STUDIES ON PROBIOTIC *LACTOBACILLUS* SPP. IN THE SUPPRESSION OF TUMOR NECROSIS FACTOR-α AND/ OR INHIBITION OF *HELICOBACTER PYLORI*

A THESIS

BY

BOONYARUT LADDA

Presented in Partial Fulfillment of the Requirements for the

Master of Science in Molecular Biology

Srinakharinwirot University

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AN ABSTRACT

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Boonyarut Ladda. (2012). *Studies on probiotic Lactobacillus spp. in the suppression of tumor necrosis factor-α and/ or inhibition of Helicobacter pylori*. Master thesis, M.Sc. (Molecular biology). Bangkok: Graduate School, Srinakharinwirot University. Advisor Committee: Dr. Malai Taweechotipatr, Dr. Piyatida Tangteerawatana

Lactobacillus species have beneficial to the health effects as probiotics. In this study, *Lactobacillus* strains isolated from infant feces and fermented food in Thailand to investigate ability to inhibit *Helicobacter pylori* growth and suppress TNF-α secretion. Four hundred *Lactobacillus* isolates were tested for antagonistic activity against *H. pylori* by using agar spot method. The suppression of tumor necrosis factor-α production was tested by lipoplysaccharide-activated THP-1 monocytic cells in condition with and without selected *Lactobacillus* isolates. The results demonstrated that 2 isolates displayed strong inhibitory activities against *H. pylori*. Thirty nine isolates significantly inhibited TNF-α production in varying degrees. Interestingly, CM128 and F19 strains exhibited the most potent TNF-α inhibition by 82% and 98%, respectively. However, five isolates which showed the most potent TNF-α inhibition (81-98%) were selected to analyze their characteristic.

The identification of selected *Lactobacillus* strains were determined by API 50 CHL revealed that, 2 anti- *H. pylori* strains were identified as *L. paracasei*. The two strains (CM128 and F19) with the most potent TNF-α inhibition were also identified as *L. paracasei*. CM187 strains was identitical as *L. fermentum*. F1 and F19 strains were identitical to *L. plantarum.* Our founding indicated that the *Lactobacillus* isolates from feces and fermented food in this study may use as probiotics to inhibit *H. pylori* growth and suppress inflammation as well as promoting human health.

การศึกษาโพรไบโอติกแลคโตบาซิลลัสสปีชีส์ในการลด tumor necrosis factor-α
และหรือยับยั้ง Helicobacter pylori

บทคัดย่อ

ของ

บุญรัตน์ ลัดดา

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษาตาม
หลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาอณูชีววิทยา

พฤษภาคม 2012

บุญรัตน์ ลัดดา. (2012). *การศึกษาโพรไบโอติกแลคโตบาซิลลัสสปีชีส์ในการลด tumor necrosis factor-α และหรือยับยั้ง Helicobacter pylori.* ปริญญานิพนธ์ วท. ม. (อณูชีววิทยา). กรุงเทพ : บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ.คณะกรรมการควบคุม: ดร. มาลัย ทวีโชติภัทร์, ดร. ปิยะธิดา ตั้งธีระวัฒนะ

แลคโตบาซิลลัสสปีชีส์มีประโยชน์ต่อร่างกายในการนำมาใช้เป็นโพรไบโอติก ในการศึกษาครั้งนี้ได้แยกเชื้อแลคโตบาซิลลัสจากอุจจาระของทารกและอาหารหมักดองในประเทศไทย เพื่อศึกษาความสามารถในการยับยั้งการเจริญของเชื้อ *Helicobacter pylori* และความสามารถในด้านการยับยั้งการสร้าง tumor necrosis factor-α (TNF-α) จากการนำแลคโตบาซิลลัสประมาณ 400 สายพันธุ์ มาทดสอบความสามารถในการยับยั้งการเจริญของเชื้อ *H. pylori* โดยวิธี spot test ทดสอบความสามารถในการยับยั้งการสร้าง TNF-α ในเซลล์โมโนไซต์ THP-1 ที่กระตุ้นด้วย lipopolysaccharide (LPS) ผลการศึกษาพบว่า เชื้อแลคโตบาซิลลัส 2 สายพันธุ์ สามารถยับยั้งเชื้อ *H. pylori* ได้ดี มีแลคโตบาซิลลัส 39 สายพันธุ์สามารถยับยั้งการสร้าง TNF-α ได้ต่างๆ กันอย่างมีนัยสำคัญ และเป็นที่น่าสนใจว่าสายพันธุ์ CM128 และ F19 สามารถยับยั้งการสร้าง TNF-α ได้สูงสุดประมาณ 81-98% อย่างไรก็ตาม มีจำนวน 5 สายพันธุ์ที่ยับยั้ง TNF-α ได้สูงที่สุดจึงคัดเลือกมาศึกษาคุณลักษณะของเชื้อต่อไป

การศึกษาคุณลักษณะของสายพันธุ์แลคโตบาซิลลัสที่คัดเลือกได้โดยใช้วิธี API 50 CHL พบว่า แลคโตบาซิลลัส 2 สายพันธุ์ที่ยับยั้ง *H. pylori* พบว่าเป็น *L. paracasei* มี 2 สายพันธุ์ คือ สายพันธุ์ CM128 และสายพันธุ์ F19 สามารถยับยั้งการสร้าง TNF-α ได้สูงสุดเป็น *L. paracasei* เช่นเดียวกัน สายพันธุ์CM187 เป็น *L. fermentum* สายพันธุ์ F1 และF19 เป็น *L. plantarum* ในการศึกษาครั้งนี้แสดงให้เห็นว่าสายพันธุ์ของแลคโตบาซิลลัสที่แยกได้จากอุจจาระและอาหารหมักดองอาจจะใช้เป็นโพรไบโอติกในการยับยั้งการเจริญของเชื้อ *H. pylori* และยับยั้งการอักเสบรวมถึงให้ประโยชน์ต่อร่างกาย

The thesis titled

" Studies on probiotic *Lactobacillus* spp. in the suppression of tumor necrosis factor-α

and/ or inhibition of *Helicobacter pylori* "

by

Boonyarut Ladda

has been approved by the Graduate School as partial fulfillment of the requirements for the

Master of Science degree in Molecular Biology of Srinakharinwirot University.

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Acknowledgements

I would like to express my deepest gratitude to my thesis advisor, Dr. Malai Taweechotipatr, for her excellent advice, kind guidance, support and encouragement throughout the period of this study. I would like to extend my deep gratitude to my thesis co-advisor, Dr. Piyatida Tangteerawatta, for their valuable suggestions, expert guidance, support and encouragement for the completeness of this thesis.

I am also very grateful to my advisory committee, Assistant Professor Dr. Surangrat Srisurapanon, the Chairperson, Prof. Dr. Somboon Tanasupawat, the external committee for their kindness, helpful suggestions, criticism and corrections for the completeness of this thesis.

Special acknowledgement is sincerely due to all of volunteers and the Srinakharinwirot University HRH Princess Maha Chakri Sirindhorn medical center.

I would like to thank Department of Microbiology, Faculty of Medicine, Srinakharinwirot University for providing equipments and instruments on my research. I would like to give special thanks to Miss Sirinet Phonnok, Miss Juntana Chimchang, and laboratory staff that suggested and contributed her knowledge, helpfulness and encouragement. Also, thank to my friend and senior master and doctoral degree students of the Faculty of Medicine, Srinakharinwirot University for their helpfulness and encouragement.

I would like to express my infinite grateful to my parents and every member of my family for their love, understanding, encouragement and support every time.

Boonyarut Ladda

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CHAPTER I

INTRODUCTION

Genus *Lactobacillus* includes 199 species (www.dsmz.de) that are aerobic or facultatively anaerobic or strictly anaerobic bacteria. Lactobacilli are gram-positive, non-motile, non-sporulating, non-catalase producing, rod-shaped bacteria. *Lactobacillus* species are widely use in food and dairy industrial [1, 2]. Lactobacilli are acid tolerant and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product [3]. They are microbiota of the gastrointestinal tract of humans and animals [1]. Several species have been isolated from humans [4], animals [5] and fermented foods products [6]. The colonization of each species is host specific, for example, *Lactobacillus amylovorus*, *L. gasseri*, *L. fermentum*, *L. delbrueckii*, *L. crispatus*, *L. vaginalis* and *L. ruminis* are commonly found in human feces [7].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [8]. Currently, the most common and the best studied probiotics activity against infectious diseases and inflammatory bowel disease [9, 10] are lactic acid-producing bacteria, particularly *Lactobacillus* spp. and *Bifidobacterium* spp. [11]. Lactobacilli are considered to have beneficial effects on the human and animal health. Several species of lactobacilli are used as probiotics [12]. Since, they can resist gastric and bile salts [13], adhere to intestinal tissues, colonize intestinal tract and are relatively harmless [14]. It has been reported that *Lactobacillus* can reduce the symtoms of the gastrointestinal tract infections [15]. Furthermore, *Lactobacillus* species can also inhibit the growth of *H. pylori* infection in asymptomatic and symptomatic patients [16-17] and reduce side effects of *H. pylori* antibiotics treatment in children and adults [18-19].

Infections with gastrointestinal pathogens continue to be major health problem worldwide. Gastrointestinal infection with *Helicobacter pylori* is associated with gastritis, gastric and duodenal ulcers and a risk factor for cancer [20]. This organism can be found in 70-90 % of the population in developing countries and in 25-50 % in developed countries [21]. Recenly, the treatment of *H. pylori* infection required the combination triple therapy;

1) clarithromycin, 2) amoxicillin and 3) a proton pump inhibitor. Since, *H. pylori* resistance to one antibiotic therapy. The most prevalence *H. pylori* resistance to clarithromycin found in Europian children, whereas resistance to amoxicillin has been reported in some prevalence studies in Brazilian and Chinese [22]. Thus, Probiotics have been suggested as a safer alternative to chemotherapy in the management of gastrointestinal disorders caused by infectious agents. Therefore, probiotics may have a potential for preventing such disorders [23].

