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 |

After 6 h of incubation, the LCM were heat-treated at 100°C for 10 min in order to inactivate the enzymes.

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 10-fold concentrated 48-h LCM with enzymatic-treated at the conditions shown in Table 7 were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If antagonistic substance was inactivated by the enzyme, the zone of inhibition would decrease or disappear. LCM adjusted with distilled water to the same volume as treated LCM were used as control. The experiments were performed in three independent experiments each in duplicate.

**Stability**

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 10-fold concentrated 48-h LCM which were stored at -20, 4, and 25°C each for 30, 60, and 90 days were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If antagonistic substance was deteriorated in the storage conditions, the zone of inhibition would decrease or disappear. Fresh preparations of LCM were used as experimental control. The experiments were performed in three independent experiments each in duplicate.

**Relation to lactic acid**

L- or DL-lactic acid was used to test for the inhibition of *H. pylori* growth instead of LCM in order to determine whether antagonistic substance was associated with lactic acid. L- or DL-lactic acid were prepared to pH values ranging from 2 to 5 with DDW.

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which

was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. L- or DL-lactic acid with pH adjusted to 2-5 were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). If antagonistic substance was related to lactic acid at the same pH as LCM, the zone of inhibition would show up as of LCM. The DDW was used as negative control. The experiments were performed in three independent experiments each in duplicate.

**Molecular size analysis**

The 48-h LCM were subjected to size analysis of antagonistic substance by using Amicon Ultra-4 3K, 10K, and 30K (Millipore Ireland B.V., Tullagreen, Carrigtwohill, County Cork, Ireland) according to the manufacturer's instruction. Briefly, for Amicon Ultra-4 3K, 3 mL of LCM were added into the tube, centrifuged by swinging bucket rotor at 4000 x *g*, 25°C for 10 min. For Amicon Ultra-4 10K and 30K, 3 mL of LCM were added into the tubes, centrifuged by swinging bucket rotor at 4000 x *g*, 25°C for 3 min. The retentated and filtrated recovery were collected. The retentated and filtrated recovery were further concentrated to 10 fold by speed vacuum drying and tested for their antagonistic activity.

Sterile discs of 6-mm diameter were placed on Columbia agar (Oxoid) supplemented with 7% (v/v) horse serum (Gibco) and 7% (v/v) sheep blood which was previously streaked with *H. pylori* ATCC 43504 for a lawn growth. The 10-fold concentrated 48-h LCM of retentated and filtrated recovery were dropped in a volume of 20 µL into each disc. The plate was incubated under microaerobic conditions at 37°C for 72 h. The zone of inhibition around each disc was measured by a vernier caliper (Mitutoyo). According to the size of Amicon Ultra-4 used, if the zone of inhibition took place in retentated recovery meaning that the size of antagonistic substance was larger than the size of Amicon Ultra-4 used. If the zone of inhibition took place in filtrated recovery meaning that the size of antagonistic substance was smaller than the size of Amicon Ultra-4 used. This method could be used for the analysis of molecular sizes of <3 kDa, 3-10 kDa, 10-30 kDa, and >30 kDa. Non-fractionated LCM were used as control. The experiments were performed in three independent experiments each in duplicate.

**Statistical analysis**

Data were presented as mean ± standard deviation (SD). The statistical significance of *in vitro* experiments was determined by Student's *t*-test. For *in vivo* experiments, the statistical analyses were performed by using one-way ANOVA with Bonferroni post hoc test. A *P*-value<0.05 was considered statistically significant.

# CHAPTER IV

# RESULTS

## Gastric-derived *Lactobacillus* spp. suppressed *H. pylori*-induced IL-8 production in AGS cells and the suppression was not due to the inhibition of *H. pylori* proliferation

Eleven isolates of gastric-derived *Lactobacillus* spp. were chosen to test for anti-inflammatory activities (Table 6). The phenotypic identification by API 50 CHL of these eleven isolates was summarized in Appendix A. Conditioned media of these *Lactobacillus* spp., except *L. salivarius* B37, suppressed TNF-α production in LPS-stimulated THP-1 monocytoid cells but did not stimulate TNF-α production by themselves (332). Five (*L. salivarius* B60, *L. plantarum* B90, *L. rhamnosus* B103, *L. casei* group B106, and *L. plantarum* XB7) of the eleven isolates were previously reported to suppress *H. pylori*-stimulated IL-8 production in AGS gastric adenocarcinoma epithelial cells (334). Preliminary screening of the remaining six isolates (*L. plantarum* B6, *L. salivarius* B37, *L. murinus* B57, *L. plantarum* B70, *L. salivarius* B74, and *L. salivarius* B101) showed IL-8 suppression in AGS cells stimulated by *H. pylori* in one experiment.

In this study, LCM from these eleven *Lactobacillus* spp. (Table 6) were analyzed for the ability to suppress IL-8 production in *H. pylori*-stimulated AGS gastric epithelial cells. LCM from eight of eleven *Lactobacillus* isolates, *L. salivarius* B37 (LS-B37), *L. salivarius* B60 (LS-B60), *L. salivarius* B74 (LS-B74), *L. plantarum* B90 (LP-B90), *L. salivarius* B101 (LS-B101), *L. rhamnosus* B103 (LR-B103), *L. casei* group B106 (LC-B106), and *L. plantarum* XB7 (LP-XB7), showed significantly ($P$<0.05) suppressive effect to *H. pylori*-induced IL-8 production in AGS cells when compared with MRS controls (Figure 6 and Table 8). The conditioned media of these IL-8 suppressing isolates did not stimulate IL-8 production when they were co-cultured with AGS cells in the absence of *H. pylori* (Table 8). LCM from *L. plantarum* B6 (LP-B6), *L. murinus* B57 (LM-B57), and *L. plantarum* B70 (LP-B70) did not suppress IL-8 production in *H. pylori*-stimulated AGS cells when compared to controls (Figure 6 and Table 8). LCM of these eleven *Lactobacillus* isolates did not affect killing of the AGS cells due to the cell viability test by trypan blue dye exclusion and MTT assays indicated >90% of viable cells (data not shown).

In order to determine whether IL-8 suppressive effect of LCM was associated with the inhibition of *H. pylori* proliferation, 5% (v/v) LCM as used in co-culture assay, 1- and 5-fold LCM for comparative purpose were prepared and tested by disc diffusion assay. It was found that 5% (v/v) LCM used in co-culture assay did not cause the inhibition zone as shown in Table 9, suggesting that IL-8 suppressive effect was not due to the inhibition of *H. pylori* proliferation.

Taken together, these results suggested that the immunomodulatory substance secreted by *Lactobacillus* presenting in LCM suppress IL-8 production in *H. pylori*-stimulated AGS cells. The eight positive strains, LS-B37, LS-B60, LS-B74, LP-B90, LS-B101, LR-B103, LC-B106, and LP-XB7, were studied further in IL-8 transcription analysis.

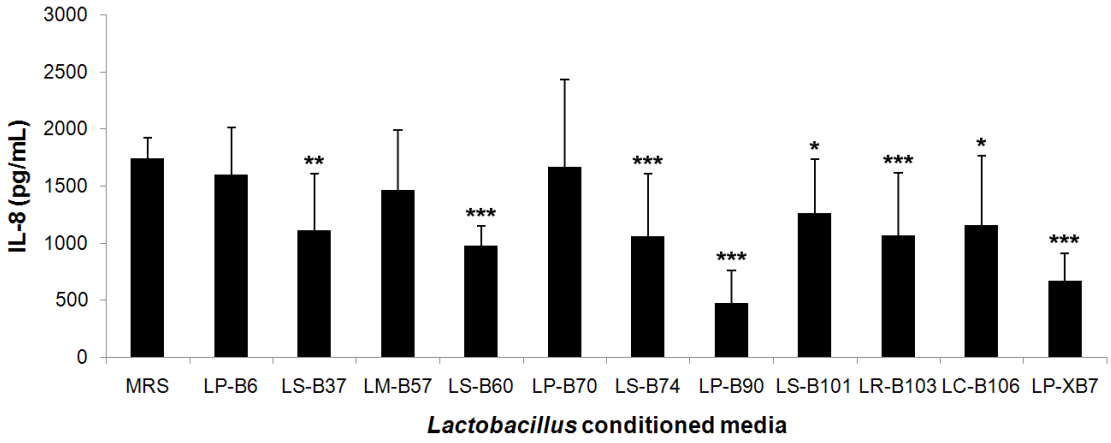



**Figure 6.** Immunomodulatory effects of LCM from 11 gastric-derived *Lactobacillus* spp. on *H. pylori*-stimulated IL-8 production by AGS cells. The results were expressed as the mean of three independent experiments each in triplicate. The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Cell viability >90% measuring by trypan blue dye exclusion and MTT assays.

**Table 8.** Immunomodulatory effects of LCM from 11 gastric-derived *Lactobacillus* spp. on IL-8 production in *H. pylori*-stimulated AGS cells

| Subject | IL-8 concentration (pg/mL) | | % IL-8 suppression | *P*-value |
|---|---|---|---|---|
| | No *H. pylori* | Plus *H. pylori* | | |
| MRS media control | 128.10 ± 24.97 | 1749.98 ± 174.11 | - | - |
| *L. plantarum* B6 | 97.01 ± 14.29 | 1603.14 ± 415.17 | 4.76 | 0.3412 |
| *L. salivarius* B37 | 90.25 ± 40.76 | 1115.78 ± 492.30 | 35.51 ** | 0.0053 |
| *L. murinus* B57 | 101.88 ± 12.60 | 1467.62 ± 524.95 | 12.81 | 0.1709 |
| *L. salivarius* B60 | 89.73 ± 13.54 | 980.42 ± 174.57 | 44.56 *** | 0.00002 |
| *L. plantarum* B70 | 109.19 ± 13.34 | 1672.04 ± 760.51 | 0.67 | 0.4846 |
| *L. salivarius* B74 | 116.53 ± 44.40 | 1061.56 ± 550.33 | 47.34 *** | 0.00006 |
| *L. plantarum* B90 | 79.01 ± 28.81 | 483.33 ± 275.32 | 71.15 *** | 0.00000001 |
| *L. salivarius* B101 | 87.78 ± 39.36 | 1267.97 ± 467.21 | 24.30 * | 0.0237 |
| *L. rhamnosus* B103 | 114.56 ± 14.26 | 1074.04 ± 545.06 | 44.51 *** | 0.0003 |
| *L. casei* group B106 | 95.05 ± 25.70 | 1165.55 ± 600.12 | 33.70 * | 0.0117 |
| *L. plantarum* XB7 | 92.42 ± 34.16 | 676.53 ± 237.59 | 53.08 *** | 0.0003 |

The results of IL-8 concentration were presented as mean ± SD. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. The experiments were performed in three independent experiments each in triplicate.

**Table 9.** Effect of various concentrations of LCM from 11 gastric-derived *Lactobacillus* spp. on the inhibition of *H. pylori* ATCC 43504 proliferation

| Subject | Zone of inhibition (mm) [a] | | |
|---|---|---|---|
| | 5% (0.05X) LCM | 1X LCM | 5X LCM |
| MRS media control | - [b] | - | 8.2 ± 0.5 [c] |
| *L. plantarum* B6 | - | - | 8.4 ± 0.6 |
| *L. salivarius* B37 | - | 6.5 ± 0.3 | 10.3 ± 0.4 |
| *L. murinus* B57 | - | - | 8.3 ± 0.5 |
| *L. salivarius* B60 | - | - | 8.6 ± 0.7 |
| *L. plantarum* B70 | - | - | 8.7 ± 0.6 |
| *L. salivarius* B74 | - | - | 8.8 ± 0.7 |
| *L. plantarum* B90 | - | - | 8.7 ± 0.7 |
| *L. salivarius* B101 | - | - | 8.4 ± 0.6 |
| *L. rhamnosus* B103 | - | - | 8.2 ± 0.4 |
| *L. casei* group B106 | - | - | 8.6 ± 0.7 |
| *L. plantarum* XB7 | - | - | 8.4 ± 0.7 |

[a] Including disc, 6 mm.

[b] No zone of inhibition.

[c] Results indicated mean ± SD of three independent experiments each in duplicate.

**Gastric-derived *Lactobacillus* spp. suppressed IL-8 mRNA expression**

To determine the effect of eight IL-8 suppressing *Lactobacillus* isolates on IL-8 transcription, quantitative real-time RT-PCR was performed. Co-culture of LCM of these isolates and *H. pylori* ATCC 43504 in AGS cells for 4 h revealed that LS-B101, LR-B103, and LP-XB7 could significantly ($P$<0.05) inhibit IL-8 mRNA expression (relative to GAPDH, down-regulated approximately 0.55 fold) in AGS cells (Figure 7). LCM from the remaining 5 isolates, LS-B37, LS-B60, LS-B74, LP-B90, and LC-B106, did not have an effect on IL-8 mRNA expression in this assay (Figure 7) and were not studied further in the signaling pathway. The three positive strains, LS-B101, LR-B103, and LP-XB7, were studied further in cell signaling analysis.

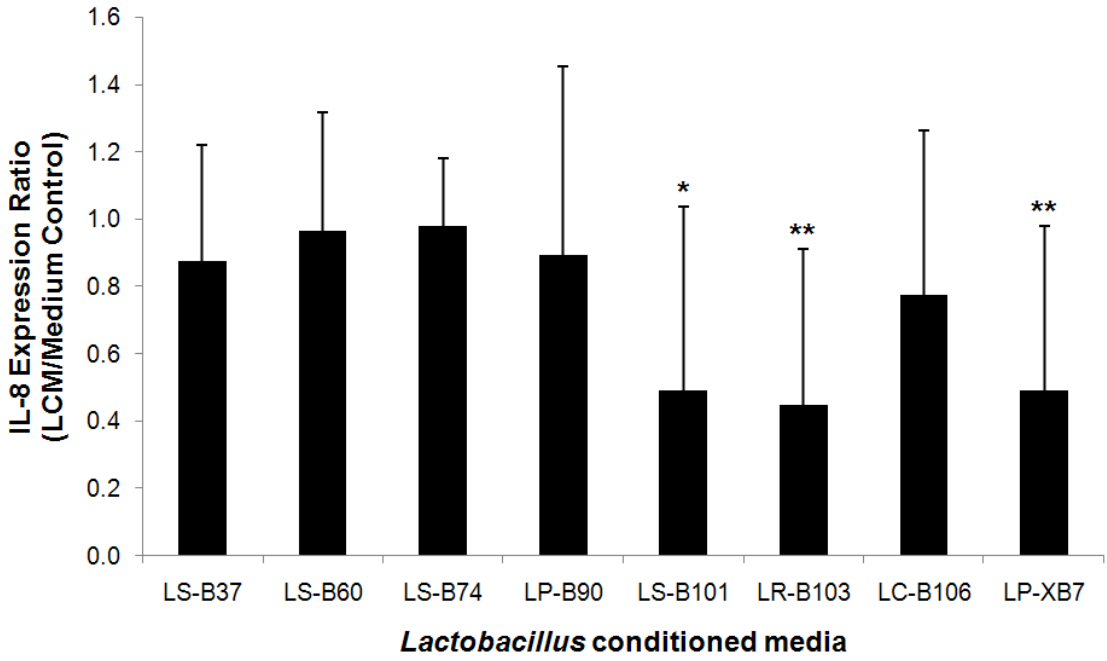



**Figure 7.** Effects of LCM from 8 anti-inflammatory gastric-derived *Lactobacillus* spp. on *H. pylori*-stimulated IL-8 mRNA expression in AGS cells. After co-incubation of LCM and *H. pylori* in AGS cells for 4 h, quantitative real-time RT-PCR analysis was performed to detect IL-8 and GAPDH mRNA levels. IL-8 mRNA levels were normalized to housekeeping gene GAPDH mRNA levels, and analyzed by $2^{-\Delta\Delta CP}$ method. The results were expressed as the mean of IL-8 expression ratio (LCM/medium control) of three independent experiments each in triplicate. The bars indicated standard deviation. An asterisk meant a statistically significant difference compared to the theoretical mean of 1.0. * $P<0.05$, ** $P<0.01$.

***L. salivarius* B101 (LS-B101) and *L. rhamnosus* B103 (LR-B103) inhibited NF-κB activation, and *L. plantarum* XB7 (LP-XB7) inhibited NF-κB and c-Jun activation**

NF-κB and AP-1 activation result in an increasing level of IL-8 gene expression (28, 30-32). To determine *Lactobacillus*-mediated signaling pathway in IL-8 suppression in gastric epithelial cells, LCM from *L. salivarius* B101 (LS-B101), *L. rhamnosus* B103 (LR-B103), and *L. plantarum* XB7 (LP-XB7) were co-cultured with *H. pylori*-stimulated AGS cells for 0.5, 1, 2, and 4 h. The effects of LCM at each time point were determined by western blot analysis using antibodies corresponding to p-NF-κB p65 (Ser 536), NF-κB p65, β-actin, p-c-Jun, and c-Jun.

Co-culture of LCM from LS-B101, LR-B103, and LP-XB7 with *H. pylori* ATCC 43504 in AGS cells for 0.5 h showed no suppressive effect of p-NF-κB and p-c-Jun (Figure 8). Co-culture for 1 h revealed that LS-B101 could suppress p-NF-κB (0.144 fold change, $P<0.05$) but not p-c-Jun (Figure 8). Co-culture for 2 h revealed that LS-B101, LR-B103, and LP-XB7 could obviously suppress p-NF-κB (0.541 fold change, $P<0.01$ for LS-B101; 0.584 fold change, $P<0.01$ for LR-B103; 0.675 fold change, $P<0.01$ for LP-XB7) but not p-c-Jun (Figure 8). Co-culture for 4 h revealed that LS-B101 and LR-B103 could suppress p-NF-κB (0.497 fold change, $P<0.05$ for LS-B101; 0.567 fold change, $P<0.05$ for LR-B103) but not p-c-Jun while LP-XB7 demonstrated the suppressive effect both p-NF-κB (0.645 fold change, $P<0.05$) and p-c-Jun (0.586 fold change, $P<0.01$) (Figure 8). Therefore, *L. plantarum* XB7 (LP-XB7) was selected for further testing of anti-inflammatory activity in Sprague-Dawley rat model due to its ability to suppress both NF-κB and c-Jun activation.

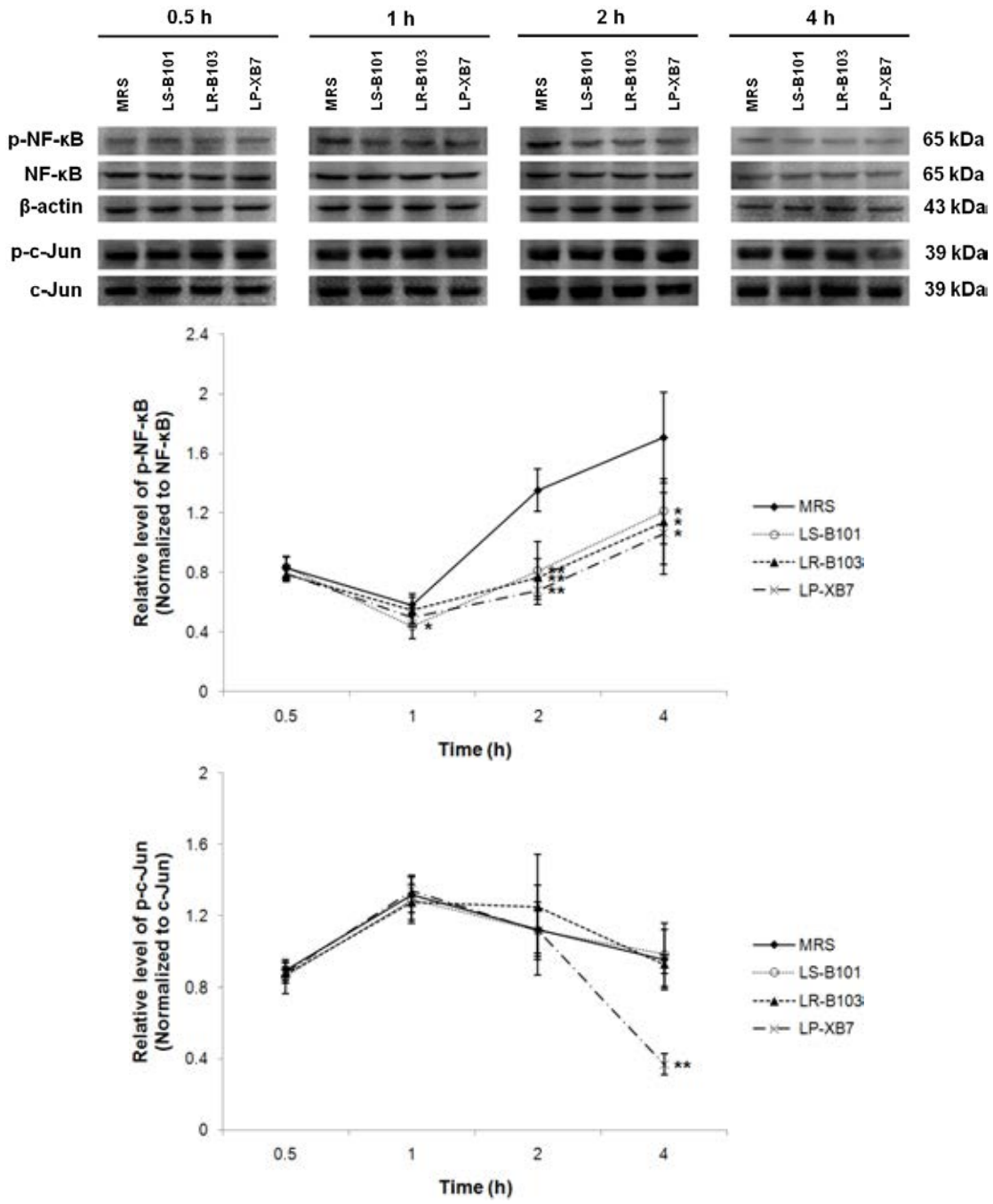



**Figure 8.** Suppressive effect of LCM on *H. pylori*-activated transcription factors in AGS cells. After co-incubation of LCM and *H. pylori* in AGS cells for 0.5, 1, 2, or 4 h, whole cell extracts were prepared and subjected to western blot analysis with antibodies corresponding to p-NF-κB p65 (Ser 536), NF-κB p65, β-actin, p-c-Jun, and c-Jun. Relative level of p-NF-κB p65 (Ser 536), NF-κB p65, p-c-Jun, and c-Jun was performed by densitometric analysis using ImageJ software version 1.45s. p-NF-κB p65 (Ser 536) levels were normalized to NF-κB p65 levels. p-c-Jun levels were normalized to c-Jun levels. The blots were representative of three independent experiments. The results of densitometric analysis were expressed as the mean. The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with control. * $P$<0.05, ** $P$<0.01.

**Cytokine profiles of anti-inflammatory gastric-derived *Lactobacillus* spp.**

LCM of cell signaling characterized strains *L. salivarius* B101 (LS-B101), *L. rhamnosus* B103 (LR-B103), and *L. plantarum* XB7 (LP-XB7) as well as *L. salivarius* B37 (LS-B37) which suppressed IL-8 production as demonstrated by co-culture assay were subjected to test for cytokines/chemokines other than IL-8 in order to determine immunomodulatory effects to other cytokines/chemokines in gastric epithelial cells. The analysis of cytokine profiles showed that LS-B37, LS-B101, LR-B103, and LP-XB7 could significantly ($P$<0.001) suppress only IL-8 production in *H. pylori*-stimulated AGS cells (Figure 9 and Table 10). Other cytokines/chemokines, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, MCP-1, and TNF-α, showed very low detectable level or below detection limit (Figure 9 and Table 10) suggesting that *H. pylori* could not stimulate these cytokines/chemokines in the AGS cells.

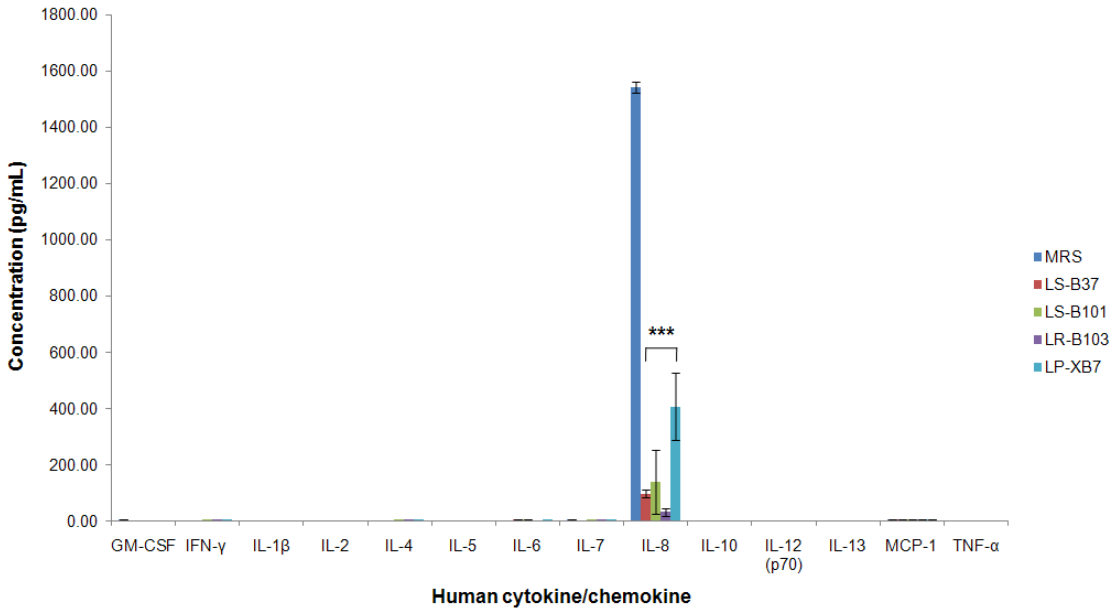



**Figure 9.** Cytokine profiles from LCM of anti-inflammatory gastric-derived *Lactobacillus* spp. in *H. pylori*-stimulated AGS cells. GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-7, interleukin-7; IL-8, interleukin-8; IL-10, interleukin-10; IL-12 (p70), interleukin-12 (p70); IL-13, interleukin-13; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α. The results were expressed as the mean of three independent experiments each in duplicate. The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with control. *** $P<0.001$.

**Table 10.** Cytokine profiles from LCM of anti-inflammatory gastric-derived *Lactobacillus* spp. in *H. pylori*-stimulated AGS cells

| Human cytokine/ chemokine | Concentration (pg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No *H. pylori* | | | | | Plus *H. pylori* | | | | |
| | MRS | B37 | B101 | B103 | XB7 | MRS | B37 | B101 | B103 | XB7 |
| GM-CSF | 1.61 ± 0.07 | 1.29 ± 0.05 | 1.34 ± 0.11 | 1.11 ± 0.05 | 1.84 ± 0.15 | 3.83 ± 0.19 | 1.56 ± 0.21 | 1.66 ± 0.41 | 1.23 ± 0.12 | 2.53 ± 0.25 |
| IFN-γ | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 |
| IL-1β | 1.50 ± 0.04 | 1.46 ± 0.03 | 1.47 ± 0.02 | 1.44 ± 0.02 | 1.46 ± 0.03 | 1.66 ± 0.04 | 1.47 ± 0.02 | 1.48 ± 0.03 | 1.45 ± 0.02 | 1.50 ± 0.04 |
| IL-2 | 1.45 ± 0.03 | 1.45 ± 0.02 | 1.47 ± 0.03 | 1.45 ± 0.04 | 1.47 ± 0.03 | 1.47 ± 0.00 | 1.47 ± 0.04 | 1.47 ± 0.03 | 1.45 ± 0.02 | 1.50 ± 0.05 |
| IL-4 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 |
| IL-5 | 1.97 ± 0.00 | 1.96 ± 0.02 | 1.96 ± 0.02 | 1.95 ± 0.01 | 1.95 ± 0.02 | 1.97 ± 0.00 | 1.95 ± 0.01 | 1.94 ± 0.02 | 1.95 ± 0.02 | 1.96 ± 0.02 |
| IL-6 | 2.71 ± 0.02 | 4.09 ± 0.20 | 3.59 ± 0.34 | 2.84 ± 0.07 | 3.57 ± 0.34 | 2.75 ± 0.01 | 3.90 ± 0.49 | 3.60 ± 0.34 | 3.12 ± 0.19 | 3.22 ± 0.08 |
| IL-7 | <3.20 | <3.20 | <3.20 | <3.20 | <3.20 | 5.30 ± 1.99 | <3.20 | <3.20 | <3.20 | <3.20 |
| IL-8 | 94.83 ± 1.47 | 32.75 ± 2.25 | 38.94 ± 11.65 | 10.03 ± 2.11 | 79.69 ± 4.88 | 1541.78 ± 21.38 | 97.06 ± 13.58 *** | 138.02 ± 113.58 *** | 31.75 ± 12.36 *** | 406.27 ± 119.99 *** |
| IL-10 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 | <1.75 |
| IL-12 (p70) | 1.44 ± 0.11 | 1.35 ± 0.12 | 1.41 ± 0.07 | 1.24 ± 0.09 | 1.18 ± 0.04 | 1.42 ± 0.15 | 1.25 ± 0.09 | 1.19 ± 0.05 | 1.19 ± 0.08 | 1.20 ± 0.00 |
| IL-13 | 2.63 ± 0.13 | 2.72 ± 0.20 | 2.96 ± 0.18 | 2.72 ± 0.16 | 2.48 ± 0.15 | 2.45 ± 0.13 | 2.54 ± 0.11 | 2.60 ± 0.09 | 2.48 ± 0.15 | 2.48 ± 0.19 |
| MCP-1 | 5.86 ± 0.02 | 5.84 ± 0.00 | 5.84 ± 0.02 | 5.83 ± 0.02 | 5.85 ± 0.01 | 5.86 ± 0.02 | 5.84 ± 0.02 | 5.84 ± 0.00 | 5.82 ± 0.02 | 5.84 ± 0.02 |
| TNF-α | 1.90 ± 0.01 | 1.99 ± 0.03 | 1.97 ± 0.04 | 1.91 ± 0.01 | 2.06 ± 0.05 | 2.07 ± 0.03 | 2.04 ± 0.06 | 2.04 ± 0.05 | 1.96 ± 0.05 | 2.07 ± 0.02 |

The results of human cytokine/chemokine concentration were presented as mean ± SD. LCM, *Lactobacillus* conditioned media; MRS, bacterial media control; SD, standard deviation; significantly different from MRS media control, *** $P<0.001$. The experiments were performed in three independent experiments each in duplicate.

**The anti-inflammatory substance secreted by gastric-derived *Lactobacillus* spp. was not associated with histamine**

Histamine derived from *L. reuteri* ATCC PTA 6475 has been reported to suppress TNF (338). In order to determine whether IL-8 suppressing strains of *Lactobacillus* spp. secreted the anti-inflammatory substance related to histamine, the IL-8 suppressing strains, *L. plantarum* B7 (LP-B7), *L. salivarius* B37 (LS-B37), *L. salivarius* B101 (LS-B101), *L. rhamnosus* B103 (LR-B103), and *L. plantarum* XB7 (LP-XB7), as well as non-IL-8 suppressing strains, *L. salivarius* B21 (LS-B21), and *L. plantarum* B67 (LP-B67), were subjected to test in histamine assay. The $OD_{600}$ determination of 24 h cultures of *Lactobacillus* spp. in LDM revealed that LR-B103 had low $OD_{600}$ value (0.95 ± 0.13) compared to the other *Lactobacillus* isolates which had $OD_{600}$ value between 2.46 ± 0.32 to 5.53 ± 0.21 (Table 11), suggesting that LR-B103 could not grow well in LDM. Thus, LR-B103 was excluded for testing in histamine ELISA due to low value of $OD_{600}$ in LDM reflecting the slow growth in this media. The results of histamine concentration in LDM revealed that all of tested isolates, LS-B101, LS-B37, LS-B21, LP-XB7, LP-B7, and LP-B67, produced histamine concentration ranging from 0.035 ± 0.007 µg/mL to 0.051 ± 0.012 µg/mL while positive control strain *L. reuteri* ATCC PTA 6475, which is histamine-producing isolate, produced histamine in a concentration of 63.342 ± 19.157 µg/mL (Table 12). In this case, when histamine concentrations of gastric-derived *Lactobacillus* spp. were compared with that of *L. reuteri* ATCC PTA 6475, it suggested that anti-inflammatory substance secreted by gastric-derived *Lactobacillus* spp. to suppress IL-8 production was not related with histamine due to low level of histamine production by gastric-derived *Lactobacillus* isolates.

**Table 11.** $OD_{600}$ of 24 h cultures of gastric-derived *Lactobacillus* spp. in LDM

| Bacterial strain | IL-8 suppression | $OD_{600}$ in MRS | $OD_{600}$ in LDM |
|---|---|---|---|
| *L. salivarius* B101 | Yes | 8.02 ± 0.07 | 3.14 ± 0.30 [c] |
| *L. salivarius* B37 [a] | Yes | 9.98 ± 0.01 | 2.46 ± 0.32 |
| *L. salivarius* B21 | No | 9.09 ± 0.19 | 3.17 ± 0.07 |
| *L. rhamnosus* B103 | Yes | 7.96 ± 0.23 | 0.95 ± 0.13 [d] |
| *L. plantarum* XB7 | Yes | 8.28 ± 0.53 | 5.42 ± 0.12 |
| *L. plantarum* B7 | Yes | 8.61 ± 0.28 | 5.53 ± 0.21 |
| *L. plantarum* B67 [b] | No | 8.44 ± 0.05 | 4.72 ± 0.10 |

[a] Antagonistic strain to *H. pylori*, but 5% (v/v) LCM in IL-8 production co-culture assay did not inhibit *H. pylori* growth.

[b] This isolate killed AGS cells in IL-8 production co-culture assay.

[c] Results indicated mean ± SD.

[d] *L. rhamnosus* B103 was excluded for testing in histamine ELISA due to low value of $OD_{600}$ in LDM which means it could not grow well in this media.

**Table 12.** Histamine concentration in LDM secreted by gastric-derived *Lactobacillus* spp.

| Bacterial strain | IL-8 suppression | Histamine concentration (μg/mL) |
|---|---|---|
| *L. reuteri* ATCC 6475 [a] | No | 63.342 ± 19.157 [d] |
| *L. salivarius* B101 | Yes | 0.045 ± 0.010 |
| *L. salivarius* B37 [b] | Yes | 0.051 ± 0.012 |
| *L. salivarius* B21 | No | 0.045 ± 0.019 |
| *L. plantarum* XB7 | Yes | 0.049 ± 0.037 |
| *L. plantarum* B7 | Yes | 0.044 ± 0.018 |
| *L. plantarum* B67 [c] | No | 0.035 ± 0.007 |

[a] Positive control strain, *L. reuteri* ATCC PTA 6475.

[b] Antagonistic strain to *H. pylori*, but 5% (v/v) LCM in IL-8 production co-culture assay did not inhibit *H. pylori* growth.

[c] This isolate killed AGS cells in IL-8 production co-culture assay.

[d] Results indicated mean ± SD.

**_L. plantarum_ XB7 attenuated level of urease production, gastric inflammation, and _H. pylori_ colonization in _H. pylori_-infected Sprague-Dawley rat model**

*H. pylori*-infected Sprague-Dawley rat model (283) was used to determine the anti-inflammatory activity of *L. plantarum* XB7 *in vivo*. The rats consisted of three groups (8 rats in each group) as control, *H. pylori*-infected, and *L. plantarum* XB7-treated groups. *H. pylori* infection in the rats could be detected by using urease test which gave positive result within 48 h in case of *H. pylori* infection occurred. *H. pylori* colonization was evaluated using histopathology in addition to the urease test for detection of *H. pylori*. Level of gastric inflammation and *H. pylori* colonization were assessed using histopathology and scored by a blind pathologist.