Helicobacter pylori induces production of the pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) which leading causes of the inflammatory diseases in the gastrointestinal tract such as gastritis, peptic ulcers and gastric adenocarcinoma [24]. Several studies have been shown that there was a significantly reduction in the density of *H. pylori* and the severity of gastric inflammation in volunteers infected with *H. pylori* following the intake of fermented milk containing *L. johnsonii* LAI [25]. *L. salivarius* was found to be potentially effective against *H. pylori* and reduce the inflammatory response in *H. pylori*-infected gnotobiotic murine model after oral administration [26]. Furthermore, *Lactobacillus* can also reduce inflammation of the stomach caused by infection with *H. pylori* [27-28], inhibit secretion of interleukin-8 [29], and reduce the symptoms of inflammatory bowel disease [30].

Tumor necrosis factor alpha represents a pro-inflammatory cytokine produced by intestinal epithelial cells, In addition, it is largely derived from activated macrophages in the intestinal mucosa. Over production of TNF-α during *H. pylori* infection is implicated in pathogenesis of chronic intestinal inflammation. *Lactobacillus* species have been demonstrated to effectively diminish TNF- α production by *L. rhamnosus* GG-conditioned media in LPS-activated macrophages [31]. Interestingly, *L. rhamnosus* GG-conditioned media also decreases TNF-α production of *H. pylori* and *H. hepaticus*- activated macrophages [32]. *L. plantarum* Lp91 down regulate TNF- α in colitis mouse model [33]. *L. delbruekii* and *L. fermentum* decrease pro-inflammatory cytokines (TNF- α, IL-6) in ulcerative colitis [34]. *L. reuteri* strains suppressed human TNF production by LPS-activated monocytoid cells [35].

Lactobacillus species are considered to have beneficial effects on the human health by interference with gastrointestinal pathogens and able to suppress pathologic inflammatory responses in the gastrointestinal tract. Therefore, several species of lactobacilli are used as probiotics for treatment and prevention of gastrointestinal disorders. However, the inhibitory effect of *Lactobacillus* is variable even within the same or different species and the underlying mechanism remain unclear. Consequently, selection of optimal *Lactobacillus* strains against gastrointestinal pathogens and anti-inflammatory cytokines responses are required for further investigation.

In this study, *Lactobacillus* strains isolated from infant feces and fermented food products to investigate for two main probiotic properties which are the inhibitory effect against pathogens and the suppression of TNF-α production.

Hypothesis

Healthy infant feces and fermented foods probiotic *Lactobacillus* strains play a role in suppression of TNF-α production and/or inhibition of *H. pylori* growth.

Objectives

1. To search for antagonistic activities of *Lactobacillus* isolates against *Helicobacter pylori*
2. To search for anti-inflammatory activities of *Lactobacillus* isolates on the suppression of TNF-α production
3. To study the acid and bile tolerances of selected *Lactobacillus* candidates
4. To study the phenotypic characteristics of selected *Lactobacillus* candidates

CHAPTER II

LITERATURE REVIEW

Genus *Lactobacillus*

The genus *Lactobacillus* belong to family Lactobacillaceae (shown in Figure 1). They are Gram positive rod or coccobacilli shaped, non-sporulating and anaerobic bacteria. Lactobacilli are found in gastrointestinal tract, female urogenital tract and human feces. Lactobacilli play an important role in normal gut function and protection against infectious disease [36]. Lactobacilli are commonly isolated from human feces including *L. acidophilus, L. crispatus, L. delbrueckii (*ssp. *bulgaricus* and ssp.*lactis), L. fermentum, L. gasseri, L. jensenii, L. paracasei (*ssp. *paracasei and* ssp. *tolerans), L. plantarum, L. reuteri, L. rhamnosus* and *L. salivarius* (ssp. *salicinius* and ssp. *V salivarius* [37]. Moreover, they can be found in plants or materials of plant origin, silage and fermented foods such as *L. johnsonii*, *L. casei*, *L. paracasei L. rhamnosus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. brevis*, *L. delbrueckii*, *L. sakei* and *L. curvatus* [36].

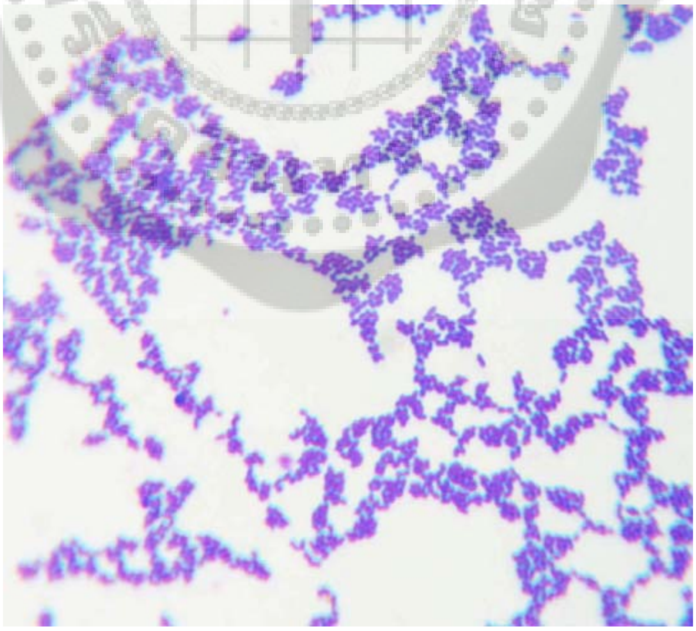


Figure 1 Genus *Lactobacillus* [36]

Lactobacillus as probiotics

Definition of probiotics

The term "probiotics" comes from the Greek meaning "for life". Probiotics were first used to described substances produced by one microorganism that stimulate the growth of another microorganism [38]. Fuller defined a probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" [39]. Recently, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" by Food and Agriculture Organization (FAO) and World Health Organization (WHO) [8].

Probiotic properties

For the selection of potential probiotics, the research recommended that microorganisms should have some predefined criteria in order to be considered as probiotics. These criteria were summarized in Table 1 [40]. Probiotic strains of human origin are most suitable because health promoting benefits may be species specific and microorganisms may perform optimally in the species from which they were isolated [41]. All probiotic strains should have generally recognized as safe (GRAS) status [42]. To survival during passage through the human gut, probiotics must tolerate to gastric acid and bile salt conditions and able to grow in the presence of bile and acids. Adherent probiotics strains are desirable because they have a greater chance of prolonged residence in the gastrointestinal tract for promoting health [43]. The production of antimicrobial substances is regarded as important selection criteria for probiotics. The report showed that *L. reuteri* able to produce reuterin which is antimicrobial substances and inhibit the growth of different enteric pathogens *in vitro* [44]. The ability of probiotic bacteria to modulate immune responses is important for protection of immune disorder. Probiotic microorganisms should be technologically suitable for incorporation into food industry and commercial fermented food products [41].

Table1 Criteria for an ideal probiotic strains

Desirable characteristics of an ideal probiotic microorganisms
Human origin
Generally recognized as safe (GRAS) status
Resistance to gastric acidity and bile toxicity
Adherence to gut epithelial tissue
Ability to colonize the gastrointestinal tract
Production of antimicrobial substances
Ability to modulate immune responses
Amenable to large scale fermentation and commercial production

Mechanism of action of probiotics

There are several proposed mechanisms of actions of probiotics against infectious diseases and intestinal disorder, but the main mechanisms remained unclear. The general mechanisms underlying the health promoting capacities of *Lactobacillus* include antimicrobial activity, epithelial barrier protection and immunomodulation (Figure 2).

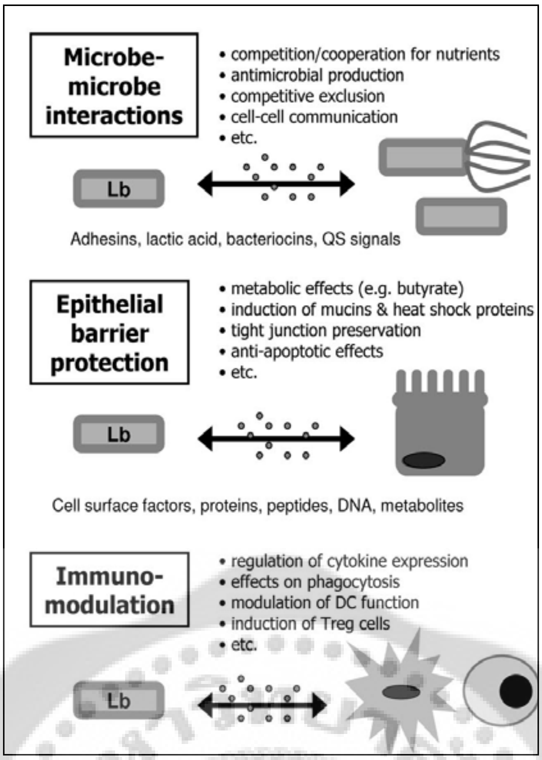


Figure 2 Summary of probiotics mechanisms [36]

Antimicrobial activity (microbe-microbe interaction)

Probiotic bacteria have been antagonized pathogenic bacteria by reducing luminal pH, producing antimicrobial substances and competitive exclusion [45]. It has been demonstrated that probiotic *Bacillus breve* produced a high concentration of acetic acid and lowered the pH of the intestine, results in the protection of mice from Shiga toxin-producing *E. coli* O157:H7 (STEC) infections [46].