The urease test of *H. pylori*-infected group was turned to pink color within 24 h (positive) indicating for *H. pylori* infection in the rats (Table 13). Interestingly, in the *L. plantarum* XB7-treated group, the urease test was delayed by turning to pink within 30 h (positive) indicated lower amount of *H. pylori* colonization (Table 13). The urease test of control group did not showed changing in color over 48 h (negative) indicated no *H. pylori* infection (Table 13). The indication of lower amount of *H. pylori* colonization in the *L. plantarum* XB7-treated group was confirmed by histopathology. For *H. pylori* colonization scores in *H. pylori*-infected group, 7 rats were scored 1, and 1 rat was scored 2 while in *L. plantarum* XB7-treated group, 5 rats were scored 0, 2 rats were scored 1, and 1 rat was scored 2 suggesting reduction of *H. pylori* colonization in this group (Table 13). For gastric inflammation scores in *H. pylori*-infected group, 1 rat was scored 0, and 7 rats were scored 1 while in *L. plantarum* XB7-treated group, 5 rats were scored 0, and 3 rats were scored 1 suggesting attenuation by improvement of gastric inflammation in this group (Table 13). The 8 rats in the control group demonstrated scored 0 in both case of *H. pylori* colonization and gastric inflammation (Table 13).

In conclusion, gastric inflammation and *H. pylori* colonization assessed by histopathology in *H. pylori*-infected group was showed moderate scores while in *L. plantarum* XB7-treated group demonstrated attenuation of scores indicated that improvement of gastric inflammation and reduction of *H. pylori* colonization. The control group showed no histopathological change of gastric inflammation as well as no *H. pylori* colonization. The urease test results, gastric inflammation, and *H. pylori*

colonization scores were summarized in Table 13. The histopathology of gastric inflammation and *H. pylori* colonization were shown in Figure 10.

**Table 13.** Summary of urease test for detection of *H. pylori* infection, gastric inflammation, and *H. pylori* colonization score

| Group [a] | Number | Urease test | | | Gastric inflammation [b] | | | | *H. pylori* colonization [c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | + <24 h | + <30 h | - >48 h | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| Control | 8 | - | - | 8 | 8 | - | - | - | 8 | - | - | - |
| Hp | 8 | 8 | - | - | 1 | 7 | - | - | 0 | 7 | 1 | - |
| Hp + LP-XB7 | 8 | - | 8 | - | 5 | 3 | - | - | 5 | 2 | 1 | - |

[a] Control, Control group; Hp, *H. pylori*-infected group; Hp + LP-XB7, *L. plantarum* XB7-treated group.

[b] Gastric inflammation was estimated and scored by blind pathologist according to the updated Sydney System. Infiltration of mononuclear and polymorphonuclear leukocyte in the gastric mucosa was scored from 0 to 3 represented normal, mild, moderate, and marked inflammatory changes, respectively.

[c] *H. pylori* colonization was scored by blind pathologist according to the following system; score 0, no bacteria detected; score 1, mild colonization in some gastric crypts; score 2, mild colonization in most gastric crypts; score 3, moderate colonization in all gastric crypts.

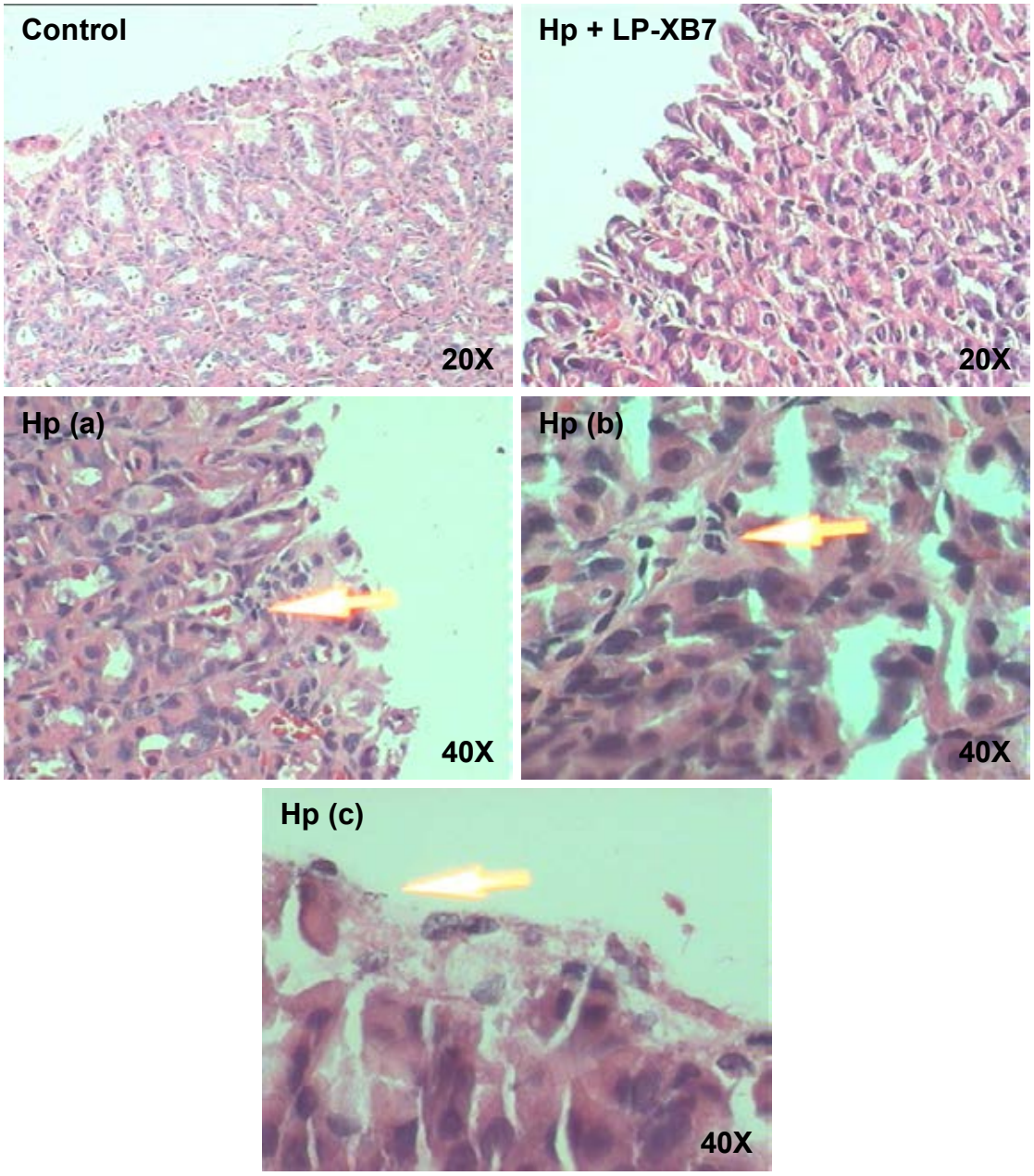



**Figure 10.** Histopathological change for assessment of gastric inflammation and *H. pylori* colonization. Control, Control group; Hp, *H. pylori*-infected group; Hp + LP-XB7, *L. plantarum* XB7-treated group. Control group showed no gastric inflammation and no *H. pylori* colonization. In *H. pylori*-infected group, (a, b) indicated moderate inflammatory cells infiltration (arrow), and (c) indicated *H. pylori* colonization (arrow). *L. plantarum* XB7-treated group showed improvement of histopathology both gastric inflammation and *H. pylori* colonization.

**_L. plantarum_ XB7 suppressed both systemic and local inflammatory cytokine production in _H. pylori_-infected Sprague-Dawley rat model**

To determine anti-inflammatory effect of *L. plantarum* XB7 (LP-XB7) to inflammatory cytokine production induced by *H. pylori* in the rats, both gastric tissue as local and serum as systemic were prepared and measured using ELISA. The detected cytokines consisted of CINC-1, IL-1β, and TNF-α. The level of TNF-α in serum of *H. pylori*-infected group was significantly ($P$<0.001) higher than control group, while treatment in the rats by LP-XB7 demonstrated significantly ($P$<0.05) decreasing of TNF-α level when compared with *H. pylori*-infected group (Figure 11). However, there was no significant difference of TNF-α level in gastric tissue between control, *H. pylori*-infected, and LP-XB7-treated group (Figure 12). The IL-1β level in both serum and gastric tissue also demonstrated no significant difference between each group (Figures 13 and 14). The level of CINC-1 in serum of *H. pylori*-infected group was significantly ($P$<0.01) higher than control group, while treatment in the rats by LP-XB7 demonstrated significantly ($P$<0.05) decreasing of CINC-1 level when compared with *H. pylori*-infected group (Figure 15). The level of CINC-1 in gastric tissue of *H. pylori*-infected group was significantly ($P$<0.05) higher than control group, while treatment in the rats by LP-XB7 demonstrated significantly ($P$<0.05) decreasing of CINC-1 level when compared with *H. pylori*-infected group (Figure 16). The level of rat cytokines in serum and gastric tissue were summarized in Table 14. In conclusion, *L. plantarum* XB7 suppressed TNF-α and CINC-1 in sera, and CINC-1 in gastric tissue of *H. pylori*-infected rats.

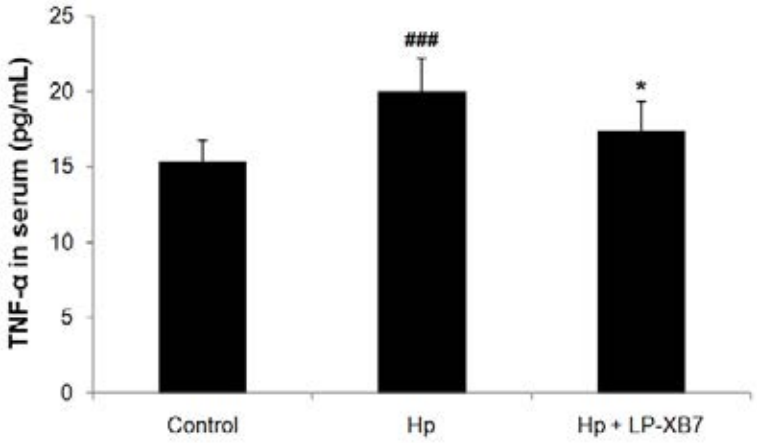



**Figure 11.** Suppressive effect of *L. plantarum* XB7 on *H. pylori*-stimulated TNF-α production in rat serum. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with *H. pylori*-infected group. * $P<0.05$. The square indicated a statistically significant difference compared with control group. ### $P<0.001$.

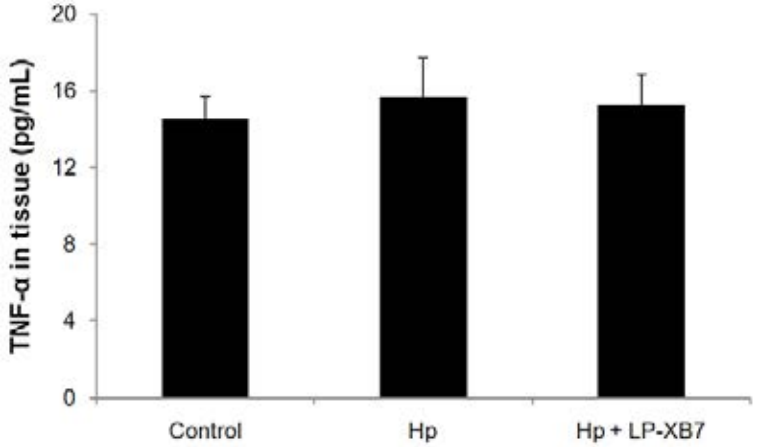



**Figure 12.** Effect of *L. plantarum* XB7 on *H. pylori*-stimulated TNF-α production in rat gastric tissue. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation.

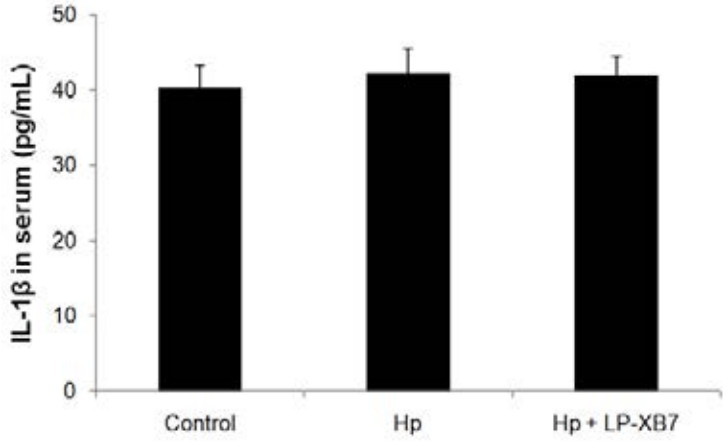



**Figure 13.** Effect of *L. plantarum* XB7 on *H. pylori*-stimulated IL-1β production in rat serum. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation.

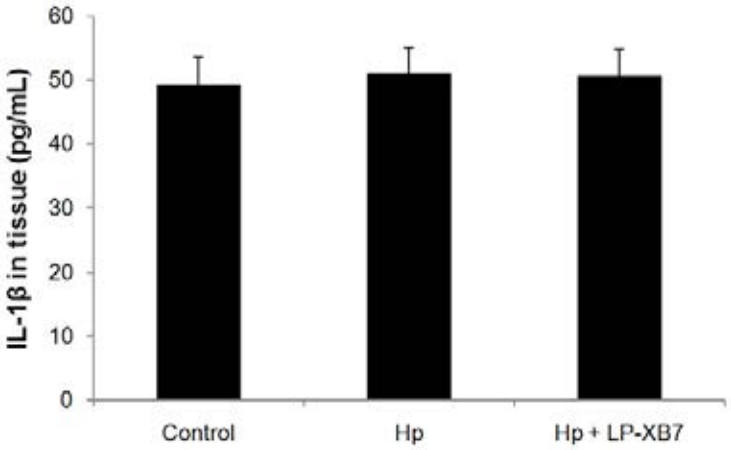



**Figure 14.** Effect of *L. plantarum* XB7 on *H. pylori*-stimulated IL-1β production in rat gastric tissue. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation.

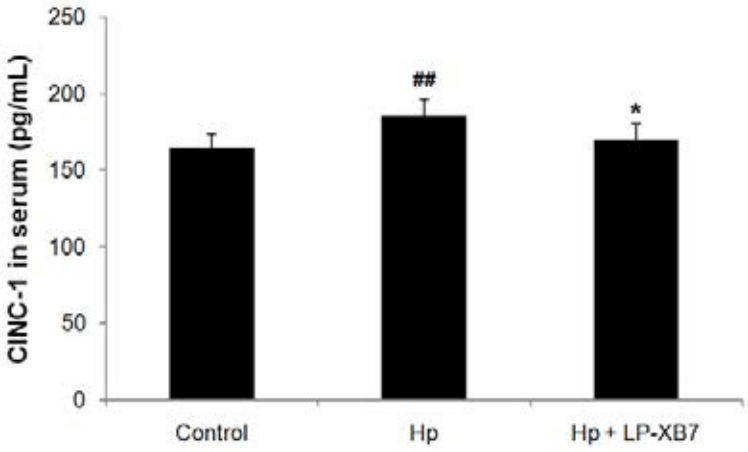



**Figure 15.** Suppressive effect of *L. plantarum* XB7 on *H. pylori*-stimulated CINC-1 production in rat serum. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with *H. pylori*-infected group. * $P<0.05$. The square indicated a statistically significant difference compared with control group. ## $P<0.01$.

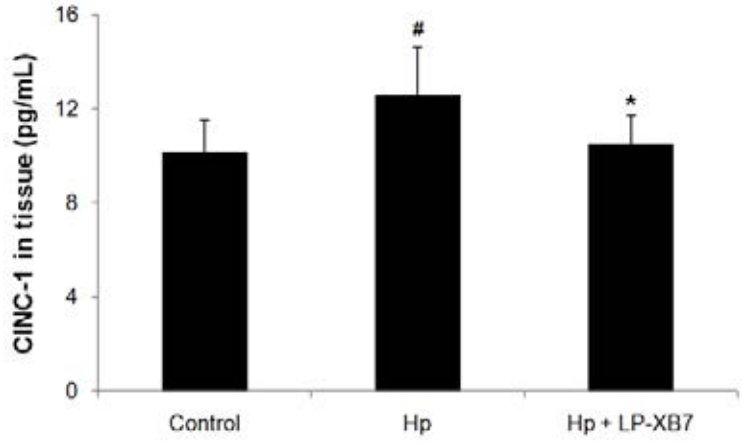



**Figure 16.** Suppressive effect of *L. plantarum* XB7 on *H. pylori*-stimulated CINC-1 production in rat gastric tissue. Control, control group; Hp, *H. pylori*-infected group; and Hp + LP-XB7, *L. plantarum* XB7-treated group. The results were expressed as the mean of eight rats in each group (n = 8). The bars indicated standard deviation. An asterisk meant a statistically significant difference compared with *H. pylori*-infected group. * $P<0.05$. The square indicated a statistically significant difference compared with control group. # $P<0.05$.

**Table 14.** Summary of rat cytokine level in serum and gastric tissue

| Cytokine / Group (n = 8) | Cytokine concentration (pg/mL) | | |
|---|---|---|---|
| | Control | Hp | Hp + LP-XB7 |
| TNF-α in serum | 15.31 ± 1.42 | 19.97 ± 2.25 ### | 17.38 ± 1.96 * |
| TNF-α in gastric tissue | 14.56 ± 1.11 | 15.69 ± 2.05 | 15.25 ± 1.60 |
| IL-1β in serum | 40.31 ± 3.02 | 42.25 ± 3.30 | 41.94 ± 2.60 |
| IL-1β in gastric tissue | 49.44 ± 4.20 | 51.25 ± 3.84 | 50.75 ± 4.11 |
| CINC-1 in serum | 164.84 ± 8.90 | 185.81 ± 10.67 ## | 170.13 ± 10.28 * |
| CINC-1 in gastric tissue | 10.16 ± 1.36 | 12.59 ± 2.04 # | 10.50 ± 1.24 * |

Effects of *L. plantarum* XB7 on *H. pylori*-stimulated inflammatory cytokine production in rat serum and gastric tissue. Control, Control group; Hp, *H. pylori*-infected group; Hp + LP-XB7, *L. plantarum* XB7-treated group. The results of cytokine concentration were presented as the mean ± SD of eight rats in each group (n = 8). An asterisk meant a statistically significant difference compared with *H. pylori* infected group. * $P<0.05$. The square indicated a statistically significant difference compared with control group. # $P<0.05$, ## $P<0.01$, ### $P<0.001$.