Probiotic bacteria produced antimicrobial substances, bacteriocins and stimulated human defensin productions. Several bacteriocins produced by the genus *Lactobacillus* have been described. Bacteriocins from *L. rhamnosus* GP1 inhibited the growth of *Bacillus brevis*, *B. pumilus*, *B. subtilis, Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio harveyi*, *Acinetobacter* sp. and *Arthrobacter* sp. *L. plantarum* AMA-K inhibits the growth of *Enterococcus* spp., *Escherichia coli, Klebsiella pneumoniae* and *Listeria* spp. [47-48].

Defensins are anti-microbial peptides found in human epithelial cells and phagocytic cells [49]. The recent report suggests that lactobacilli and VSL#3 induced the secretion of the human beta defensins-2 (hBD-2) via pro-inflammatory pathways including nuclear factor kappa B (NF-kB), activating protein-1 (AP-1) and mitogen-activated protein kinases (MAPKs) [(50)].

The competitive exclusion is blocking the sites of pathogenic bacteria colonization. Probiotic bacteria competes nutrients or produce organic acids for eject the colonization and prevent the adhesion of pathogenic bacteria to the gut [(51)].

Epithelial barrier protection

Maintenance of barrier function including promote tight contact between epithelial cells forming a functional barrier, enhancing the production of defensive molecules such as mucins. The defect in intestinal epithelial barrier function was observed in inflammatory bowel disease (IBD) patients [(52)]. A murine colitis treated with VSL#3 has ability to maintain tight junction protein expression and increase apoptotic ratio [(53)], suggesting the protection of barrier function by probiotic bacteria.

Immunomodulation

Probiotic bacteria are claimed to have immunomodulating effects. Lactobacilli were able to modulate immune cell (CD+4 cells) in the gastrointestinal mucosa after dietary supplementation with *L. reuteri* ATCC 55730 [(54)]. Enhancing immune system related beneficial in inflammatory bowel disease (IBD) including Crohn's disease and Ulcerative colitis. *L. plantarum* Lp91 significantly up regulated anti-inflammatory markers, COX1, interleukin(IL-4) and IL-6 in mouse colitis model [(55)]. Similarly, probiotic *L. reuteri* strain ATCC PTA 6475 suppressed human TNF-α production in lipopolysaccharide (LPS)-activated monocytes and primary monocyte-derived macrophages from children with Crohn's disease [(56)].

Strains used as probiotics

Several microorganisms have been used or considered for using as probiotics [(57)]. Each strain of microorganisms was prepared as probiotic in different conditions. These probiotic preparations may be presented as powders, tablets, pastes or sprays form depending on the condition for therapy or receiveing as supplement in each individuals animal and human.

The most commonly used probiotics are strains of lactic acid bacteria especially, *Lactobacillus* and *Bifidobacterium*. The beneficial effects of *Lactobacillus* and *Bifidobacterium* have been discussed for decades. Bacteria in these two genera resist gastric acid, bile salts, pancreatic enzymes, adhere to the intestinal epithelium cell lining and colonize the lumen of the tract, produce antimicrobial substances and stabilize the intestinal microflora [(57)]. *Lactobacillus species* are used as human probiotics, because easy to cultivation and have a long history of safe use in fermented food [(58)]. As shown in Table 2, Genus *Lactobacillus, Bifidobacterium* and other microorganisms are currently being used as probiotics either singly or in combination. The most commonly used probiotics against enteropathogen are the *Lactobacillus spp*. which, they resistance to bile and low pH, attachment to intestinal cells activities [(59)]. *Lactobacillus* species have been demonstrated to inhibit the pathogenic bacteria including *Klebsiella pneumoniae, Escherichia coli, Salmonella enteritidis and Pseudomonas flourescen, Salmonella enterica* ssp. enterica *in vitro* [(57)]. The recently studied have showed *L. salivarius* C3 and *L. rhamnosus* C44 able to grow in acid and bile condition and adherence to intestinal cell [(60)]. *Bifidobacterium* have been shown antagonistic activity against *Shigella sonnei*, *S. typhimurium* [(11, 61)]. In additions, *Enterococcus faecium* able to decrease duration of acute diarrhoea from gastroenteritis. *Bacteroides species* able to decrease chronic colitis, gastritis, arthritis and *Saccharomyces boulardii* also reduced recurrence of *Clostridium difficile* diarrhea [(57)]. It has been reported that VSL#3 administration in a dose-dependent able to decrease cellular invasion by the pathogenic bacteria *Salmonella enterica* serovar Dublin [(62)].

Table 2 Microorganisms considered as probiotics [57]

Lactobacillus species	*Bifidobacterium species*	Others
L. casei	*B. longum*	*Enterococcus faecium*
L. rhamnosus	*B. lactis*	*Bacteroides species*
L. fermentum	*B. animalis*	*Saccharomyces boulardii*
L. acidophilus	*B. bifidum*	VSL#3 (*L.bulgaricus, L. plantarum, B. longum, B. infantis, B. breve, S. salivarius* subsp. *thermophilus*)
L. plantarum		
L. paracasei		
L. salivarius		

The role of probiotics in gastrointestinal disorders

***Helicobacter pylori* gastroenteritis**

Helicobacter pylori is a gram negative bacterium with a helical rod shape and micro-aerophilic found on surface of the gastric epithelium (shown in Figure 3). *H. pylori* have been shown to be an important aetiologic agent of gastritis, peptic ulcer and a risk factor for the development of gastric adenocarcinoma and lymphoma in humans. The mixture of live probiotics *L. rhamnosus* and *L. acidophilus* reduced *H. pylori* colonization in infected mice [63]. Probiotics combination including *L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* subsp. *shermanii* Js, *Bifidobacterium breve* Bb99 were inhibited *H. pylori* adhesion in Caco-2 cells [64]. The report in human showed that probiotic *Bifidobacterium bifidum* BF-1 suppressed *H. pylori* inducing IL- 8 in the gastric mucosa [65].

Figure 3 *H. pylori* isolated from a patient's biopsy (Gram-staining, 1000x) [66].

Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) including two clinical forms of Crohn's disease (CD) and ulcerative colitis (UC) [67]. The etiology of IBD is not clear understood. IBD has inflamed small and large intestine. The most common clinical manifestation of UC is an inflammation of the colon. No specific treatment is available for either disease [68]. The factors initiating to IBD has four basic viewpoints including genetics, immune dysregulation, barrier dysfunction and microbial flora [69]. The genetic factor showed that whole genome scans have found susceptibility genes for UC on chromosomes 1 and 4, although these loci have not been confirmed. The immune factor, the cytokine profile including IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) elevated in IBD patients both CD and UC. Especially, TNF-α was upregulated in serum and mucosa of both IBD conditions [67]. The barrier dysfunction factor, intestinal mucosa form a barrier between luminal contents and the interstitium which is compromised in a number of intestinal diseases, most notably in IBD. Evidence increased epithelial permeability suggests that the immune system plays an important role in modulating intestinal permeability. The two cytokine including interferon- γ (IFN-γ) and TNF-α were found in high levels in intestinal mucosa involved in IBD and resulting in decreasing barrier function. Imbalance in TNF-α and TNF-α inhibitors plays an important role in gut inflammation in patients with IBD. The microbial flora factor, enterobacteria were observed significantly more frequently in CD than in healthy human [70].

However, some probiotics including *L. casei* Shirota inhibited IL-6 production in lipopolysaccharide (LPS)-stimulated lamina propria mononuclear cells (LPMCs) isolated from murine IBD [71]. The probiotic mixture VSL#3 decreased expression of the tight junction resulting in protecting the epithelial barrier in acute colitis of murine model [53] and has efficacy on maintenance remission in active UC [72]. The oral administration with lysate of *L. casei* DN-114001 decreases production of pro-inflammatory cytokines TNF-α, IFN-γ and anti-inflammatory IL-10 in Peyer's patches and large intestine resulting in decreasing the severity of intestinal inflammation in a murine model of IBD [73].

CHAPTER III

Materials and methods

Materials

1 MRS agar and MRS broth (Oxoid, Basingstoke, Hampshire, UK)
2 Brain heart infusion (BHI; Himedia, India)
3 Transport medium (Oxoid, Basingstoke, Hampshire, UK)
4 Amphotericin B (Sigma, USA)
5 Horse serum (Gibco-Invitrogen, USA)
6 Lipopolysaccharide from *E.coli* (Sigma, USA)
7 Albumin bovine fraction V powder (Sigma, USA)
8 Microaerobic and Anerobic gas package (MGC, Japan)
9 Cell lines: THP-1 monocytic cells (ATCC, TIB 202)
10 Fetal bovine serum (Gibco-Invitrogen, USA)
11 Penicillin-Streptomycin (Gibco-Invitrogen, USA)
12 RPMI 1640 (Gibco-Invitrogen, USA)
13 Hemocytometer (Hausser Scientific, USA)
14 Counter (Fisher Scientific, USA)
15 ELISA plate: 96- well plate: High binding (Corning, USA)
16 Recombinant human TNF-α (R&D Systems, USA)
17 ELISA kit (R&D Systems, USA)
18 BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA)

Methods

Subjects and fermented food samples

Infant feces

Three hundred healthy infant feces were obtained from HRH princess Maha Chakri Sirindhorn Medical Center. Each individual gave informed consent. Fresh fecal samples were taken by sterile cotton swab and inserted into a modified Cary-Blair transport medium (Oxoid, Hampshire, UK). The samples were processed immediately upon receipt.

Fermented foods

Seven samples of fermented foods including Naem (fermented sausage made with pork and beef), Koh mak (fermented rice), pickled tare, pickled vegetable-like garlic, fermented onions, pickled cabbage and Kimchi.