**Antagonistic activity of *Lactobacillus* against *H. pylori* reference strains**

**Spot-overlay method**

Eighty-five gastric-derived *Lactobacillus* isolates were screened for antagonistic activity against *H. pylori* by spot-overlay method. *H. pylori* growth was inhibited resulting in a clear zone around each specific strain of *Lactobacillus* spot. *L. salivarius* B23 and B37 showed clear zones to all *H. pylori* reference strains ATCC 43504, 51110, 51932, and the results were reproducible (Table 15). *L. salivarius* B37 also demonstrated the largest inhibition zone. For other *Lactobacillus* isolates, they sometimes gave the positive results but were not reproducible as shown in Table 15. Due to *H. pylori* growth was faint on the media, it was very difficult to take the photo of antagonistic result.

**Disc diffusion (LCM-disc) method**

*L. salivarius* B23 and B37 which reproducibly inhibited the growth of all *H. pylori* ATCC reference strains by spot-overlay method were further tested by LCM-disc against *H. pylori* ATCC 43504 using 1- or 5-fold concentrated LCM. In addition, the LCM of other *Lactobacillus* with the same and different pH that gave negative results from spot-overlay method were also prepared for using as controls. The results demonstrated that *L. salivarius* B37 showed the largest zone of inhibition to *H. pylori* ATCC 43504 (Table 16). Five-fold concentrated LCM of other *Lactobacillus* which had pH 3.6 could also inhibit *H. pylori* (Table 16). *L. salivarius* B23 (LS-B23), B37 (LS-B37), *L. plantarum* B87 (LP-B87) as representative of *L. plantarum*, and *L. gasseri* XB68 (LG-XB68) as representative of *L. gasseri* were selected for further characterization experiments. The example of image of the inhibition zone by LCM-disc was shown in Figure 17.

**Table 15.** Ability of *Lactobacillus* in growth inhibition of *H. pylori* reference strains by spot-overlay method

| Number | Isolate | Species | Zone of inhibition [a] | | |
|---|---|---|---|---|---|
| | | | ATCC 43504 | ATCC 51932 | ATCC 51110 |
| 1 | B2 | *L. fermentum* | - | - | - |
| 2 | B4/2 | *L. salivarius* | ++ | ++ | ++ |
| 3 | B5 | *L. fermentum* | - | - | - |
| 4 | B6 | *L. plantarum* | - | - | + |
| 5 | B7 | *L. plantarum* | - | + | + |
| 6 | XB7 | *L. plantarum* | - | - | - |
| 7 | B8 | *L. salivarius* | - | - | - |
| 8 | B9 | *L. fermentum* | - | - | - |
| 9 | B13 | *L. casei* group | - | - | - |
| 10 | B14 | *L. fermentum* | - | - | ++ |
| 11 | B15 | *L. mucosae* | - | - | - |
| 12 | B16 | *L. salivarius* | - | - | - |
| 13 | B18 | *L. oris* | - | - | - |
| 14 | XB19 | *L. gasseri* | - | - | - |
| 15 | B20 | *L. fermentum* | - | - | - |
| 16 | B21 | *L. salivarius* | - | - | - |
| 17 | B22 | *L. oris* | - | - | - |
| 18 | B23 | *L. salivarius* | ++ | ++ | + |
| 19 | B24 | *L. fermentum* | - | - | - |
| 20 | B25 | *L. fermentum* | - | - | - |
| 21 | B26 | *L. mucosae* | - | - | - |
| 22 | B29 | *L. fermentum* | - | - | - |
| 23 | XB30 | *L. gasseri* | - | - | - |
| 24 | B31 | *L. fermentum* | - | - | - |
| 25 | B32 | *L. salivarius* | + | + | - |
| 26 | B33 | *L. fermentum* | | + | - |
| 27 | B35 | *L. fermentum* | - | - | - |
| 28 | B36 | *L. agilis* | - | - | - |
| 29 | B37 | *L. salivarius* | +++ | ++ | + |
| 30 | B38 | *L. fermentum* | - | - | - |

**Table 15.** Ability of *Lactobacillus* in growth inhibition of *H. pylori* reference strains by spot-overlay method (continued)

| Number | Isolate | Species | Zone of inhibition [a] | | |
|---|---|---|---|---|---|
| | | | ATCC 43504 | ATCC 51932 | ATCC 51110 |
| 31 | B39 | *L. fermentum* | - | - | - |
| 32 | XB40 | *L. gasseri* | - | - | - |
| 33 | XB41 | *L. gasseri* | - | - | - |
| 34 | B42 | *L. fermentum* | - | - | - |
| 35 | B43 | *L. salivarius* | - | - | + |
| 36 | B44 | *L. fermentum* | - | - | - |
| 37 | XB45 | *L. gasseri* | - | - | - |
| 38 | B46 | *L. fermentum* | - | - | - |
| 39 | B47 | *L. salivarius* | - | - | + |
| 40 | XB48 | *L. gasseri* | - | - | - |
| 41 | XB49 | *L. gasseri* | - | - | - |
| 42 | B52 | *L. salivarius* | - | - | + |
| 43 | B53 | *L. salivarius* | - | - | - |
| 44 | B54 | *L. mucosae* | - | - | - |
| 45 | B55 | *L. salivarius* | + | ++ | + |
| 46 | B57 | *L. murinus* | - | - | - |
| 47 | XB58 | *L. gasseri* | - | - | - |
| 48 | B59 | *Weissella confusa* | - | - | - |
| 49 | B60 | *L. salivarius* | - | - | - |
| 50 | B61 | *L. fermentum* | - | - | - |
| 51 | B62 | *L. salivarius* | - | - | - |
| 52 | B64 | *L. plantarum* | - | - | + |
| 53 | B66 | *L. fermentum* | - | - | - |
| 54 | B67 | *L. plantarum* | - | - | - |
| 55 | XB68 | *L. gasseri* | - | - | - |
| 56 | B70 | *L. plantarum* | - | - | - |
| 57 | B71 | *Weissella cibaria* | - | - | - |
| 58 | B72 | *L. fermentum* | - | - | - |
| 59 | B73 | *L. salivarius* | - | - | - |
| 60 | B74 | *L. salivarius* | ++ | ++ | ++ |

**Table 15.** Ability of *Lactobacillus* in growth inhibition of *H. pylori* reference strains by spot-overlay method (continued)

| Number | Isolate | Species | Zone of inhibition [a] | | |
|---|---|---|---|---|---|
| | | | ATCC 43504 | ATCC 51932 | ATCC 51110 |
| 61 | B75 | *L. fermentum* | - | - | - |
| 62 | B76 | *L. fermentum* | - | - | - |
| 63 | XB77 | *L. gasseri* | - | - | - |
| 64 | B78 | *L. salivarius* | - | - | - |
| 65 | B79 | *L. mucosae* | - | - | - |
| 66 | B82 | *L. fermentum* | - | - | - |
| 67 | B83 | *L. fermentum* | - | - | - |
| 68 | B84 | *L. fermentum* | - | - | - |
| 69 | B85 | *L. salivarius* | - | - | - |
| 70 | B87 | *L. plantarum* | - | - | - |
| 71 | B90 | *L. plantarum* | - | - | - |
| 72 | B91 | *L. salivarius* | - | - | - |
| 73 | B92 | *L. fermentum* | - | - | + |
| 74 | B93 | *L. oris* | - | - | - |
| 75 | XB94 | *L. gasseri* | - | - | - |
| 76 | XB95 | *L. gasseri* | - | - | - |
| 77 | XB96 | *L. gasseri* | - | - | - |
| 78 | B99 | *L. fermentum* | - | - | - |
| 79 | B101 | *L. salivarius* | - | - | - |
| 80 | B102 | *L. salivarius* | - | - | + |
| 81 | B105 | *L. fermentum* | - | - | - |
| 82 | B106 | *L. casei* group | - | - | - |
| 83 | B108 | *L. fermentum* | - | - | - |
| 84 | B109 | *L. salivarius* | - | - | + |
| 85 | B110 | *L. salivarius* | + | + | + |

[a] Including spot, ~4 mm. - no zone of inhibition, + slight zone of inhibition <6 mm, ++ moderate zone of inhibition 6-9 mm, +++ obvious zone of inhibition >9 mm. The results came from at least three independent experiments each in duplicate.

**Table 16.** Ability of *Lactobacillus* in growth inhibition of *H. pylori* reference strain ATCC 43504 by LCM-disc method

| Subject | pH [a] | Zone of inhibition (mm) [b] | |
|---|---|---|---|
| | | 1X | 5X |
| MRS media control | 6.0 | - [c] | 8.1 ± 0.2 [d] |
| *L. salivarius* B23 | 3.6 | - | 9.4 ± 0.6 |
| *L. salivarius* B37 | 3.6 | 6.8 ± 0.4 | 10.5 ± 0.5 |
| *L. plantarum* B67 | 3.6 | - | 9.4 ± 0.7 |
| *L. plantarum* B87 | 3.6 | - | 9.5 ± 0.9 |
| *L. plantarum* B90 | 3.6 | - | 9.4 ± 0.6 |
| *L. gasseri* XB40 | 3.6 | - | 8.5 ± 0.5 |
| *L. gasseri* XB68 | 3.6 | - | 8.7 ± 0.4 |
| *L. casei* B13 | 3.6 | - | 9.4 ± 0.6 |
| *L. agilis* B36 | 3.6 | - | 9.2 ± 0.5 |
| *L. murinus* B57 | 3.8 | - | 8.3 ± 0.3 |
| *L. fermentum* B2 | 4.1 | - | 8.0 ± 0.4 |
| *L. fermentum* B5 | 4.1 | - | 8.1 ± 0.5 |
| *L. fermentum* B25 | 4.1 | - | 8.1 ± 0.3 |
| *L. fermentum* B44 | 4.1 | - | 8.1 ± 0.5 |
| *L. mucosae* B15 | 4.1 | - | 8.0 ± 0.7 |
| *L. oris* B93 | 4.1 | - | 8.0 ± 0.6 |

[a] Average pH of LCM.

[b] Including disc, 6 mm.

[c] No zone of inhibition.

[d] Results indicated mean ± SD of three independent experiments each in duplicate.

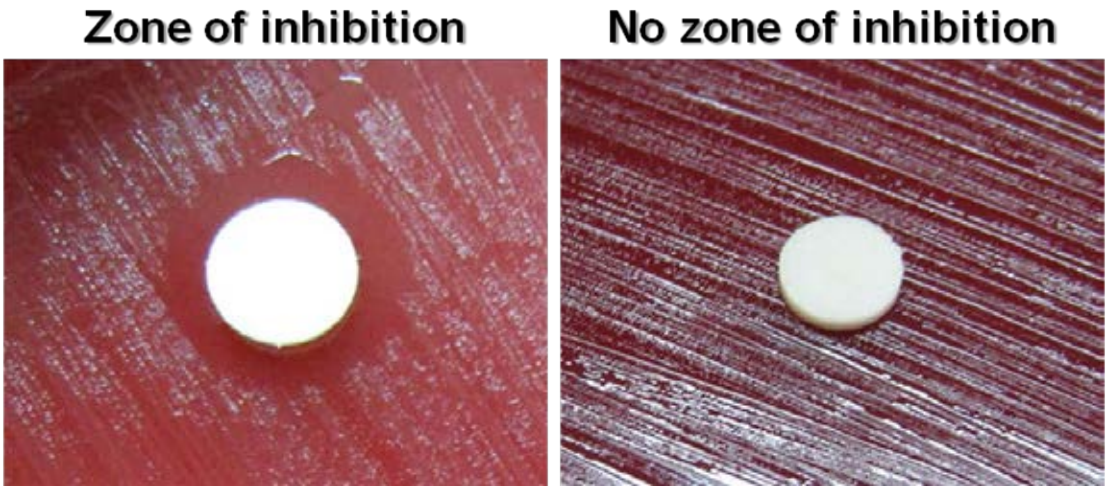



**Figure 17.** Representative image for zone of inhibition of *H. pylori* ATCC 43504 growth by LCM-disc.

**Characterization of antagonistic substance**

**Dose-dependent manner of inhibition**

Five-fold and ten-fold concentrated LCM of *L. salivarius* B37 were first tested by adding various volumes of LCM to sterile discs and subjected to antagonistic assay. Ten-fold concentrated LCM was appropriate for the test (data not shown) and selected for the experiment. As shown in Table 17, *L. salivarius* B37 gave the best result for inhibition of *H. pylori* growth and demonstrated dose dependent manner of inhibition ($P<0.05$ when compared the result of 10 µL and 15 µL, and $P<0.01$ when compared the result of 15 µL and 20 µL). Ten-fold concentrated LCM in a volume of 20 µL on disc was used for further experiments in LCM-disc assay.

**Table 17.** Dose-dependent inhibition of the growth of *H. pylori* ATCC 43504 by 10-fold concentrated LCM

| Volume | Zone of inhibition (mm) [a] | | | | |
|---|---|---|---|---|---|
| | **MRS control** | **LS-B23** | **LS-B37** | **LP-B87** | **LG-XB68** |
| **10 µL** | 7.3 ± 0.7 [b] | 7.9 ± 1.0 | 8.7 ± 0.9 | 8.3 ± 0.7 | 8.6 ± 1.7 |
| **15 µL** | 7.8 ± 0.7 | 9.4 ± 0.7 | 10.0 ± 0.5 | 9.6 ± 1.1 | 9.3 ± 0.8 |
| **20 µL** | 8.6 ± 0.5 | 10.5 ± 0.4 | 11.6 ± 0.4 | 10.7 ± 0.5 | 10.5 ± 0.5 |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

**pH sensitivity**

Antagonistic substances in LCM of all tested *Lactobacillus* spp. could tolerate pH in all tested conditions as shown in Table 18. There was no statistically significance when compared the treated to the control LCM.

**Table 18.** pH sensitivity of 10-fold concentrated LCM in the growth inhibition of *H. pylori* ATCC 43504

| pH | Zone of inhibition (mm) [a] | | | | |
|---|---|---|---|---|---|
| | **MRS control** | **LS-B23** | **LS-B37** | **LP-B87** | **LG-XB68** |
| **Control** | 8.3 ± 0.3 [b] | 10.4 ± 0.2 | 11.5 ± 0.5 | 10.5 ± 0.6 | 10.3 ± 0.3 |
| **2** | 8.1 ± 0.6 | 10.2 ± 0.2 | 11.4 ± 0.4 | 10.6 ± 0.5 | 10.4 ± 0.4 |
| **3** | 8.3 ± 0.2 | 10.2 ± 0.2 | 11.3 ± 0.5 | 10.6 ± 0.3 | 10.4 ± 0.2 |
| **4** | 8.3 ± 0.2 | 10.2 ± 0.2 | 11.2 ± 0.2 | 10.8 ± 0.3 | 10.4 ± 0.2 |
| **5** | 8.2 ± 0.2 | 10.2 ± 0.2 | 11.2 ± 0.6 | 10.7 ± 0.4 | 10.4 ± 0.3 |
| **6** | 8.4 ± 0.2 | 10.2 ± 0.3 | 11.2 ± 0.4 | 10.6 ± 0.4 | 10.2 ± 0.1 |
| **7** | 8.2 ± 0.3 | 10.2 ± 0.4 | 11.1 ± 0.5 | 10.4 ± 0.5 | 10.3 ± 0.4 |
| **8** | 8.2 ± 0.4 | 10.2 ± 0.4 | 11.2 ± 0.6 | 10.3 ± 0.4 | 10.1 ± 0.1 |
| **9** | 8.2 ± 0.1 | 10.1 ± 0.4 | 11.2 ± 0.5 | 10.4 ± 0.3 | 10.2 ± 0.2 |
| **10** | 8.2 ± 0.2 | 10.1 ± 0.3 | 11.1 ± 0.5 | 10.4 ± 0.2 | 10.2 ± 0.2 |
| **11** | 8.2 ± 0.2 | 10.2 ± 0.4 | 11.1 ± 0.6 | 10.5 ± 0.4 | 10.1 ± 0.4 |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

**Thermostability**

Antagonistic substances in LCM of all tested *Lactobacillus* spp. were heat-stable as shown in Table 19. There was no statistically significance when compared the treated to the control LCM.

**Table 19.** Thermostability of 10-fold concentrated LCM in the growth inhibition of *H. pylori* ATCC 43504

| Temperature | Zone of inhibition (mm) [a] | | | | |
|---|---|---|---|---|---|
| | **MRS control** | **LS-B23** | **LS-B37** | **LP-B87** | **LG-XB68** |
| **Control** | 8.4 ± 0.3 [b] | 10.4 ± 0.5 | 11.6 ± 0.4 | 10.7 ± 0.6 | 10.3 ± 0.5 |
| **100°C, 15 min** | 8.3 ± 0.5 | 10.5 ± 0.5 | 11.5 ± 0.3 | 10.6 ± 0.3 | 10.4 ± 0.6 |
| **100°C, 30 min** | 8.3 ± 0.4 | 10.3 ± 0.4 | 11.4 ± 0.4 | 10.6 ± 0.6 | 10.4 ± 0.4 |
| **100°C, 60 min** | 8.3 ± 0.5 | 10.5 ± 0.6 | 11.6 ± 0.4 | 10.7 ± 0.5 | 10.2 ± 0.5 |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

**Enzymatic sensitivity**

Antagonistic substances in LCM of all tested *Lactobacillus* spp. could not be inactivated by all tested enzymes as shown in Table 20. There was no statistically significance when compared the treated to the control LCM.

**Table 20.** Enzymatic sensitivity of 10-fold concentrated LCM in the growth inhibition of *H. pylori* ATCC 43504

| Treatment | Zone of inhibition (mm) [a] | | | | | |
|---|---|---|---|---|---|---|
| | MRS control | LS-B23 | LS-B37 | LP-B87 | LG-XB68 | Enzyme control |
| **Control** | 8.6 ± 0.2 [b] | 10.4 ± 0.4 | 11.5 ± 0.4 | 10.9 ± 0.5 | 10.4 ± 0.5 | |
| **Pepsin** | 8.4 ± 0.3 | 10.4 ± 0.3 | 11.2 ± 0.4 | 10.9 ± 0.3 | 10.6 ± 0.4 | - [c] |
| **Proteinase K** | 8.6 ± 0.2 | 10.4 ± 0.2 | 11.4 ± 0.2 | 11.0 ± 0.2 | 10.5 ± 0.2 | - |
| **Trypsin** | 8.6 ± 0.2 | 10.5 ± 0.3 | 11.5 ± 0.1 | 11.1 ± 0.2 | 10.5 ± 0.2 | - |
| **α-amylase** | 8.5 ± 0.3 | 10.3 ± 0.2 | 11.3 ± 0.4 | 11.2 ± 0.2 | 10.5 ± 0.3 | - |
| **Lipase** | 8.3 ± 0.3 | 10.3 ± 0.2 | 11.3 ± 0.3 | 10.9 ± 0.4 | 10.6 ± 0.3 | - |
| **Lysozyme** | 8.6 ± 0.1 | 10.5 ± 0.2 | 11.5 ± 0.3 | 11.0 ± 0.4 | 10.5 ± 0.3 | - |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

[c] No zone of inhibition.

**Stability**

LCM of all tested *Lactobacillus* spp. still had antagonistic activity even when it was kept for 90 days as shown in Table 21. There was no statistically significance when compared the treated to the control LCM.

**Table 21.** Stability over times of 10-fold concentrated LCM in the growth inhibition of *H. pylori* ATCC 43504

| Storage condition | Zone of inhibition (mm) [a] | | | | |
|---|---|---|---|---|---|
| | MRS control | LS-B23 | LS-B37 | LP-B87 | LG-XB68 |
| **Control** | 8.6 ± 0.2 [b] | 10.3 ± 0.4 | 11.5 ± 0.2 | 10.8 ± 0.5 | 10.3 ± 0.3 |
| **30 days, -20°C** | 8.5 ± 0.2 | 10.4 ± 0.4 | 11.4 ± 0.2 | 10.7 ± 0.5 | 10.3 ± 0.3 |
| **30 days, 4°C** | 8.5 ± 0.1 | 10.3 ± 0.2 | 11.4 ± 0.3 | 10.8 ± 0.4 | 10.4 ± 0.3 |
| **30 days, 25°C** | 8.5 ± 0.2 | 10.3 ± 0.4 | 11.4 ± 0.2 | 10.8 ± 0.4 | 10.4 ± 0.3 |
| **Control** | 8.5 ± 0.4 | 10.4 ± 0.5 | 11.6 ± 0.4 | 10.7 ± 0.4 | 10.3 ± 0.5 |
| **60 days, -20°C** | 8.4 ± 0.3 | 10.4 ± 0.4 | 11.5 ± 0.3 | 10.7 ± 0.4 | 10.4 ± 0.3 |
| **60 days, 4°C** | 8.4 ± 0.3 | 10.5 ± 0.5 | 11.4 ± 0.4 | 10.8 ± 0.4 | 10.4 ± 0.4 |
| **60 days, 25°C** | 8.5 ± 0.4 | 10.4 ± 0.4 | 11.4 ± 0.4 | 10.8 ± 0.3 | 10.4 ± 0.4 |
| **Control** | 8.4 ± 0.4 | 10.4 ± 0.4 | 11.6 ± 0.4 | 10.6 ± 0.4 | 10.5 ± 0.5 |
| **90 days, -20°C** | 8.5 ± 0.4 | 10.4 ± 0.4 | 11.6 ± 0.3 | 10.7 ± 0.4 | 10.3 ± 0.5 |
| **90 days, 4°C** | 8.5 ± 0.3 | 10.4 ± 0.3 | 11.6 ± 0.3 | 10.6 ± 0.4 | 10.6 ± 0.4 |
| **90 days, 25°C** | 8.4 ± 0.3 | 10.5 ± 0.4 | 11.5 ± 0.3 | 10.7 ± 0.5 | 10.4 ± 0.4 |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

**Relation to lactic acid**

As shown in Table 22, only L- and DL-lactic acid with pH value of 2.0 showed inhibitory effect to *H. pylori*. Since LCM of *L. salivarius* B37 (LS-B37) and other *Lactobacillus* isolates with antagonistic activity had pH value of 3.6, the result suggested that their secreted antagonistic substances were not associated with lactic acid.

**Table 22.** L-, DL-lactic acid of various pH in the growth inhibition of *H. pylori* ATCC 43504

| pH | Zone of inhibition (mm) [a] | |
|---|---|---|
| | **L-lactic acid** | **DL-lactic acid** |
| **2.0** | 8.6 ± 0.1 [b] | 8.3 ± 0.1 |
| **3.0** | - [c] | - |
| **3.6** | - | - |
| **4.0** | - | - |
| **5.0** | - | - |
| **DDW (negative control)** | - | - |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

[c] No zone of inhibition.

**Molecular size analysis**

The molecular size of a specific antagonistic substance of *L. salivarius* B37 (LS-B37) was found to be 3-10 kDa (Table 23). As shown in Table 23, the major activity resembling control remained in retentated recovery of Amicon Ultra-4 3K ($P$=0.633 when compared the result of control and retentated recovery, and $P$<0.001 when compared the result of control and filtrated recovery) suggested the molecular size of more than 3 kDa and those remained in filtrated recovery of Amicon Ultra-4 10K ($P$<0.001 when compared the result of control and retentated recovery, and $P$=0.483 when compared the result of control and filtrated recovery) and 30K ($P$<0.001 when compared the result of control and retentated recovery, and $P$=0.851 when compared the result of control and filtrated recovery) suggested the molecular size of less than 10 kDa. In other *Lactobacillus* isolates, antagonistic activity was distributed in both retentated and filtrated recovery of Amicon Ultra-4 3K, 10K, and 30K. Interestingly, the antagonistic substance in 10-fold concentrated MRS control was found to be greater than 30 kDa.

**Table 23.** Molecular size analysis of antagonistic substance in 10-fold concentrated LCM by Amicon Ultra-4 in the growth inhibition of *H. pylori* ATCC 43504

| Fraction | Zone of inhibition (mm) [a] | | | | |
|---|---|---|---|---|---|
| | **MRS control** | **LS-B23** | **LS-B37** | **LP-B87** | **LG-XB68** |
| **Control** | 8.5 ± 0.3 [b] | 10.4 ± 0.3 | 11.5 ± 0.3 | 10.7 ± 0.3 | 10.4 ± 0.3 |
| **3K - Retentated recovery** | 8.4 ± 0.3 | 9.7 ± 0.3 | 11.4 ± 0.3 | 10.3 ± 0.3 | 10.3 ± 0.3 |
| **3K - Filtrated recovery** | - [c] | 9.5 ± 0.3 | 10.0 ± 0.3 | 10.3 ± 0.3 | 10.1 ± 0.3 |
| **Control** | 8.4 ± 0.3 | 10.5 ± 0.4 | 11.4 ± 0.3 | 10.6 ± 0.3 | 10.4 ± 0.4 |
| **10K - Retentated recovery** | 8.4 ± 0.3 | 9.8 ± 0.3 | 10.1 ± 0.4 | 10.2 ± 0.4 | 10.0 ± 0.4 |
| **10K - Filtrated recovery** | - | 9.5 ± 0.3 | 11.3 ± 0.5 | 10.3 ± 0.5 | 10.2 ± 0.4 |
| **Control** | 8.4 ± 0.4 | 10.4 ± 0.4 | 11.5 ± 0.4 | 10.7 ± 0.4 | 10.4 ± 0.4 |
| **30K - Retentated recovery** | 8.3 ± 0.4 | 9.8 ± 0.4 | 10.2 ± 0.4 | 10.2 ± 0.5 | 10.1 ± 0.5 |
| **30K - Filtrated recovery** | - | 9.6 ± 0.4 | 11.6 ± 0.4 | 10.3 ± 0.5 | 10.3 ± 0.3 |

[a] Including disc, 6 mm.

[b] Results indicated mean ± SD of three independent experiments each in duplicate.

[c] No zone of inhibition.

In conclusion, *L. salivarius* B37 (LS-B37) showed the best antagonistic activity against *H. pylori* reference strains. The characteristics of antagonistic substance secreted by LS-B37 were summarized in Table 24.

**Table 24.** Summary of antagonistic substance characterization of *L. salivarius* B37

| **Characteristics of antagonistic substance secreted by *L. salivarius* B37** | |
|---|---|
| 1. Secreted substance(s) | 5. Stability over 3 months |
| 2. Dose dependent manner of inhibition | 6. Non-lactic acid |
| 3. Acid-alkali tolerance | 7. Molecular size between 3-10 kDa |
| 4. Heat-stable substance | |

**Inhibition of antibiotic-resistant *H. pylori* clinical isolates**

Since clarithromycin and metronidazole are the drugs of choice for treatment of *H. pylori*-infected patients, clinical isolates with known susceptibility patterns were tested with *L. salivarius* B37 (LS-B37) and other control isolates. The results showed that LS-B37 had the strongest inhibitory activity against all tested *H. pylori* isolates as shown in Table 25.

**Table 25.** Ability of 10-fold concentrated LCM in the growth inhibition of *H. pylori* reference strains and clinical isolates

| *H. pylori* strain | Source/ Patient | Antibiotic susceptibility [a] | | Zone of inhibition (mm) [b] | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CLA | MET | MRS control | LS-B23 | LS-B37 | LP-B87 | LG-XB68 |
| **43504 (control)** | ATCC | S | R | 8.4 ± 0.3 [c] | 10.4 ± 0.4 | 11.5 ± 0.4 | 10.6 ± 0.4 | 10.4 ± 0.4 |
| **51110** | ATCC | S | S | 8.4 ± 0.4 | 11.3 ± 0.5 | 13.9 ± 0.4 | 11.9 ± 0.5 | 11.8 ± 0.5 |
| **51932** | ATCC | S | S | 8.1 ± 0.4 | 10.2 ± 0.4 | 11.8 ± 0.4 | 10.4 ± 0.4 | 10.6 ± 0.4 |
| **FM4** | Gastric ulcer | R | R | 8.4 ± 0.3 | 10.5 ± 0.3 | 12.1 ± 0.4 | 10.7 ± 0.4 | 10.5 ± 0.4 |
| **T1433** | Duodenal ulcer and gastritis | R | R | 8.1 ± 0.4 | 11.4 ± 0.5 | 13.2 ± 0.5 | 11.0 ± 0.4 | 10.9 ± 0.4 |
| **T1264** | Gastric ulcer and duodenitis | S | R | 8.6 ± 0.4 | 11.2 ± 0.5 | 13.3 ± 0.5 | 11.6 ± 0.4 | 11.5 ± 0.5 |
| **BT246** | Normal | S | R | 8.1 ± 0.3 | 9.8 ± 0.3 | 11.7 ± 0.3 | 10.3 ± 0.4 | 9.7 ± 0.3 |
| **BT260** | Gastritis | S | R | 7.2 ± 0.3 | 9.1 ± 0.3 | 11.7 ± 0.4 | 10.2 ± 0.3 | 10.1 ± 0.3 |
| **BT274** | Gastritis | S | R | - [d] | 8.6 ± 0.5 | 11.9 ± 0.5 | 10.0 ± 0.4 | 9.3 ± 0.5 |
| **BT280** | Atrophic erosion | S | R | - | 8.9 ± 0.3 | 11.5 ± 0.4 | 9.6 ± 0.5 | 10.1 ± 0.3 |
| **BT289** | Normal | S | R | - | 9.6 ± 0.4 | 11.7 ± 0.4 | 9.5 ± 0.5 | 10.0 ± 0.3 |
| **BT263** | Gastritis | S | S | - | 9.8 ± 0.3 | 11.6 ± 0.4 | 10.7 ± 0.4 | 10.6 ± 0.4 |
| **BT324** | Gastritis | S | S | - | 9.4 ± 0.4 | 11.4 ± 0.4 | 10.1 ± 0.4 | 10.2 ± 0.3 |
| **BT345** | Gastric erosion | S | S | - | 10.9 ± 0.4 | 14.6 ± 0.5 | 11.5 ± 0.4 | 11.6 ± 0.4 |
| **BT349** | Gastric erosion | S | S | 7.5 ± 0.3 | 10.3 ± 0.4 | 11.6 ± 0.4 | 10.3 ± 0.4 | 10.3 ± 0.5 |
| **BT393** | Atrophic gastritis | S | S | - | 8.2 ± 0.3 | 11.5 ± 0.4 | 10.7 ± 0.4 | 10.6 ± 0.4 |

[a] CLA, clarithromycin; MET, metronidazole; R, resistant; S, susceptible.

[b] Including disc, 6 mm.

[c] Results indicated mean ± SD of three independent experiments each in duplicate.

[d] No zone of inhibition.

# CHAPTER V

# DISCUSSION

Lactobacilli are indigenous bacteria among other microorganisms that reside in the gastric micro-environment of humans (341). Previous studies demonstrated that these lactobacilli were successfully isolated from gastric biopsies of humans (332, 342). Several lines of evidence showed the beneficial effects of *Lactobacillus* spp. in the reduction of *H. pylori* colonization and improvement of *H. pylori*-induced gastric inflammation both *in vitro* and *in vivo*. However, they are not originated from the stomach in most cases (40, 41, 43, 44, 343). Lactobacilli isolated from gastric biopsy are promising candidates as adjuncts in the treatment of *H. pylori*-associated diseases. In this study, we then searched for gastric-derived lactobacilli with anti-inflammatory and/or antagonistic activity against *H. pylori*.

For the study of anti-inflammatory activity, our results demonstrated that conditioned media of eight *Lactobacillus* isolates could suppress IL-8 production stimulated by *H. pylori* in gastric epithelial cells (Figure 6 and Table 8) without impairing *H. pylori* proliferation (Table 9). This suggests the possibility that *Lactobacillus* spp. secreted immunomodulatory substance interfering with IL-8 production in the host cells. Three of eight anti-inflammatory *Lactobacillus* isolates suppressed IL-8 gene expression in *H. pylori*-stimulated gastric epithelial cells via the inhibition of NF-κB and/or c-Jun activation (Figures 7 and 8). Other five isolates that did not suppress IL-8 mRNA expression at 4 h in this study may suppress IL-8 mRNA expression at other time points, for example at 2 or 8 h of incubation in the co-culture assay. Although we cannot exclude the possibility that LP-XB7 down regulated the expression of virulence factors associated with IL-8 production, the inhibition of host signaling pathway is the possible mechanism. Recent finding supported our results by demonstrating that *L. acidophilus* LA5® played role in the reduction of *H. pylori*-induced inflammation through mechanism of NF-κB inactivation (344). Exploration of cytokines/chemokines modulating ability other than IL-8 of *Lactobacillus* spp. revealed that IL-8 was the only cytokine that LS-B37, LS-B101, LR-B103, and LP-XB7 could inhibit in AGS gastric epithelial cells (Figure 9 and Table 10). Previous studies reported that LS-B101, LR-B103, and LP-XB7 could inhibit TNF-α in LPS-stimulated THP-1 monocytoid cells (332, 334). Unfortunately, in the present study,

LS-B101, LR-B103, and LP-XB7 could not show suppressive effect on *H. pylori*-induced TNF-α in AGS cells due to the very low detectable level near the detection limit of this assay (Table 10) suggesting the previous TNF-α lowering effect is specific to stimulation of LPS in THP-1 cells. Probiotic *L. reuteri* ATCC PTA 6475 has been reported for production of histamine leading to inhibit TNF production in THP-1 cells by using the mechanism through PKA and ERK signaling (338). On the other hand, our TNF-suppressing gastric-derived strains, LS-B101, LR-B103, and LP-XB7, did not produce histamine (Table 12), thus they must work via using a different mechanism rather than the same with *L. reuteri*.

Due to the ability to inhibit both NF-kB and c-Jun activation as well as downstream IL-8 production, the anti-inflammatory properties of *L. plantarum* XB7 (LP-XB7) were further determined in *H. pylori*-infected Sprague-Dawley rat model. LP-XB7 reduced *H. pylori* colonization, and both local and systemic inflammation in this model. While LP-XB7 did not inhibit *H. pylori* proliferation *in vitro* (Table 9), LP-XB7 could delay changing in color of urease test to 30 h, and *H. pylori* colonization scores were decreased in the rats treated with LP-XB7 (Table 13). We cannot completely exclude the possibility that LP-XB7 negatively effects viability of *H. pylori in vivo*, but the presence of LP-XB7 resulting in decreased *H. pylori* colonization could possibly be attributed due to competitive exclusion. The improvement of histopathological change in LP-XB7-treated rats demonstrated the anti-inflammatory effect by LP-XB7 (Figure 10). Recently, *in vivo* study of *L. johnsonii* MH-68 and *L. salivarius* subsp. *salicinius* AP-32 showed the reduction of *H. pylori* load and lymphocyte infiltration but these inhibitory effects could be due to suppression of *H. pylori* viability (345). LP-XB7 was also evaluated for the inflammatory cytokines suppressive effect in the rats. The inflammatory cytokines consisted of TNF-α, IL-1β, and CINC-1 (rat IL-8) were selected for testing in the rats due to these cytokines have been reported to express higher in *H. pylori* infected patients than in *H. pylori* negative patients (346, 347). The results showed that LP-XB7 could attenuate level of TNF-α production in serum, and CINC-1 production in both serum and gastric tissue of the *H. pylori*-infected rats (Figures 11, 15, 16 and Table 14). These cytokine-suppressing effects of LP-XB7 might help elucidate some part of histopathology in gastric inflammation scores. Because LP-XB7 could suppress inflammatory cytokines, TNF-α and CINC-1, so it was not surprising that why gastric inflammation showed decreasing tendency scores, and also improvement of

histopathological change in LP-XB7-treated rats. Other proinflammatory cytokines are modulated by probiotic bacteria *in vivo*. For instance, *L. johnsonii* La1 could attenuate proinflammatory chemokines macrophage inflammatory protein 2 (MIP-2) and keratinocyte-derived cytokine (KC) in the serum and gastric tissue of *H. pylori*-infected mouse model (41). MIP-2 and KC are known as IL-8-like chemokines in the murine model (42, 348) which are homolog of IL-8 in humans (349). Although LP-XB7 could show inhibitory effect to proinflammatory cytokine production *in vitro*, we cannot conclude that the decreased gastric inflammation *in vivo* was solely contributed by the anti-inflammatory properties of LP-XB7. In the presence of LP-XB7, less gastric inflammation could probably be due to the reduction in colonization of *H. pylori*.

In summary, three human gastric-derived lactobacilli were identified with the ability to secrete factors interfering with IL-8 transcription in the course of inflammation. Specifically, LP-XB7 attenuated gastric inflammation in the rats infected with *H. pylori*. LP-XB7 is thus a promising agent for the use in adjunctive therapy against *H. pylori* infection.