Cultivation of samples

The fecal samples and fermented foods were diluted in normal saline solution (NSS) and 10-fold serial dilutions were diluted to 10^5-10^6 times. Finally 200 µl of each dilution were spreaded onto deMan-Rogosa-Sharpe (MRS) agar plates (Oxoid, Basingstoke, Hampshire, UK). The plates were incubated at $37^{\circ}C$ for 24-48 hr under anaerobic condition generated by using an anaerobic GasPak (Mitsubishi, Japan) in an anaerobic box (Mitsubishi, Japan). Colonies of different morphologies were isolated on MRS agar plates. After anaerobic incubation, single pure colonies were isolated and subcultured for experimental use.

Selection of *Lactobacillus* isolates

Each of the isolate was presumptively identified by Gram reaction and catalase test. Catalase test was examined by place 3% H_2O_2 on the cell of isolates. Immediate formation of bubbles were indicated the presence of catalase in the cells. Select isolates of catalase-negative, gram- positive, regular rods, short rods or coccobacilli were maintained as frozen cultures in MRS broth with 20% glycerol at -80°C for experimental use.

Antagonistic activities of *Lactobacillus* isolates against *Helicobacter pylori* strain H40

Antimicrobial activities of *Lactobacillus* isolates were tested against *H. pylori* H 40 by agar spot method. This method was carried out as previously described [74]. Briefly, overnight cultures of *Lactobacillus* isolates in each well of 96-well plate were spotted (2 µl) onto the surface of BHI agar supplemented with 20 mM glucose in 15 mm plate. The spots were developed by incubated anaerobic condition at 37°C for 24-48 hr. After incubation, each plate was overlaid with appropriate soft agar (0.8%, v/v) containing *Helicobacter pylori* 1 x 10^8 cells/ml (0.5 McFarland) and plates were incubated under micro-aerobic condition at 37°C for 3 days. Inhibition zones were recorded as clear zone of equal or more than 1 mm around a spot will be scored as positive. These experiments were performed two times in duplicate.

Assay for immunomodulatory effects of *Lactobacillus* isolates on TNF-α Production in LPS-activated THP-1 monocytic cells

Preparation of conditioned media

Lactobacillus isolates from human fecal samples and fermented foods were selected and recovered from -80°C to test for TNF-α production in THP-1 human monocytic cell line. The *Lactobacillus* conditioned media (LCM) were prepared as previously described [9]. In brief, *Lactobacillus* isolates were grown anaerobically in MRS broth at 37 °C overnight. The overnight cultures of lactobacilli were diluted in MRS broth (10^8 cells/ml) and were grown anaerobically at 37°C for 48 hr. *Lactobacillus* cell-free conditioned media was collected by centrifugation at 4,000 x g for 10 min and was filter-sterilized using a 0.22 µm pore size filter (Sigma, USA).The conditioned media were concentrated by speed-vacuum drying (Speed vacuum:

Rotational Vacuum Concentrator RVC 2-18, Germany). The residual pellet was re-suspended in an equal volume of serum-free culture media. All *Lactobacillus*-conditioned media were frozen at -20°C until analysis.

THP-1 cell culture and bioassay

Cell culture and *in vitro* bioassays were performed as previously described [9] Briefly, the THP-1 human monocytic cell lines (ATCC, TIB 202) were maintained in RPMI 1640 (Gibco-Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen, USA) in 96-well flat-bottomed tissue culture plates (Corning, USA) and were incubated at 37°C in humidified 5% CO_2 incubator. For bioassays, cells were counted with hemacytometer (Hausser Scientific, USA) under an inverted microscope (Nikon TMS No. 300679: Nikon, Japan) and diluted to desired cell densities. Two hundred microliters of THP-1 cells were seeded in 96-well plates at a final density 2.5×10^5 cells/ml. Cells were incubated with 10 µl of *Lactobacillus* conditioned media (5% v/v) and 5 µl of (final concentration 100 ng/ml) purified lipopolysaccharide (LPS) from *Escherichia coli* serotype O127:B8 (Sigma, USA). After 3.5 hr at 37°C in a humidified 5% CO_2 incubator, supernatants were collected by centrifugation at 1,000 x g for 9 min in 4°C for TNF-α measurement. Cell viability was assessed by the Trypan-blue (Gibco-Invitrogen, USA) stain exclusion assay.

TNF-α measurement

TNF-α production in THP-1 monocytic cell culture supernatants were measured with cytokine-specific sandwich quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, USA). Briefly, 96-well microtiter plates (Corning, USA) were coated overnight with 100 µl mouse anti-human TNF-α antibodies as capture antibodies diluted in phosphate-buffered saline (PBS) pH 7.4. Plates were washed three times with PBS containing 0.05%

Tween 20 (PBST) to remove excess capture antibody. To reduce non-specific binding, wells were blocked with 300 µl of 1% (w/v) bovine serum album (BSA: Sigma, USA) in PBS (reagent diluent) for 2 hr and were washed three times with PBST. Recombinant human TNF-α were used as standard diluted in reagent diluent at the concentration of 15.625, 31.5, 62.5, 125, 250, 500, and 1,000 pg/ml. Reagent diluent were used for blank. Standard or samples were added (100 µl volume) to appropriate wells and plates were incubated overnight. After washing three times with PBST, 100 µl per well of biotinylated goat anti-human TNF-α antibodies, diluted in reagent diluent were added as detection antibodies and incubated for 2 hr. The plates were washed three times with PBST and were incubated with 100 µl per well of streptavidin-horseradish peroxidase conjugate for 20 min. After washing three times, 100 µl per well of TMB (tetramethyl benzidine) substrate were added to the plates as color indicator and were incubated for 20 min. Stopping reagent consisting of H_2SO_4 were added (50 µl to each well) to stop the reaction. Absorbance was measured at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). All procedures were performed at room temperature. Results of cytokine concentration were quantified from standard curve and expressed as pg/ml of culture medium.

Statistical analysis

Results were expressed as means and standard deviations (± SD). The percentage of TNF-α inhibition and cell viability was calculated by the formula as follows.

$$\%\ \text{TNF-}\alpha\ \text{inhibition} = 100 \times ((\text{observed} \div \text{baseline}) - 1).$$

Where observed = secreted TNF-α of experiment (pg/ml) and baseline = secreted TNF-α of MRS bacterial culture medium (pg/ml).

$$\%\ \text{Cell viability} = 100 \times (1 - (\text{dead cells} \div \text{total cells}).$$

Statistical significance or differences were evaluated by GraphPad Prism version 5.01 using Student's *t*-test with one-tailed distribution. A *p*-value $\leq$ 0.05 was considered statistically significantly.

Acid and bile tolerance test

Seven *Lactobacillus* isolates which showed significantly TNF-α inhibitory activities and displayed the highest antagonistic activities against *H. pylori* H40 were tested for acid and bile tolerance in order to evaluate the relative survivability of candidate probiotics in the gastrointestinal tract.

Acid tolerance test

MRS broth was adjusted with 1 N HCL to pH 2, 3, and 4 respectively. Unadjusted MRS broth was used as control. Overnight cultures of candidate probiotic *Lactobacillus* was diluted to an initial bacterial concentration of 10^8 cells/ml in each 10 ml of adjusted pH MRS broth in duplicated and was incubated at 37°C for 3 hr under anaerobic condition. *Lactobacillus* isolates were taken and serial 10-fold dilutions (in PBS, pH 7.2) of each sample was prepared (10^{-1}-10^{-5}). Viable bacterial counts were determined by spreading 100 µl of each serial dilution onto MRS agar plates. The plates were incubated at 37°C for 24-48 hr under anaerobic condition generated by using an anaerobic GasPak (Mitsubishi, Japan) in an anaerobic jar (Mitsubishi, Japan). Viable bacteria were displayed as cell numbers and the log10 of colonies grown on MRS agar compared to the initial bacterial concentration.

Bile tolerance test

Candidated probiotic *Lactobacillus* was inoculated in 10 ml MRS broth supplemented with 1%, 2%, 3% and 4% bovine and ovine bile (Sigma, USA) and MRS control cultures without bovine and ovine bile. The initial bacterial concentration was 10^8cells/ml. All samples were incubated for 3 hr at 37°C under anaerobic conditions. After incubation, *Lactobacillus* cultures were taken and 10-fold serial dilutions of each sample was prepared (10^{-1}-10^{-5}). Viable cell counts were determined by spreading 100 μl of each dilution onto MRS agar plates. The plates were incubated under anaerobic conditions at 37°C for 24-48 hr in under anaerobic condition generated by using an anaerobic GasPak (Mitsubishi, Japan) in an anaerobic jar (Mitsubishi, Japan). Viable bacteria were displayed as cell numbers and the log 10 of colonies grown on MRS agar compared to the initial bacterial concentration.

Phenotypic characterization of selected *Lactobacillus* isolates by API 50CHL

Carbohydrate fermentation patterns of candidate *Lactobacillus* strains were determined by the API 50 CH rapid fermentation test strips (BioMerieux, France) and the *Lactobacillus* identification (API 50 CHL) medium (BioMerieux, France) according to the manufacturer's instructions. Briefly, the overnight *Lactobacillus* cultures were collected cell pellets by centrifugation at 2,000 x g for 5 min and then re-suspended in NSS or distilled water and diluted in API 50 CHL medium (2 McFarland standard). The diluted cultures were loaded to the API 50 CH test strips and covered with paraffin oil, and the test strips were incubated at 37°C for 48 hours. The test strip information was read and recorded at 24 and 48 hr. The carbohydrate fermentation experiment was performed in duplicate. Species were determined by characterizing their abilities to ferment 49 carbohydrates, and comparing profiles with known carbohydrate fermentation profiles of lactobacilli in the API 50 CH version 5.1 database (http://apiweb.biomerieux.com).