For the study of antagonistic activity, the results showed that specific strains of gastric-derived *Lactobacillus* spp. could inhibit the growth of *H. pylori* (Table 15). In addition, the antagonistic effect demonstrated by LCM-disc method in Table 16 suggested the presence of antagonistic substances in the culture supernatant. Among the antagonistic strains, *L. salivarius* B37 (LS-B37) showed the strongest antagonistic activity against *H. pylori*. This inhibition was dose-dependent with increased inhibitory effect by increased volume used (Table 17), and the antagonistic activity still existed in the storage conditions for 90 days (Table 21). The nature of antagonistic substances in these strains was tentative to be non-biological molecules because they were resistant to acid-base (Table 18), heat (Table 19), and enzymatic treatment (Table 20). Normally, if antagonistic substance was protein, it would be heat-labile. However, there was an antagonistic substance which was protein and heat-stable such as the bacteriocin of *L. plantarum* NC8 (350). If the antagonistic substance was protein, it would be inactivated by the enzymes such as pepsin, proteinase K, and trypsin. If the antagonistic substance was carbohydrate, it would be inactivated by amylase. If the antagonistic substance was lipid, it would be inactivated by lipase. It was difficult to purify non-biological molecule for further study

of its structure. Previous studies found that antagonistic substances for *H. pylori* growth were lactic acid (48, 323), heat-labile substance (328), heat-stable substance (49, 50), autolysin (51), extracellular secretory substance (47), unidentified substance (53, 330), non-proteinaceous substance (329), and bacteriocin (bulgaricin BB18) (331).

Although previous studies showed that lactic acid could inhibit *H. pylori* growth (48, 323), this study demonstrated that L-and DL-lactic acid with pH value of 3.6 could not (Table 22). On the contrary, LCM with pH value of 3.6 of LS-B37 could weakly inhibit, whereas its 5-fold concentrated LCM increasingly inhibited the growth of *H. pylori* (Table 16). In addition, 5-fold concentrated LCM of other *Lactobacillus* isolates with pH values of 3.6 could inhibit *H. pylori* growth in various magnitudes as shown in Table 16. This suggested that the antagonism of these *Lactobacillus* strains was not associated with lactic acid.

Molecular size analysis of antagonistic substances in LCM of these *Lactobacillus* isolates as shown in Table 23 revealed the presence of various substances of different sizes. The antagonistic substance in 10-fold concentrated MRS was found to be greater than 30 kDa. On the contrary, the specific antagonistic substance found only in LCM of LS-B37 had molecular size of 3-10 kDa. Since its antagonistic substance could tolerate a wide range of pH and still display the bactericidal effect at low pH, LS-B37 has the promising role for the application in the gastric environment of humans. Previous studies demonstrated that *L. salivarius* CECT5713 was safe to be used in humans (351, 352), suggesting the safety use of *L. salivarius* as supplement agent. Identification of LS-B37 by phenotypic method as shown in Appendix A and previous genotypic method (332) gave similar result as *L. salivarius* which is an autochthonous (indigenous) species and stable life-long in the human body (353). In addition, LCM of LS-B37 could inhibit antibiotic-resistant *H. pylori* strains as shown in Table 25. This finding strengthened LS-B37 as a promising *Lactobacillus* candidate for an adjunct in the treatment of clarithromycin- and/or metronidazole-resistant *H. pylori* strains.

In summary of the study for antagonism against *H. pylori* by gastric-derived *Lactobacillus*, LS-B37 was found to have the strongest antagonistic activity against *H. pylori* by spot-overlay and LCM-disc method. The characterization of antagonistic substance of LS-B37 revealed that it was acid-alkali tolerant, heat-stable, stable over

3 months, non-lactic acid, and had molecular size between 3-10 kDa. In addition, the antagonistic substance of LS-B37 showed the strongest inhibition of clarithromycin- and/or metronidazole-resistant *H. pylori* clinical strains.

In conclusion, human gastric-derived *L. plantarum* XB7 (LP-XB7) demonstrated the anti-inflammatory properties both *in vitro* and *in vivo*, and *L. salivarius* B37 (LS-B37) showed the strongest antagonistic activity against antibiotic-resistant *H. pylori*. Therefore, the present studies suggest the potential of LP-XB7 and LS-B37 as promising adjunctive therapeutic agents for treatment of *H. pylori*-associated diseases.

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# APPENDICES

# APPENDIX A

## PHENOTYPIC IDENTIFICATION BY API 50 CHL

### Phenotypic identification of eleven gastric-derived *Lactobacillus* spp.

In addition to the results of previous genotypic identification (332), phenotypic identification of 11 *Lactobacillus* isolates was performed by API 50 CHL with database version 5.1 (https://apiweb.biomerieux.com/servlet/Identify). Different species of *Lactobacillus* has different pattern of carbohydrate biochemical profile by API 50 CHL. Therefore, API 50 CHL could be used for identification of *Lactobacillus* to the species level. Results of identification of 11 *Lactobacillus* isolates by API 50 CHL were shown in Figures 18-28 and Tables 26-36. The species identifications of 11 gastric-derived *Lactobacillus* isolates by API 50 CHL were summarized in Table 37.

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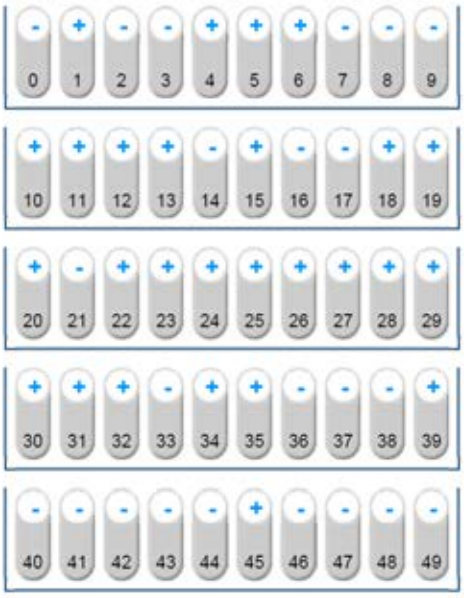
API 50 CHL V5.1





REFERENCE
B6

DATE
4/8/11

COMMENT

ACCEPTABLE IDENTIFICATION

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | - + - - + + + - - - + + + + + - + - - + + + - + + + + + + + + + + - + + - - - + - - - - - + - - - - |
| Note | |

| Significant taxa | % ID | T | Tests against | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lactobacillus plantarum 1 | 84.9 | 0.54 | GLY | 1% | DXYL | 2% | | |

| Next taxon | % ID | T | Tests against | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lactobacillus pentosus | 15.0 | 0.41 | RHA | 25% | MDM | 1% | MLZ | 25% | DARL | 0% |

**Figure 18.** Identification result of B6 isolate by API 50 CHL.

**Table 26.** Carbohydrate biochemical profile of B6 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | + |
| Glycerol | + | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | + | Glycogen | - |
| L-Arabinose | + | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | + | Amygdalin | + | D-Turanose | - |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | + | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | + |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | + | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - 0 | - 1 | - 2 | - 3 | - 4 | - 5 | - 6 | - 7 | - 8 | - 9 |
| - 10 | + 11 | + 12 | + 13 | - 14 | - 15 | - 16 | - 17 | + 18 | + 19 |
| - 20 | - 21 | + 22 | - 23 | - 24 | - 25 | - 26 | - 27 | + 28 | + 29 |
| - 30 | + 31 | + 32 | - 33 | - 34 | + 35 | - 36 | - 37 | - 38 | - 39 |
| - 40 | - 41 | - 42 | - 43 | - 44 | - 45 | - 46 | - 47 | - 48 | - 49 |


**REFERENCE** B37 **DATE** 3/28/11

**COMMENT**

**VERY GOOD IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | ----------+++----+++--+-----+++-+++--+---------- |
| Note | |

| Significant taxa | % ID | T | Tests against | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lactobacillus salivarius | 99.8 | 0.82 | GAL | 85% | MEL | 85% | | |

| Next taxon | % ID | T | Tests against | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lactobacillus delbrueckii ssp lactis 1 | 0.1 | 0.43 | MAN | 3% | SOR | 0% | RAF | 6% |

**Figure 19.** Identification result of B37 isolate by API 50 CHL.

**Table 27.** Carbohydrate biochemical profile of B37 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | - |
| Glycerol | - | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | - | N-AcetylGlucosamine | + | Gentiobiose | - |
| D-Xylose | - | Amygdalin | - | D-Turanose | - |
| L-Xylose | - | Arbutin | - | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | - | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | - | D-Fucose | - |
| D-Galactose | - | D-Cellobiose | - | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | - | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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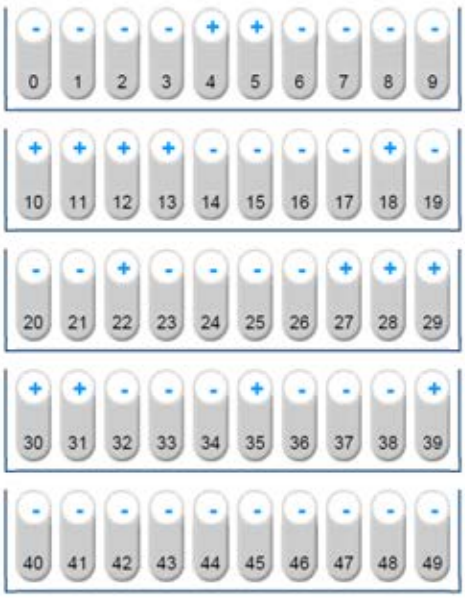
API 50 CHL V5.1





**REFERENCE** **DATE**
B57 4/8/11
**COMMENT**

**GOOD IDENTIFICATION**

| | |
|---|---|
| **Strip** | API 50 CHL V5.1 |
| **Profile** | ----+----+++++----+---+----+++++---+---+---------- |
| **Note** | |

| **Significant taxa** | **% ID** | **T** | **Tests against** | | | |
|---|---|---|---|---|---|---|
| **Leuconostoc lactis** | **94.5** | **0.49** | **LARA 15%** | **RIB 10%** | **MAN 5%** | **CEL 10%** |
| | | | **GEN 11%** | | | |

| **Next taxon** | **% ID** | **T** | **Tests against** | | | |
|---|---|---|---|---|---|---|
| **Lactobacillus salivarius** | **3.7** | **0.4** | **SOR 98%** | **CEL 14%** | **TRE 99%** | **GEN 14%** |

**Figure 20.** Identification result of B57 isolate by API 50 CHL.

**Table 28.** Carbohydrate biochemical profile of B57 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | - |
| Glycerol | - | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | - | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | + | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | - | Amygdalin | - | D-Turanose | - |
| L-Xylose | - | Arbutin | - | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | - | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | - | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | - | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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API 50 CHL V5.1

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| - | - | - | - | - | - | - | - | - | - |
| 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| + | + | + | + | - | - | - | - | + | - |
| 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
| - | - | + | - | - | - | - | - | + | + |
| 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| + | + | + | - | - | + | - | - | - | - |
| 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
| - | - | - | - | - | + | - | - | - | - |


**REFERENCE** **DATE**
B60 4/8/11
**COMMENT**

**DOUBTFUL PROFILE**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | ----------++++-----+---+-----++++++--+---------+---- |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus salivarius | 77.1 | 0.61 | SOR 98% | DARL 1% | | |
| Leuconostoc lactis | 22.1 | 0.5 | MAN 5% | DARL 0% | | |

| Next taxon | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus delbrueckii ssp lactis 1 | 0.6 | 0.32 | MAN 3% | MEL 10% | RAF 6% | DARL 0% |

| Complementary test(s) | GLUCOSEg | 15°C | 45°C | COCCI |
|---|---|---|---|---|
| Lactobacillus salivarius | - | - | + | - |
| Leuconostoc lactis | + | + | - | + |

**Figure 21.** Identification result of B60 isolate by API 50 CHL.

**Table 29.** Carbohydrate biochemical profile of B60 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | - |
| Glycerol | - | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | - | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | - | N-AcetylGlucosamine | + | Gentiobiose | - |
| D-Xylose | - | Amygdalin | - | D-Turanose | - |
| L-Xylose | - | Arbutin | - | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | - | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | - | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | - | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | + |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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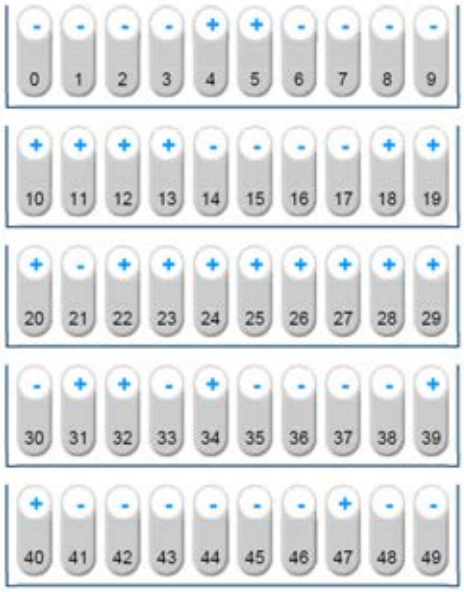
API 50 CHL V5.1





**REFERENCE** B70 **DATE** 4/8/11

**COMMENT**

**EXCELLENT IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | ----+----++++----+++-+++++++++-+-+-+----++------+-- |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus plantarum 1 | 99.9 | 0.84 | MEL 94% | | | |

| Next taxon | % ID | T | Tests against | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lactobacillus brevis 1 | 0.1 | 0.4 | SOR 14% | MDM 0% | MLZ 14% | TUR 14% |

**Figure 22.** Identification result of B70 isolate by API 50 CHL.

**Table 30.** Carbohydrate biochemical profile of B70 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | + |
| Glycerol | - | D-Mannitol | + | D-Raffinose | - |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | + | Glycogen | - |
| L-Arabinose | + | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | - | Amygdalin | + | D-Turanose | + |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | + | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | - | Potassium Gluconate | + |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

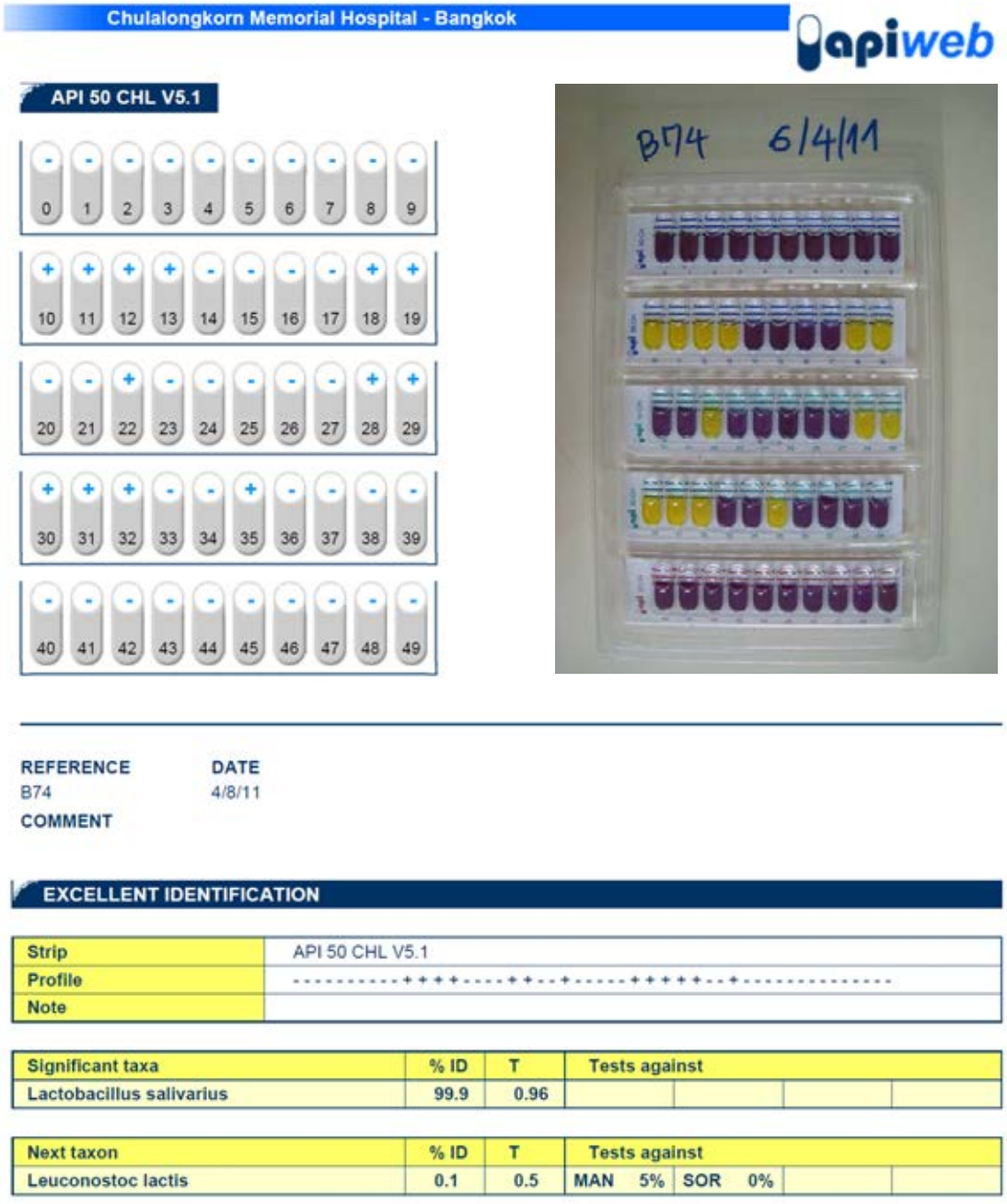



**Figure 23.** Identification result of B74 isolate by API 50 CHL.

**Table 31.** Carbohydrate biochemical profile of B74 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | - |
| Glycerol | - | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | - | N-AcetylGlucosamine | + | Gentiobiose | - |
| D-Xylose | - | Amygdalin | - | D-Turanose | - |
| L-Xylose | - | Arbutin | - | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | - | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | - | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | - | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - 0 | - 1 | - 2 | - 3 | + 4 | + 5 | - 6 | - 7 | - 8 | - 9 |
| + 10 | + 11 | + 12 | + 13 | - 14 | + 15 | - 16 | - 17 | + 18 | + 19 |
| - 20 | - 21 | + 22 | + 23 | + 24 | + 25 | + 26 | + 27 | + 28 | + 29 |
| + 30 | + 31 | + 32 | - 33 | + 34 | - 35 | - 36 | - 37 | - 38 | + 39 |
| + 40 | - 41 | - 42 | - 43 | - 44 | - 45 | - 46 | + 47 | - 48 | - 49 |


**REFERENCE** **DATE**
B90 4/1/11
**COMMENT**

**VERY GOOD IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | ----++----++++-+--++--++++++++++-+----++------+-- |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus plantarum 1 | 99.6 | 0.92 | | | | |

| Next taxon | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus pentosus | 0.3 | 0.61 | GLY 75% | DXYL100% | RHA 25% | MLZ 25% |
| | | | RAF 75% | | | |

**Figure 24.** Identification result of B90 isolate by API 50 CHL.

**Table 32.** Carbohydrate biochemical profile of B90 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | + |
| Glycerol | - | D-Mannitol | + | D-Raffinose | - |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | + | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | - | Amygdalin | + | D-Turanose | + |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | + | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | + |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | + | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - 0 | - 1 | - 2 | - 3 | - 4 | - 5 | - 6 | - 7 | - 8 | - 9 |
| + 10 | + 11 | + 12 | + 13 | - 14 | - 15 | - 16 | - 17 | + 18 | + 19 |
| - 20 | - 21 | + 22 | - 23 | - 24 | - 25 | - 26 | - 27 | + 28 | + 29 |
| + 30 | + 31 | + 32 | - 33 | - 34 | + 35 | - 36 | - 37 | - 38 | - 39 |
| - 40 | - 41 | - 42 | - 43 | - 44 | - 45 | - 46 | - 47 | - 48 | - 49 |


**REFERENCE** B101 **DATE** 4/1/11

**COMMENT**

**EXCELLENT IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | ----------++++----++--+-----+++++--+-------------- |
| Note | |

| Significant taxa | % ID | T | Tests against | | | |
|---|---|---|---|---|---|---|
| Lactobacillus salivarius | 99.9 | 0.96 | | | | |

| Next taxon | % ID | T | Tests against | | | | | |
|---|---|---|---|---|---|---|---|---|
| Leuconostoc lactis | 0.1 | 0.5 | MAN | 5% | SOR | 0% | | |

**Figure 25.** Identification result of B101 isolate by API 50 CHL.

**Table 33.** Carbohydrate biochemical profile of B101 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | - |
| Glycerol | - | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | - | N-AcetylGlucosamine | + | Gentiobiose | - |
| D-Xylose | - | Amygdalin | - | D-Turanose | - |
| L-Xylose | - | Arbutin | - | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | - | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | - | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | - | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - 0 | - 1 | - 2 | - 3 | - 4 | + 5 | - 6 | - 7 | - 8 | - 9 |
| + 10 | + 11 | + 12 | + 13 | - 14 | - 15 | + 16 | + 17 | + 18 | + 19 |
| - 20 | + 21 | + 22 | + 23 | + 24 | + 25 | + 26 | + 27 | + 28 | - 29 |
| - 30 | + 31 | + 32 | - 33 | + 34 | - 35 | - 36 | - 37 | - 38 | + 39 |
| + 40 | - 41 | + 42 | - 43 | - 44 | - 45 | - 46 | + 47 | - 48 | - 49 |


**REFERENCE** B103 **DATE** 4/3/11

**COMMENT**

**VERY GOOD IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | -----+----++++--++++-+++++++++--++-+----++-+----+-- |
| Note | |

| Significant taxa | % ID | T | Tests against |
|---|---|---|---|
| Lactobacillus paracasei ssp paracasei 2 | 99.8 | 0.94 | |

| Next taxon | % ID | T | Tests against | | | | | |
|---|---|---|---|---|---|---|---|---|
| Lactobacillus paracasei ssp paracasei 1 | 0.1 | 0.62 | DUL | 13% | INO | 6% | LAC | 99% |

**Figure 26.** Identification result of B103 isolate by API 50 CHL.

**Table 34.** Carbohydrate biochemical profile of B103 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | + | D-Melezitose | + |
| Glycerol | - | D-Mannitol | + | D-Raffinose | - |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | + | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | - | Amygdalin | + | D-Turanose | + |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | + | D-Tagatose | + |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | - | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | - | Potassium Gluconate | + |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | + | Inulin | - | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - | - | - | - | - | + | - | - | + | - |
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| + | + | + | + | - | - | - | - | + | + |
| 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| - | + | + | + | + | + | + | + | + | + |
| 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
| - | + | + | + | + | - | - | - | - | + |
| 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| + | - | + | - | - | - | - | + | - | - |
| 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |


**REFERENCE** B106 **DATE** 4/1/11

**COMMENT**

**VERY GOOD IDENTIFICATION**

| | |
|---|---|
| Strip | API 50 CHL V5.1 |
| Profile | -----+--+-++++----++-++++++++++-++++----++-+----+-- |
| Note | |

| Significant taxa | % ID | T | Tests against |
|---|---|---|---|
| Lactobacillus paracasei ssp paracasei 1 | 99.5 | 0.86 | ADO 13% |

| Next taxon | % ID | T | Tests against |
|---|---|---|---|
| Lactobacillus paracasei ssp paracasei 2 | 0.4 | 0.67 | LAC 0% |

**Figure 27.** Identification result of B106 isolate by API 50 CHL.

**Table 35.** Carbohydrate biochemical profile of B106 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | + |
| Glycerol | - | D-Mannitol | + | D-Raffinose | - |
| Erythritol | - | D-Sorbitol | + | Amidon | - |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | - | Glycogen | - |
| L-Arabinose | - | Methyl-αD-Glucopyranoside | + | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | - | Amygdalin | + | D-Turanose | + |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | + | Esculin ferric citrate | + | D-Tagatose | + |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | - |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | - | Potassium Gluconate | + |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | - | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | + | | |

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API 50 CHL V5.1

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| - | + | - | - | + | + | + | - | - | - |
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| + | + | + | + | - | + | - | - | + | + |
| 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| + | - | + | + | + | + | + | + | + | + |
| 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
| + | + | + | - | + | + | + | + | - | + |
| 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| - | - | - | - | - | + | - | - | - | - |
| 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |


**REFERENCE** **DATE**
XB7 4/3/11
**COMMENT**

**GOOD IDENTIFICATION**

| | |
|---|---|
| **Strip** | API 50 CHL V5.1 |
| **Profile** | - + - - + + + - - - + + + + - + - - + + + - + + + + + + + + + + - + + + + - + - - - - - + - - - - |
| **Note** | |

| Significant taxa | % ID | T | Tests against | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lactobacillus plantarum 1 | 99.9 | 0.32 | GLY | 1% | DXYL | 2% | AMD | 7% | GLYG | 7% |

| Next taxon | % ID | T | Tests against | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lactobacillus pentosus | 0.1 | 0.0 | RHA | 25% | MDM | 1% | MLZ | 25% | AMD | 0% |
| | | | GLYG | 0% | DARL | 0% | | | | |

**Figure 28.** Identification result of XB7 isolate by API 50 CHL.

**Table 36.** Carbohydrate biochemical profile of XB7 isolate by API 50 CHL

| Substrate | Result | Substrate | Result | Substrate | Result |
|---|---|---|---|---|---|
| Control | - | Inositol | - | D-Melezitose | + |
| Glycerol | + | D-Mannitol | + | D-Raffinose | + |
| Erythritol | - | D-Sorbitol | + | Amidon | + |
| D-Arabinose | - | Methyl-αD-Mannopyranoside | + | Glycogen | + |
| L-Arabinose | + | Methyl-αD-Glucopyranoside | - | Xylitol | - |
| D-Ribose | + | N-AcetylGlucosamine | + | Gentiobiose | + |
| D-Xylose | + | Amygdalin | + | D-Turanose | - |
| L-Xylose | - | Arbutin | + | D-Lyxose | - |
| D-Adonitol | - | Esculin ferric citrate | + | D-Tagatose | - |
| Methyl-βD-Xylopyranoside | - | Salicin | + | D-Fucose | - |
| D-Galactose | + | D-Cellobiose | + | L-Fucose | - |
| D-Glucose | + | D-Maltose | + | D-Arabitol | + |
| D-Fructose | + | D-Lactose | + | L-Arabitol | - |
| D-Mannose | + | D-Melibiose | + | Potassium Gluconate | - |
| L-Sorbose | - | D-Saccharose | + | Potassium 2-KetoGluconate | - |
| L-Rhamnose | + | D-Trehalose | + | Potassium 5-KetoGluconate | - |
| Dulcitol | - | Inulin | - | | |

**Table 37.** Phenotypic identification of 11 gastric-derived *Lactobacillus* isolates by API 50 CHL

| Isolate | Species | % identification |
|---|---|---|
| B6 | *L. plantarum* 1 | 84.9 |
| B37 | *L. salivarius* | 99.8 |
| B57 | *Leuconostoc lactis* | 94.5 |
| B60 | *L. salivarius* | 77.1 |
| B70 | *L. plantarum* 1 | 99.9 |
| B74 | *L. salivarius* | 99.9 |
| B90 | *L. plantarum* 1 | 99.6 |
| B101 | *L. salivarius* | 99.9 |
| B103 | *L. paracasei* subsp. *paracasei* 2 | 99.8 |
| B106 | *L. paracasei* subsp. *paracasei* 1 | 99.5 |
| XB7 | *L. plantarum* 1 | 99.9 |

# APPENDIX B

## MATERIALS AND EQUIPMENTS

### 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)
- 6 mm membrane discs (Whatman, UK)
- 100% ethanol (Merck, Germany)
- 100% isopropanol (Merck, Germany)
- AGS human gastric adenocarcinoma epithelial cells, CRL-1739 (ATCC, USA)
- Amicon Ultra-4 3K, 10K, and 30K (Millipore Ireland B.V., Ireland)
- Anaerobic indicator (Oxoid, UK)
- AnaeroPack-Anaero (Mitsubishi Gas Chemical Company, Inc, Japan)
- AnaeroPack-MicroAero (Mitsubishi Gas Chemical Company, Inc, Japan)
- API 50 CHL (BioMérieux, France)
- 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, UK)
- Conical centrifuge tubes (Nunc, USA)
- 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, pepsin, proteinase K, trypsin, α-amylase, lipase, and lysozyme (Sigma-Aldrich, USA)
- Fetal bovine serum (Gibco-Invitrogen, New Zealand)
- Glycerol (Merck, Germany)

- Halt phosphatase inhibitor single-use cocktail (Pierce Biotechnology, USA)
- Halt protease inhibitor single-use cocktail EDTA-free (Pierce Biotechnology, USA)
- Histamine ELISA kit (Neogen, USA)
- Horse serum (Gibco-Invitrogen, New Zealand)
- Human IL-8 ELISA kit (DuoSet, R&D Systems, USA)
- Hydrochloric acid (HCl) (Merck, Germany)
- L- and DL-lactic acid (Sigma-Aldrich, USA)
- LightCycler Capillary (Roche, Germany)
- LightCycler® FastStart DNA MasterPLUS SYBR Green I (Roche, Germany)
- Methanol (Merck, Germany)
- Microcentrifuge tube (Eppendorf, USA)
- MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 14 Plex (Millipore, 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-κB p65 (Ser 536), NF-κB p65, β-actin, p-c-Jun, and c-Jun (Santa Cruz Biotechnology, Inc, USA)
- Rat CINC-1, IL-1β, and TNF-α ELISA kits (Quantikine, R&D Systems, USA)
- RPMI 1640 medium (Gibco-Invitrogen, USA)
- Secondary antibody, goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Inc, USA)
- Serological pipettes (Corning, USA)
- Sheep blood (Faculty of Veterinary Medicine, Chulalongkorn University, Thailand)
- Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, Germany)
- Sodium chloride (NaCl) (Sigma-Aldrich, USA)
- Sodium hydroxide (NaOH) (Merck, Germany)
- SuperScript® VILO cDNA synthesis kit (Invitrogen, USA)

- SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, USA)
- Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, USA)
- Tissue culture flasks (Nunc, Denmark)
- Tissue culture plates (Nunc, Denmark)
- Tris base (Sigma-Aldrich, USA)
- TRIzol® reagent (Invitrogen, USA)
- Tween 20 (Merck, Germany)
- Yeast extract (Difco, USA)

**Equipments**

- Anaerobic chamber (Concept Plus, Ruskinn Technology Ltd, UK)
- Autoclave (Hirayama, Japan)
- Biological safety cabinet (Astec-Microflow, Bioquell UK Ltd, UK)
- ChemiDoc™ XRS (Bio-Rad Laboratories, Inc, USA)
- $CO_2$ incubator (BINDER GmbH, Germany)
- Frogger (DAN-KAR CCRP, USA)
- Heat block (Scientific Industries, Inc, USA)
- Hemocytometer (Boeco, Germany)
- Incubator (Memmert GmbH, Germany)
- Inverted microscope (Olympus, Japan)
- LightCycler® 2.0 instrument (Roche, Germany)
- Luminex 100 platform (Luminex Corporation, USA)
- Micropipettes (Gilson, France)
- Mini-PROTEAN® 3 Cell (Bio-Rad Laboratories, Inc, USA)
- Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc, USA)
- NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc, USA)
- pH meter (Thermo Fisher Scientific, Inc, USA)
- PowerPac™ Universal Power Supply (Bio-Rad Laboratories, Inc, USA)
- Rectangular jar (Mitsubishi Gas Chemical Company, Inc, Japan)
- Savant SpeedVac (Savant instruments, USA)

- Spectrophotometer (Bio-Rad Smart Spec$^{TM}$ Plus, Bio-Rad Laboratories, Inc, USA)
- Vernier caliper (Mitutoyo, Japan)
- Vortex mixer (Scientific Industries, Inc, USA)
- Water bath (Memmert GmbH, Germany)

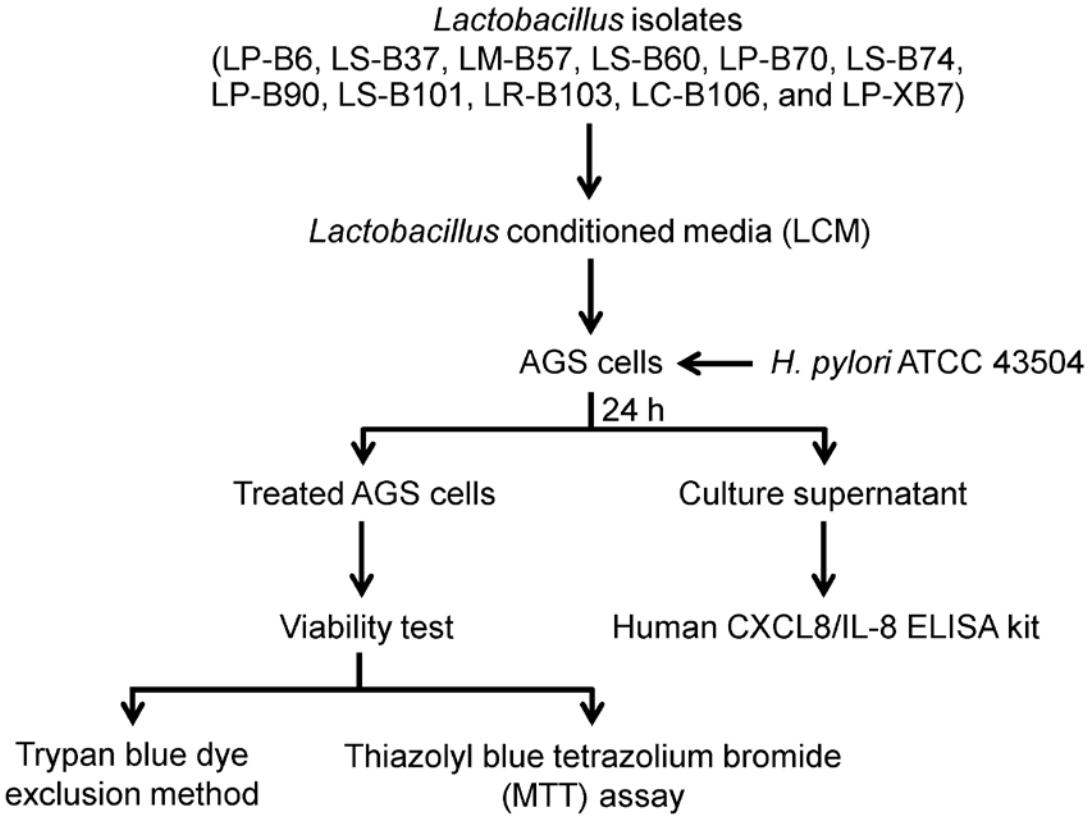
**Software and program**

- API database version 5.1 (https://apiweb.biomerieux.com/servlet/Identify)
- ImageJ version 1.45s