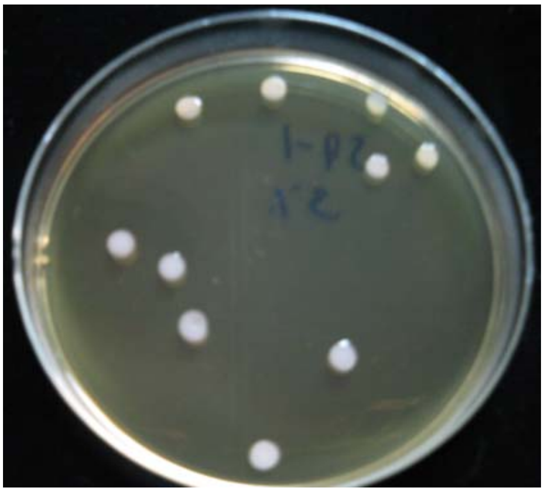
CHAPTER IV

Results

Isolation of *Lactobacillus* Isolates

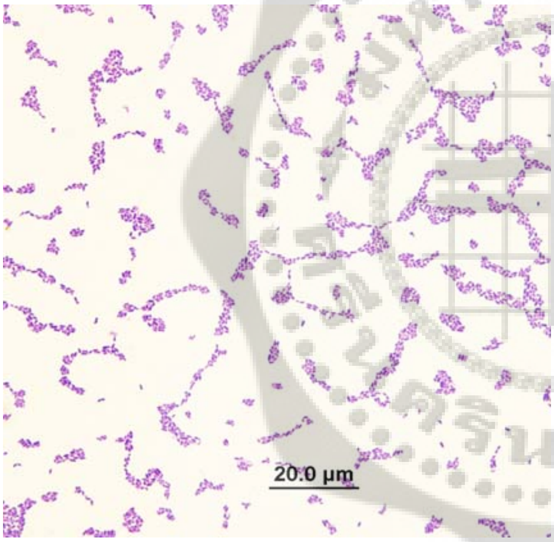
The *Lactobacillus* strains were isolated from 300 fecal samples and 20 fermented foods by presumptive tests including Gram's stain, catalase test. Four hundred *Lactobacillus* isolates were obtained. The colonies varied from small to big colonies with white or yellow, circular, smooth and convex colonial morphologies (Figure 4). They are all gram-positive and catalase-negative. Cell morphology in each isolate varied such as cocci, coccobacilli, short bacilli, long bacilli with granule. Most isolates grew well under anaerobic conditions. Most *Lactobacillus* isolates were obligate anaerobes, while some isolates were facultative anaerobes.

Colony characteristics on MRS agar for strain CM187 and F19 of *Lactobacillus* were demonstrated in Figure 4. Colony morphologies for CM187 (Figure 4A) and F19 (Figure 4B) strains were characterized by 1-2 mm diameters, white color, smooth margins, round and convex contours, but colony of CM187 was bigger than F19 strain. For Gram's staining test, CM187 strain represent short rod (Figure 4C), while F19 strain represent slender rod (Figure 4D).

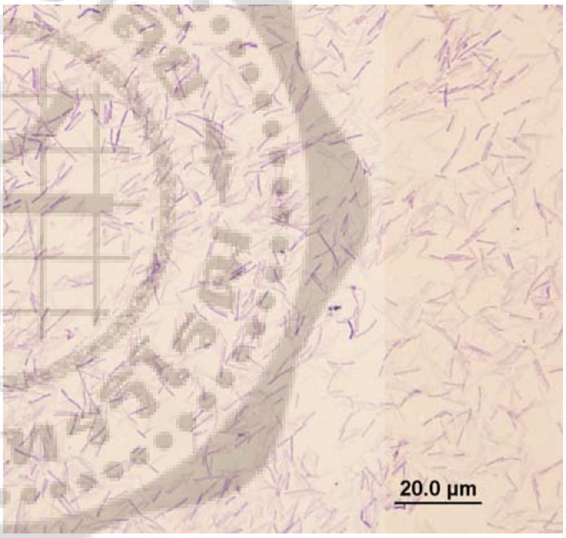


A. Colony of CM187 strain

B. Colony of F19 strain



C. CM187 strain

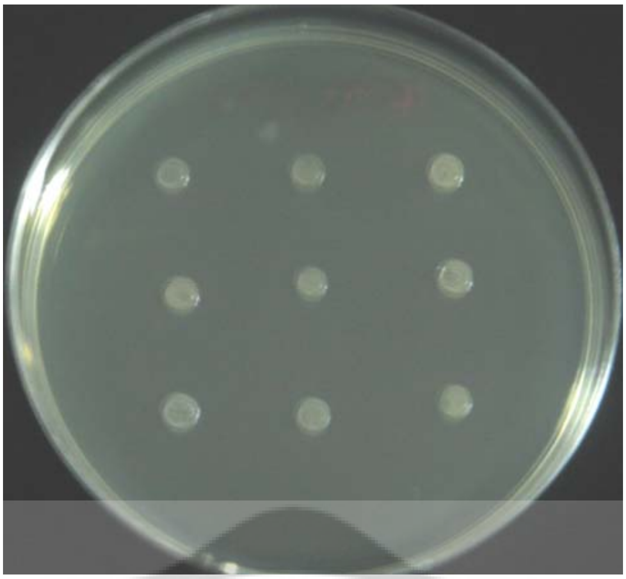


D. F19 strain

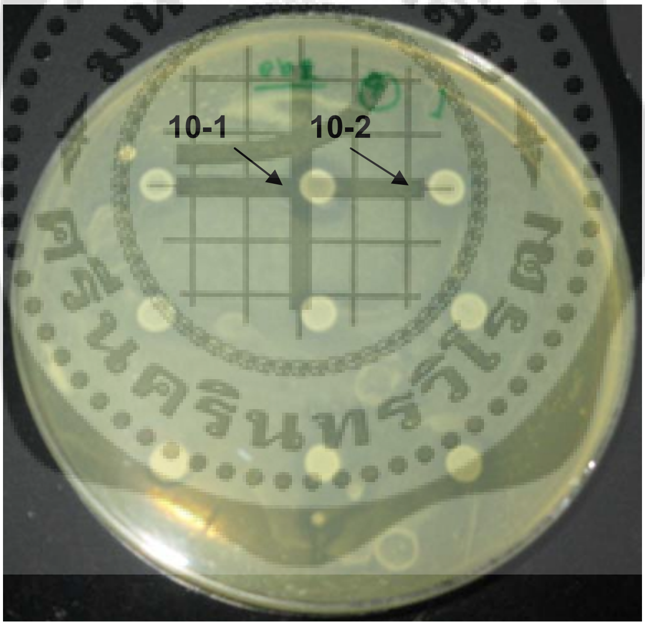
Figure 4 Representative colony morphologies and gram staining of CM128 and F19 strains

Antagonistic activities of *Lactobacillus* isolates against *Helicobacter pylori* strain H40

The antimicrobial activities of *Lactobacillus* isolates against *Helicobacter pylori* H40 were detected by agar spot method. This method was simply tested antimicrobial substances which were secreted directly to the surround *Lactobacillus* isolate spots and were seen as clear zone or inhibition zone. *Lactobacillus* isolates were spotted (Figure 5A) onto the surface of BHI agar supplemented with 20 mM glucose and subsequently overlaid with appropriate soft agar (0.8%, v/v) containing *H. pylori* H40. A clear zone of inhibition > 4 mm surround a spot as demonstrated in Figure 5B was scored as positive. The results indicated that 100 of 400 *Lactobacillus* isolates showed no inhibitory activities. Two hundred and twenty seven *Lactobacillus* isolates showed weak inhibitory activities. Seventy one *Lactobacillus* isolates showed moderate inhibitory activities and two strains showed strong inhibitory activities (Tables 3-7 and Figure 6). Candidated *Lactobacillus* strains such as 10-1 and 10-2 isolates showed strong inhibitory activities to *H. pylori* H40 when compared to *Escherichia coli* and *Pseudomonas*, which were used as negative control as present in Figure 6.

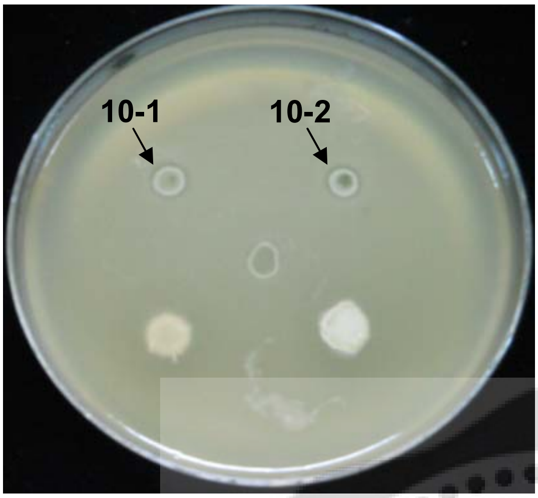


A. Spot of *Lactobacillus*

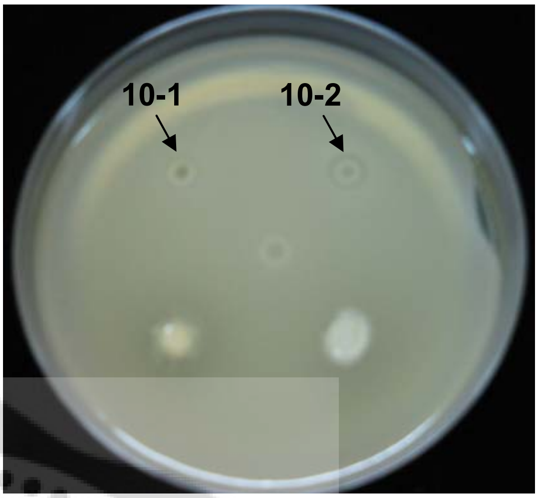


B. 10-1 and 10-2 strains

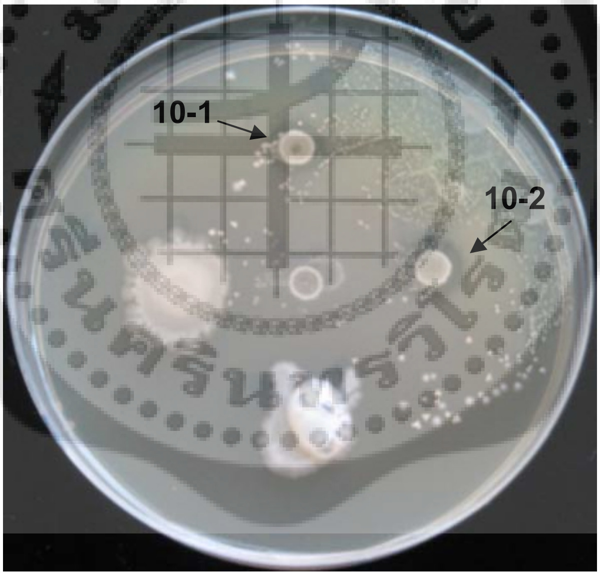
Figure 5 Antagonistic activity of *Lactobacillus* isolates against *H. pylori* H40 using the agar spot method. A) Spot of *Lactobacillus*, B) *Lactobacillus* strain 10-1 and 10-2 inhibit *H. pylori* H40 growth