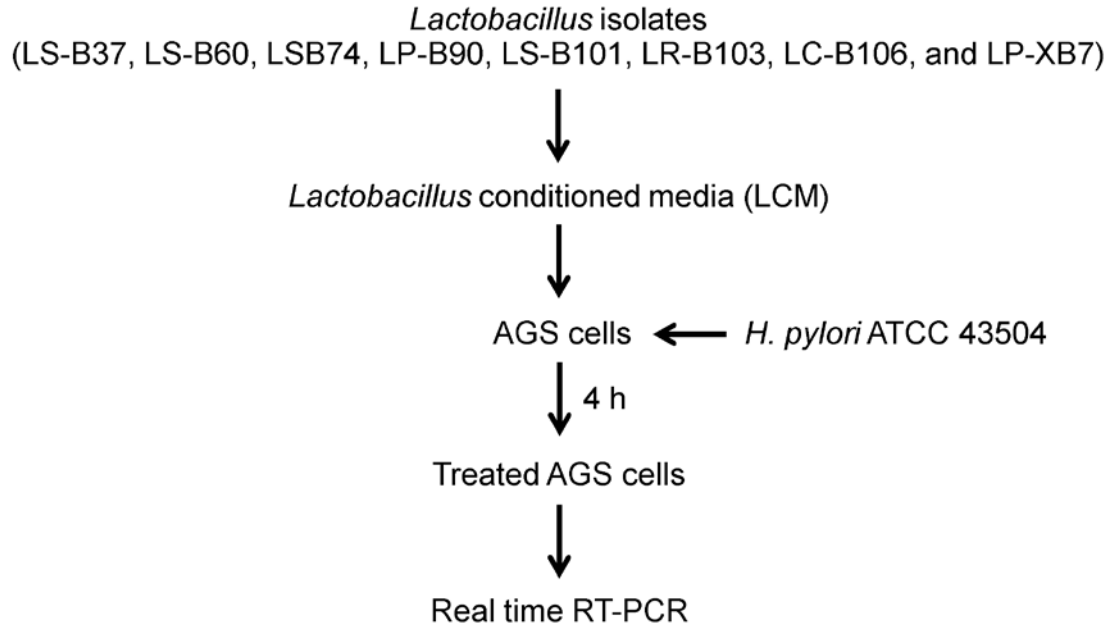
## APPENDIX C

## FLOW CHART

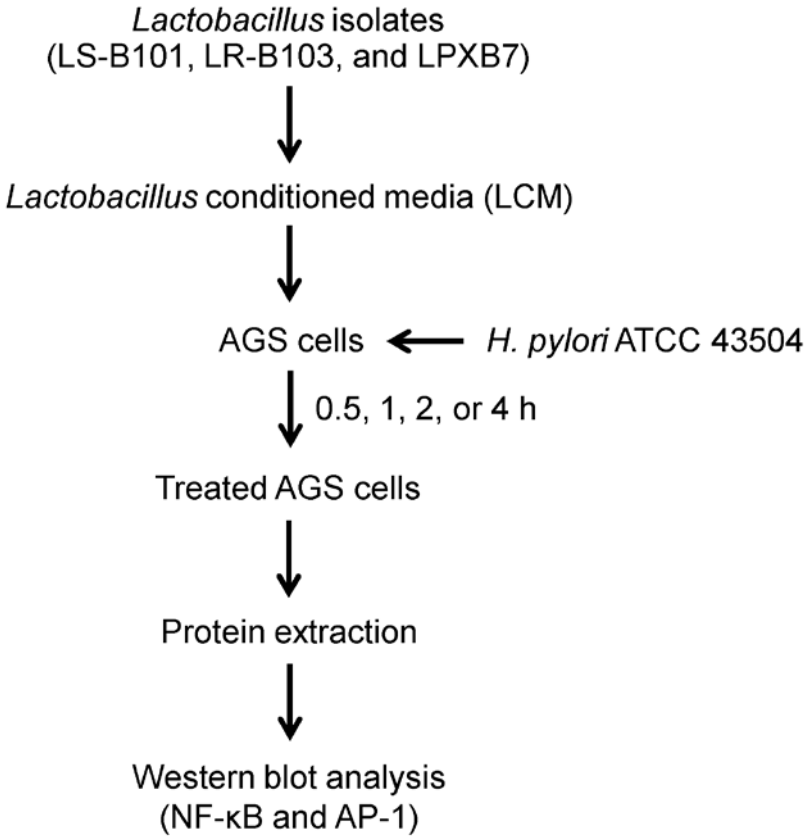
**Identification of *Lactobacillus* isolates that suppress IL-8 production in translation level**



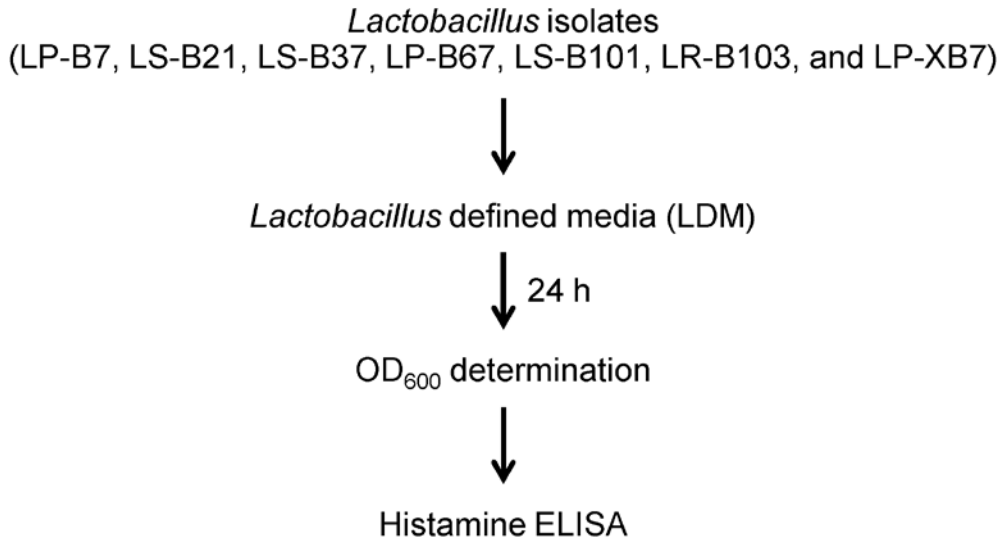
**Identification of *Lactobacillus* isolates that suppress IL-8 production in transcription level**



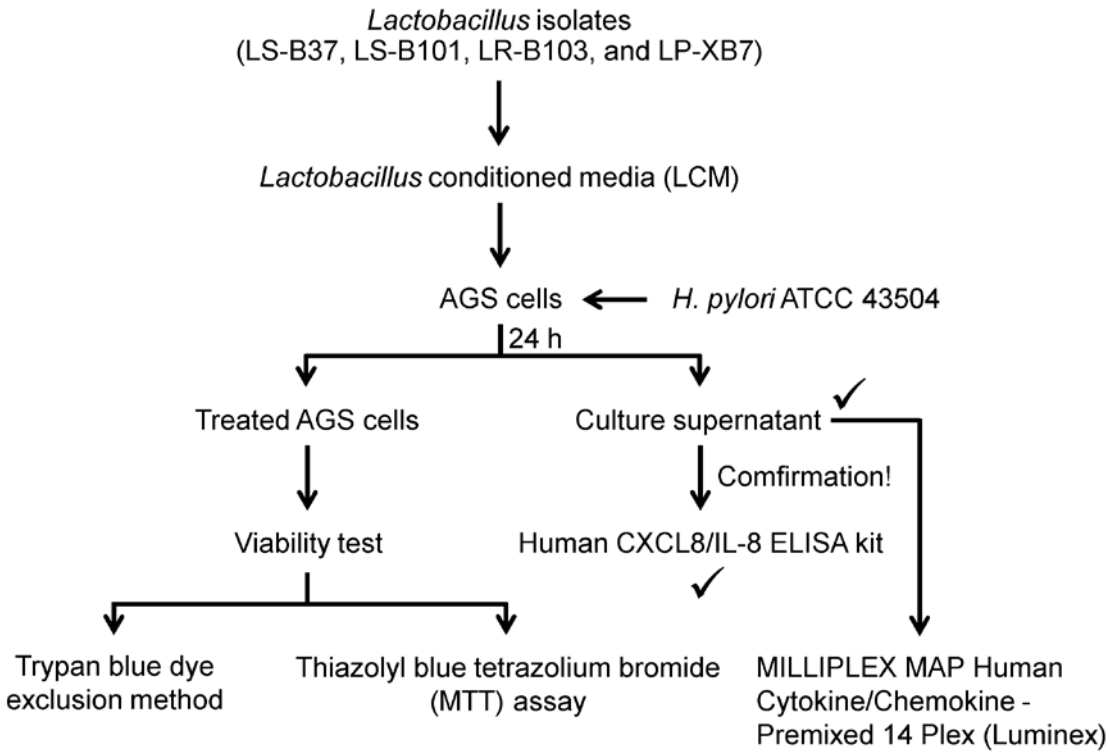
**Determination of *Lactobacillus*-mediated signaling pathway in IL-8 suppression in gastric epithelial cell**



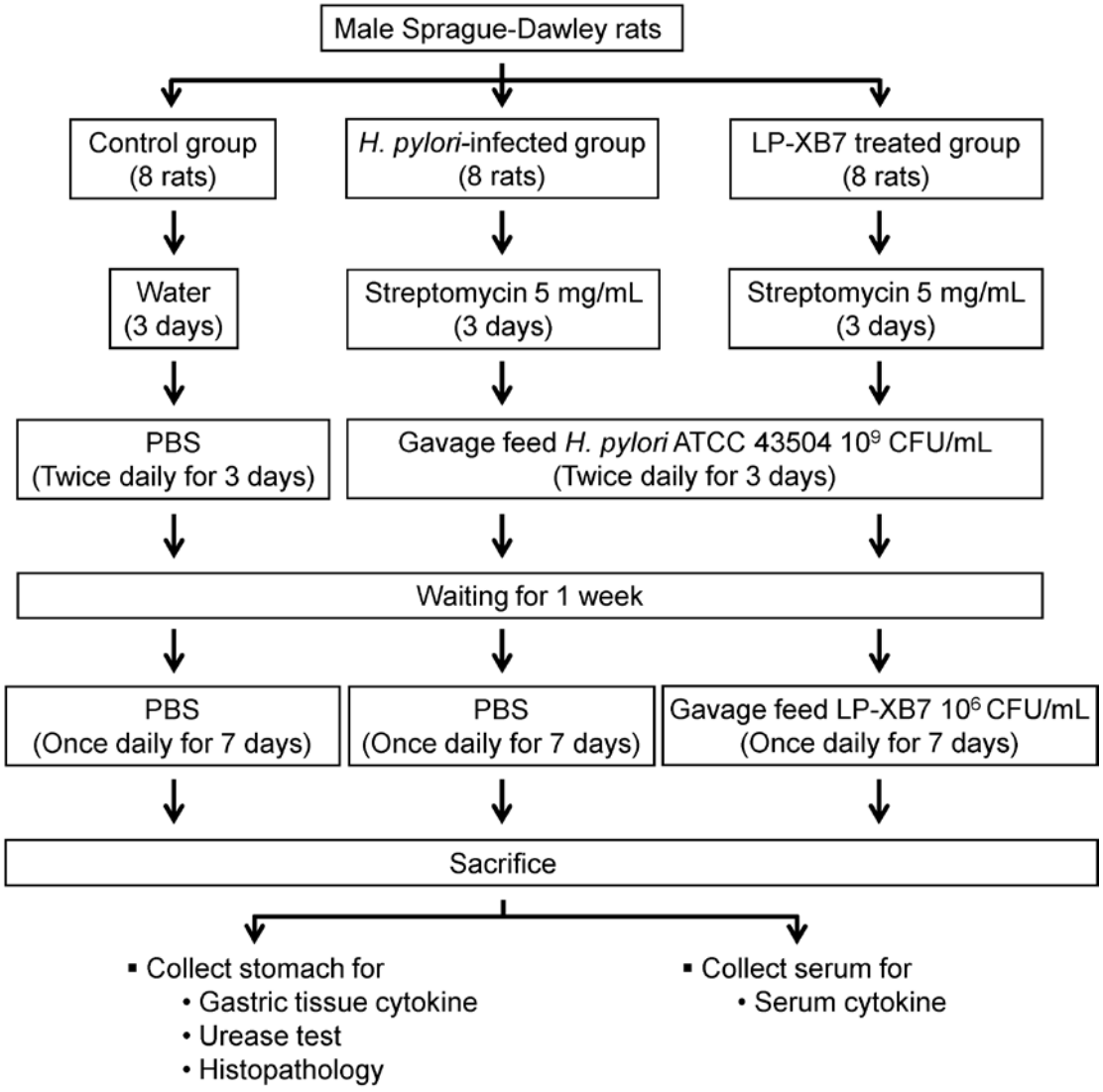
**Histamine assay**



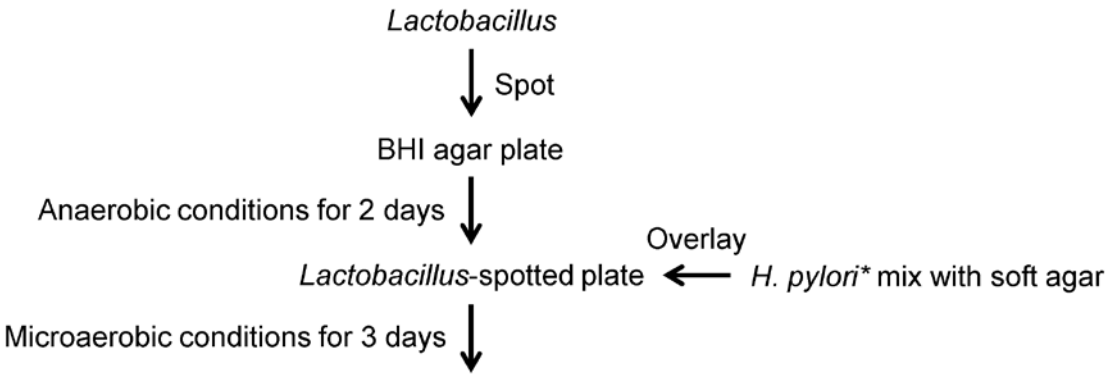
**Cytokine profiles**



***In vivo* work flow**



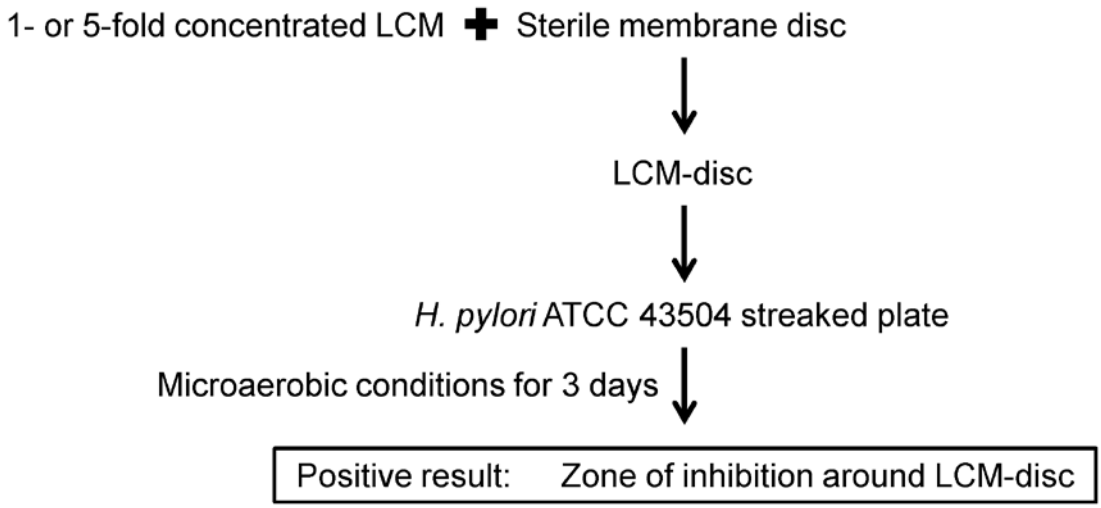
**Screening of *Lactobacillus* for antagonistic activity to *H. pylori* by spot-overlay method**




| | |
|---|---|
| Positive result: | Zone of inhibition around spot of *Lactobacillus* (Isolates LS-B23 and LS-B37 gave positive results) |
| Negative result: | No zone of inhibition around spot of *Lactobacillus* |

* *H. pylori* ATCC 43504 or ATCC 51110 or ATCC 51932

**Confirmation of results of *Lactobacillus* for antagonistic activity to *H. pylori* by LCM-disc method**




- Advantage of LCM-disc method
  - Easy to study for further characterization of antagonistic substance

# APPENDIX D

## MEDIA FORMULA

### Media for *H. pylori*

**Columbia agar with 7% horse serum and 7% sheep blood**

| | | |
|---|---|---|
| Columbia blood agar (Oxoid) | 39 | g |
| Horse serum (Gibco) | 70 | mL |
| Sheep blood | 70 | mL |
| Distilled water | 860 | mL |
| Total | 1,000 | mL |

**Columbia agar with 10% horse serum**

| | | |
|---|---|---|
| Columbia blood agar (Oxoid) | 39 | g |
| Horse serum (Gibco) | 100 | mL |
| Distilled water | 900 | mL |
| Total | 1,000 | mL |

**Brain heart infusion broth with 10% horse serum**

| | | |
|---|---|---|
| Brain heart infusion broth (BBL$^{TM}$) | 37 | g |
| Horse serum (Gibco) | 100 | mL |
| Distilled water | 900 | mL |
| Total | 1,000 | mL |

**Soft agar (brain heart infusion broth with 10% horse serum)**

| | | |
|---|---|---|
| Brain heart infusion broth (BBL$^{TM}$) | 37 | g |
| Agar | 7.5 | g |
| Horse serum (Gibco) | 100 | mL |
| Distilled water | 900 | mL |
| Total | 1,000 | mL |

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

| | | |
|---|---|---|
| Brain heart infusion broth (BBL$^{TM}$) | 3.7 | g |
| Yeast extract | 0.15 | g |
| Horse serum (Oxoid) | 5 | mL |
| Glycerol | 30 | mL |
| Distilled water | 65 | mL |
| Total | 100 | mL |

**Media for lactobacilli**

**MRS agar**

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

**MRS broth**

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

**Brain heart infusion agar**

| | | |
|---|---|---|
| Brain heart infusion agar (BBL™) | 52 | g |
| Distilled water | 1,000 | mL |
| Total | 1,000 | mL |

**Brain heart infusion broth**

| | | |
|---|---|---|
| Brain heart infusion broth (BBL™) | 37 | g |
| Distilled water | 1,000 | mL |
| Total | 1,000 | mL |

**Stock media (MRS broth with 20% glycerol)**

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

# BIOGRAPHY

Mr. Thien Thiraworawong was born on May 30, 1985 in Bangkok, Thailand. He graduated with Bachelor's degree of Science (1st Class Honors) in Biology from the Faculty of Science, Srinakharinwirot University in 2006. During his undergraduate study, he received the scholarship "Human Resource Development in Science Project (Science Achievement Scholarship of Thailand, SAST)" from Office of the Higher Education Commission (OHEC). During his study in Ph.D. program, he received the scholarship from The Royal Golden Jubilee Ph.D. Program, Thailand Research Fund (TRF).

## FIELDS OF INTEREST

Molecular Bacteriology, Cell Biology, Probiotic, *Helicobacter pylori*

## TRAINING

Training on Animal Experimentation for Scientific Purposes. 1/2012. 25-27 April 2012, Chulalongkorn University, Thailand.

## PUBLICATION

Panpetch P, **Thiraworawong T**, Tumwasorn S. Human gastric biopsy-derived lactobacilli suppress *Helicobacter pylori*-induced interleukin-8 production from gastric epithelial cells *in vitro*. International Journal of Interferon, Cytokine and Mediator Research. 2011. 3:43-49.

## POSTER PRESENTATION

Panpetch P, **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). 15-17 June 2011. Thailand.