A. *Escherichia coli*



B. *Pseudomonas*



C. *Helicobacter pylori*

Figure 6 Antagonistic activity of *Lactobacillus* isolates against *H. pylori* H40 when compared to *E. coli* and *Pseudomonas*, which were negative control by using the agar spot method.

Table 3 Antagonistic activities of *Lactobacillus* isolates against *H. pylori* H40

***Lactobacillus* isolates**	**Anti-*H. pylori***	***Lactobacillus* isolates**	**Anti-*H. pylori***
1-1	+	19-3	+
1-2	+	19-4	+
2-1	-	20-1	-
3-1	+	20-2	-
3-2	+	20-3	+
4-1	+	21-1	-
4-2	+	21-2	-
4-4	-	21-3	+
5-1	-	22-1	
5-3	-	23-1	-
6-3	++	23-2	+
6-4	-	23-3	-
6-5	+	23-5	++
6-6	+	23-6	+
7-1	++	24-1	+
7-2	++	24-2	++
7-3	++	25-1	+
8-1	+	25-2	++
8-2	+	25-3	-
8-3	++	26-1	-
9-1	++	27-1	++
10-1	+++	27-2	+
10-2	+++	27-4	+
11-1	+	28-1	-
11-3	+	28-2	+
11-4	-	28-3	++
12-1	++	29-1	+
12-2	-	33-1	-
13-1	+	33-2	+
13-3	-	33-3	-
14-1	+	34-1	++
15-1	+	35-1	+
15-3	+	35-2	-
16-1	+	35-4	+
16-2	++	35-5	+
16-3	+	36-1	+
17-1	+	36-2	+
17-2	+	36-3	+
18-1	+	36-4	+
19-1	++	36-5	++
19-2	++	36-9	+

Lactobacillus spot diameter = 4 mm; -, no inhibition; +, 5-7 mm of inhibition; ++, 8-9 mm of inhibition; +++, 10-11 mm of inhibition

Table 4 Antagonistic activities of *Lactobacillus* isolates against *H. pylori* H40 (continued)

***Lactobacillus* isolates**	**Anti-*H. pylori***	***Lactobacillus* isolates**	**Anti-*H. pylori***
36-10	++	55-3	++
36-12	+	56-1	+
37-1	+	56-3	+
37-2	+	57-1	+
37-3	+	57-3	+
37-4	+	58-1	+
37-6	+	59-1	+
38-3	+	60-1	+
39-1	++	61-1	++
39-2	+	61-3	+
39-3	+	62-1	+
39-4	+	62-2	+
40-1	+	63-1	+
40-2	+	63-3	-
40-3	+	63-5	+
40-7	+	64-1	+
41-1	+	64-3	+
42-1	+	65-1	+
43-1	-	65-3	+
44-1	+	66-1	+
44-2	++	66-3	+
45-1	+	67-1	+
45-2	+	67-3	+
46-1	-	68-1	++
47-1	+	69-1	+
47-3	-	71-1	+
48-1	-	71-2	+
49-1	+	71-4	++
49-3	+	71-5	++
49-5	+	72-1	++
49-7	+	73-1	+
49-9	+	75-1	++
50-1	+	76-1	+
50-3	+	77-2	++
51-1	+	81-2	+
52-1	+	82-2	+
52-3	+	83-1	++
53-1	++	84-1	++
54-1	++	85-1	+
54-3	++	94-2	+
55-1	++	95-1	+
55-2	++	96-2	+

Lactobacillus spot diameter = 4 mm; -, no inhibition; +, 5-7 mm of inhibition; ++, 8-9 mm of inhibition; +++, 10-11 mm of inhibition

Table 5 Antagonistic activities of *Lactobacillus* isolates against *H. pylori* H40 (continued)

***Lactobacillus* isolates**	**Anti-*H. pylori***	***Lactobacillus* isolates**	**Anti-*H. pylori***
98-2	-	168-2	+
99-1	+	169-1	-
102-1	-	170-1	-
105-1	+	171-1	+
105-3	++	172-1	++
105-4	-	172-2	-
109-1	+	174-2	++
109-3	+	175-1	++
110-2	-	176-1	++
111-1	-	177-1	++
112-2	-	178-1	-
114-1	-	179-1	-
115-1	+	180-1	+
115-2	+	181-1	-
116-1	+	183-1	+
118-1	+	184-1	++
120-1	+	185-1	++
122-2	+	187-1	++
124-2	+	188-2	-
128-2	+	190-1	++
133-2	+	190-2	++
134-2	-	191-1	++
139-1	+	192-1	+
140-2	+	193-1	+
141-2	-	193-2	++
143-1	+	194-1	++
143-2	+	196-1	+
147-1	-	196-2	++
148-1	+	197-1	-
150-1	+	201-1	++
151-1	+	202-1	+
152-1	+	202-2	+
156-2	-	203-2	+
157-1	+	203-3	+
158-1	+	204-1	++
159-1	-	205-1	+
162-1	+	206-1	++
163-1	+	206-2	+
165-1	-	207-1	+
165-2	+	208-1	-
166-1	+	209-1	-
166-2	-	210-1	+

Lactobacillus spot diameter = 4 mm; -, no inhibition; +, 5-7 mm of inhibition; ++, 8-9 mm of inhibition; +++, 10-11 mm of inhibition

Table 6 Antagonistic activities of *Lactobacillus* isolates against *H. pylori* H40 (continued)

***Lactobacillus* isolates**	**Anti-*H. pylori***	***Lactobacillus* isolates**	**Anti-*H. pylori***
211-1	+	260-1	-
212-1	+	260-2	-
213-1	++	261-2	+
215-1	++	264-1	-
216-1	+	265-2	-
217-1	++	267-1	-
218-1	+	268-1	-
219-1	+	268-2	+
220-1	++	269-1	-
221-1	+	269-2	-
228-1	+	270-1	++
228-2	+	271-1	-
229-1	-	272-1	-
230-2	+	272-2	-
231-1	-	273-1	-
231-2	-	274-1	-
231-3	+	275-1	-
232-3	+	275-2	+
233-1	++	276-1	+
236-1	-	277-1	++
236-2	-	279-1	+
237-1	-	280-1	+
238-1	-	281-1	+
239-1	-	282-1	+
240-2	-	284-1	+
241-1	+	285-1	+
241-2	+	286-1	-
242-2	+	287-1	-
243-1	+	288-1	-
244-1	+	289-1	+
245-1	+	289-2	-
246-1	-	290-1	+
248-1	-	291-1	+
248-2	+	292-2	+
253-1	++	292-3	-
254-1	-	293-2	+
255-1	-	295-1	+
256-1	+	296-1	+
257-1	+	298-1	+
258-1	++	223-2	+
259-1	++	224-1	+
259-2	+	225-2	-

Lactobacillus spot diameter = 4 mm; -, no inhibition; +, 5-7 mm of inhibition; ++, 8-9 mm of inhibition; +++, 10-11 mm of inhibition

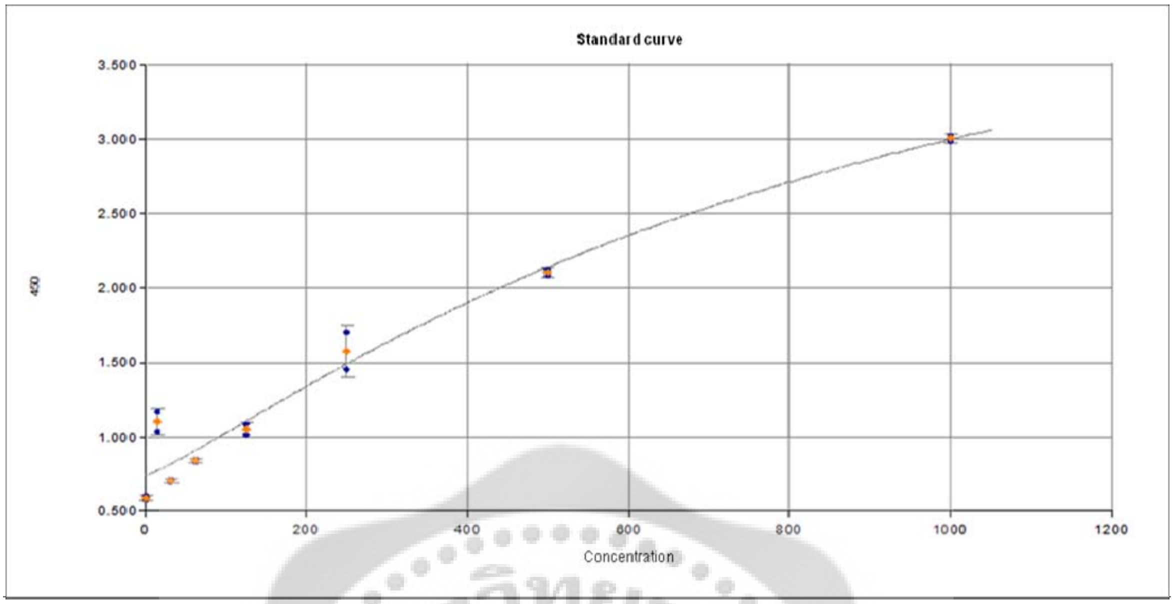
Table 7 Antagonistic activities of *Lactobacillus* isolates against *H. pylori* H40 (continued)

***Lactobacillus* isolates**	**Anti-*H. pylori***	***Lactobacillus* isolates**	**Anti-*H. pylori***
226-1	-	PB19-2	++
299-2	-	PB19-3	+
300-2	-	PB20-1	++
301-2	+	PB20-2	+
302-1	-	PB20-3	+
302-2	-	PB21-1	+
303-1	-	PB21-2	++
PB1-1	-	PB21-3	-
PB1-3	+	PB21-4	+
PB2-2	-	PB22-1	++
PB3-1	+	PB23-2	++
PB4-1	+	PB23-3	++
PB4-2	+	PB24-1	-
PB4-3	+	PB24-2	+
PB7-1	+	PB24-3	-
PB8-1	+	F1-1	+
PB9-1	-	F1-3	-
PB9-2	+	F1-4	-
PB9-3	+	F2-2	+
PB10-1	+	F4-1	+
PB10-1	+	F4-2	+
PB11-2	-	F4-4	-
PB14-1	+	F5-1	+
PB14-2	+	F5-3	+
PB16-1	+	F7-2	+
PB17-1	+	F7-3	+
PB17-2	+	F8-1	+
PB17-3	+	F9-1	+
PB18-1	-	F10-1	+
PB18-2	+	F12-2	+
PB18-3	+	F13-1	+
PB19-1	+	F14-1	+

Lactobacillus spot diameter = 4 mm; -, no inhibition; +, 5-7 mm of inhibition; ++, 8-9 mm of inhibition; +++, 10-11 mm of inhibition

Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells

A total of ninety-three *Lactobacillus* isolates were randomly selected from infant feces and fermented food to determine the immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. *Lactobacillus* isolates were grown in MRS broth for 48 hr. *Lactobacillus* conditioned media (LCM) were prepared as described in material and methods (Chapter III). Bioassays were performed by THP-1 cells incubated with *Lactobacillus* conditioned media and activated with lipopolysaccharide (LPS). TNF-α production in THP-1 monocytic cell culture supernatants were collected and measured by using cytokine-specific sandwich quantitative ELISA and cytokine concentration were quantified from standard curve and expressed as pg/ml of culture medium as shown in Figure 7.



$Y = (A-D)/(1+(X/C)^B) + D$

A	B	C	D	R^2
0.741	1.19	926	5.06	0.986

Figure 7 Standard curve of TNF-α protein determination at the concentration of 15.625, 31.5, 62.5, 125, 250, 500, and 1,000 pg/ml; R^2 = 1

Ninety-three tested isolates exhibited immunomodulatory activities as indicated in TNF-α level as shown in Tables 8-13 and Figures 8-13. These tables and figures showed the levels of TNF-α secretion by THP-1 monocytic cells incubated with *Lactobacillus* conditioned media (LCM) of each isolate in the presence or absence of lipopolysaccharide (LPS). Percentage of TNF-α inhibition was also displayed in the tables. MRS bacterial media control showed no effect on TNF-α production. LPS acted as TNF-α activator and led to increasing of TNF-α production as shown in right hand compared to no LPS on left hand of each graph. In the presence of *Lactobacillus*-conditioned media plus LPS, TNF-α production was suppressed in varying degrees among each of the *Lactobacillus* isolates. In addition, *Lactobacillus* conditioned media without LPS did not activate TNF-α production in most isolates (Figures 8-13).

The immunomodulatory effects of 93 isolates on TNF-α production in LPS-activated THP-1 monocytic cells as shown in TABLE 14 and FIGURE 14. The percentage of TNF-α inhibition demonstrated that the inhibitory activities varied among each isolate, which range from 3 to 98 % inhibition. Thirteen isolates: CM5, CM30, CM53, CM76, CM79, CM154, CM158, CM167, CM173, CM191, CM206, CM293 and CM319 displayed 3-20% TNF-α inhibition. Eleven isolates: CM1, CM4, CM9, CM16, CM25, CM29, CM71, CM100, CM152, CM199 and CM204 displayed 21-30% TNF-α inhibition. Eleven isolates: CM2, CM33, CM47, CM50, CM75, CM148, CM201, CM236, F7, F11 and F14 displayed 31-40% TNF-α inhibition. Thirteen isolates: CM6, CM7, CM18, CM21, CM73, CM89, CM102, CM106, CM139, CM146, CM339, F8 and F16 displayed 41-50% TNF-α inhibition. Thirty-nine isolates: CM10, CM12, CM17, CM36, CM61, CM90, CM98, CM104, CM107, CM114, CM118, CM119, CM120, CM121, CM122, CM123, CM128, CM130, CM134, CM141, CM184, CM186, CM187, CM189, CM261, CM283, F1, F2, F3, F4, F5, F9, F10, F13, F15, F17, F18, F19 and F20 showed > 50% TNF-α inhibition and 6 isolates: 57, 178, 182, 316, F6 and F12 showed TNF-α stimulatory activity. Interestingly, CM128 and F19 exhibited the most potent TNF-α inhibition by 82% and 98%, respectively.

From the results described above, several isolates displayed $\geq$ 50% TNF-α inhibition (Tables 8-13 and FIGURES 8-13). These isolates were selected to confirm immunomodulatory effects using the same bioassay as described above, and the test were done in duplicate. The data demonstrated that these 39 isolates: CM10, CM12, CM17, CM36, CM61, CM90, CM98, CM104, CM107, CM114, CM118, CM119, CM120, CM121, CM122, CM123, CM128, CM130, CM134, CM141, CM184, CM186, CM187, CM189, CM261, CM283, F1, F2, F3, F4, F5, F9, F10, F13, F15, F17, F18, F19 and F20 significantly inhibited TNF-α production in LPS-activated THP-1 monocytic cells when compared to MRS bacterial media control as shown in Table 15-and Figure 15. *Lactobacillus* conditioned media without LPS of these 39 isolates have no effect on TNF-α producing THP-1 monocytic cells. These data were similar to those displayed in Tables 8-13 and Figures 8-13. The TNF-α inhibitory activities of these 39 isolates were shown significantly inhibition ($p<0.01$), when compared to MRS bacterial media control. Especially, CM128, CM130, CM187, F1 and F19 isolates exhibited strong inhibitory activity when compared to MRS bacterial media control.

Table 8 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation.

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	44	0.01	MRS+ LPS	444	31.568	
CM1	37	0.19	CM1	333	1.141	25
CM2	0	0	CM2	284	4.513	36
CM4	81	4.835	CM4	310.8	8.379	30
CM5	116	1.995	CM5	355.2	17.986	20
CM6	0	0	CM6	248.64	77.701	44
CM7	0	0	CM7	248.64	12.615	44
CM9	44	1.141	CM9	333	15.887	25
CM10	24	1.141	CM10	168.12	26.366	62
CM12	0	0	CM12	177.6	5.255	60
CM16	50	45.615	CM16	333	45.615	25
CM17	0	0	CM17	137.64	34.906	69
CM18	0	0	CM18	244.2	22.886	45
CM21	0	0	CM21	244.2	7.762	45
CM23	44	1.141	CM23	205.78	86.005	21

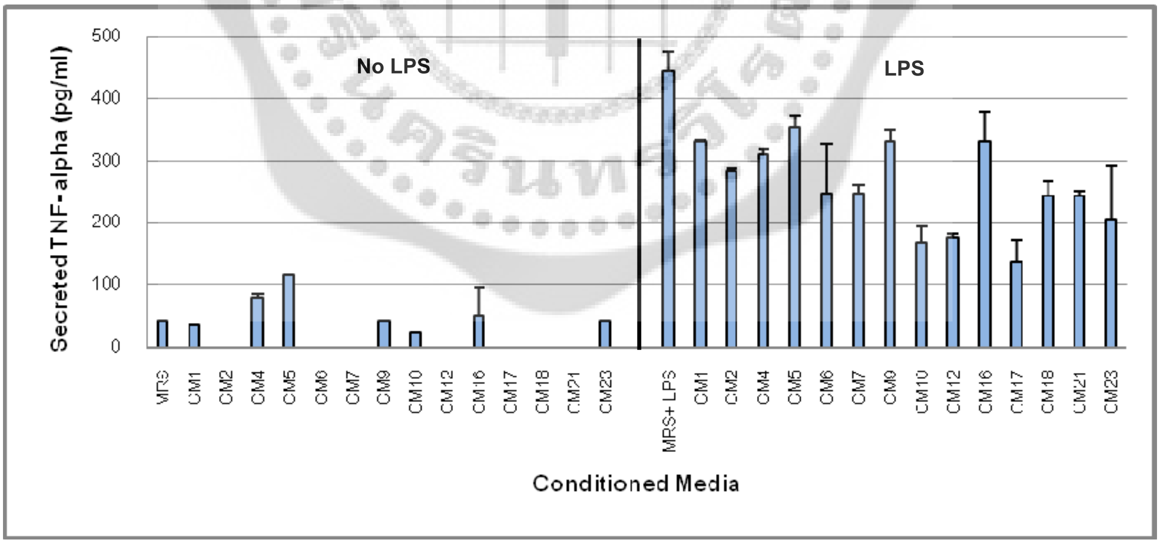


Figure 8 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 9 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	0.338	0	MRS+ LPS	398.5	64.347	
CM29	0.329	1.141	CM29	282.9	66.023	29
CM30	0.411	0.268	CM30	382.56	122.005	4
CM33	0.347	0.268	CM33	251.055	0.482	37
CM36	0.353	1.141	CM36	195.265	27.97	51
CM47	0.348	12.229	CM47	259.025	14.84	35
CM50	0.36	47.813	CM50	259.025	17.976	35
CM53	0.381	0.268	CM53	366.62	47.494	8
CM57	0.744	1.141	CM57	429.98	97.318	7.9
CM61	0.647	1.141	CM61	155.415	147.046	61
CM71	0.793	1.141	CM71	298.875	73.433	25
CM73	0.334	40.883	CM73	231.13	16.474	42
CM76	0.371	20.951	CM76	342.71	3.317	14
CM79	0.653	42.763	CM79	334.74	22.741	16
CM89	0.307	25.358	CM89	203.235	4.853	49
CM90	0.569	12.993	CM90	195.265	18.029	51

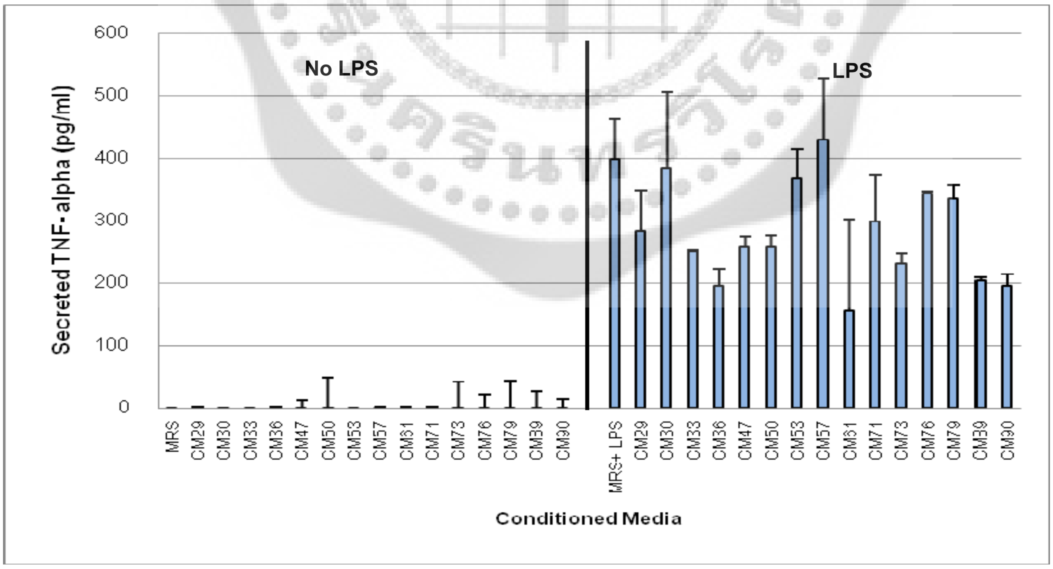


Figure 9 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 10 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	0.427	0	MRS+ LPS	664	43.161	
CM98	0.468	45.5	CM98	172.64	1.584	74
CM100	0.46	3.951	CM100	478.08	7.023	28
CM102	0.431	43.347	CM102	351.92	0.265	47
CM104	0.445	87.068	CM104	215.8	10.67	67.5
CM106	0.409	1.141	CM106	391.76	8.32	41
CM107	0.444	0.268	CM107	398.4	13.759	40
CM114	0.443	42.763	CM114	278.88	15.689	58
CM118	0.404	20.951	CM118	292.16	154.094	56
CM119	0.45	40.883	CM119	166	15.836	75
CM120	0.407	76.396	CM120	175.96	43.631	73.5
CM121	0.527	14.212	CM121	166	22.794	75
CM122	0.496	42.107	CM122	166	27.104	75

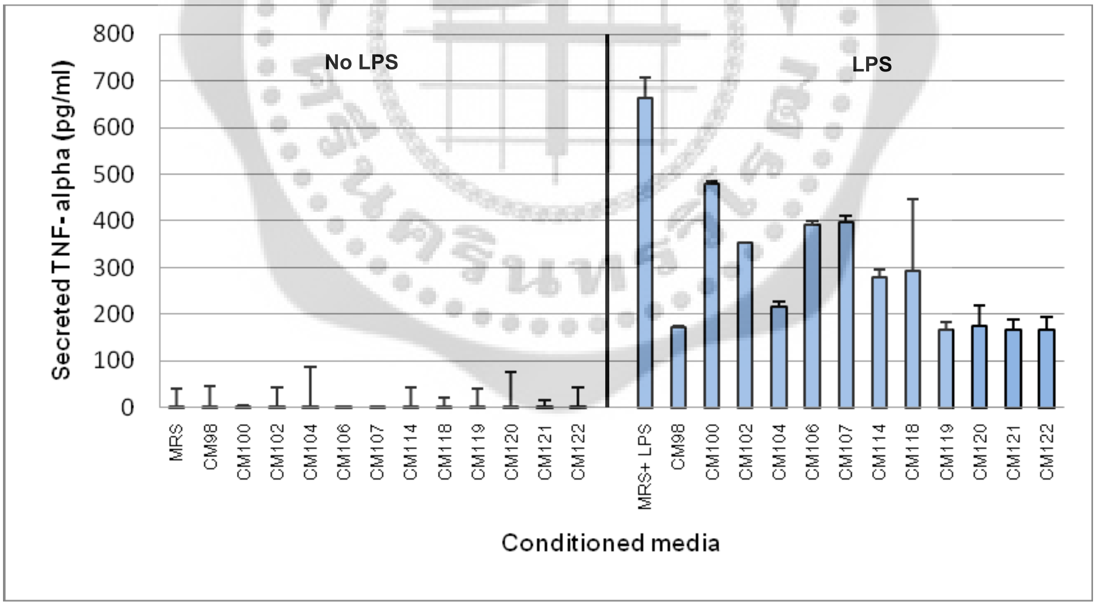


Figure 10 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 11 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS lipopolysaccharide; MRS, bacterial media control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	0.299	0	MRS+ LPS	700	59.324	
CM123	0.339	0	CM123	227.5	91.435	67.5
CM128	0.33	0.268	CM128	126	70.207	82
CM130	0.382	1.141	CM130	140	96.621	80
CM134	0.362	0	CM134	224	20.96	68
CM139	0.371	0	CM139	357	42.791	49
CM141	0.425	1.141	CM141	224	63.16	68
CM146	0.34	0.268	CM146	374.5	83.966	46.5
CM148	0.462	0.284	CM148	441	54.104	37
CM152	0.393	0.112	CM152	553	15.032	21
CM154	0.491	0.326	CM154	616	112.239	12
CM158	0.384	0.033	CM158	623	129.407	11
CM167	0.353	0	CM167	679	51.026	3
CM169	0.357	1.141	CM169	631	44.3	9
CM173	0.45	0.187	CM173	651	0.187	7
CM178	0.367	0.112	CM178	847	0.326	21
CM182	0.445	0	CM182	714	0.033	2

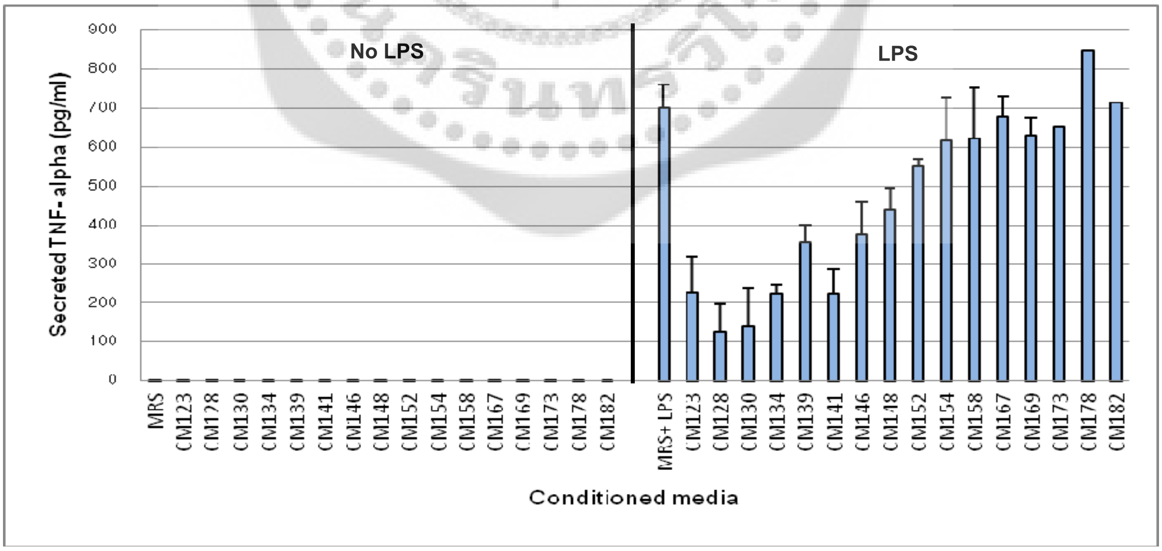


Figure 11 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 12 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	0.306	0	MRS+ LPS	486	44.569	
CM184	0.31	0.09	CM184	218.7	8.302	55
CM186	0.316	0.059	CM186	148.23	39.173	69.5
CM187	0.314	0.045	CM187	116.64	52.713	76
CM189	0.332	0.073	CM189	208.98	91.203	57
CM191	0.314	0.086	CM191	393.66	143.844	19
CM199	0.317	4.597	CM199	364.5	52.2	25
CM201	0.315	5.852	CM201	320.76	17.317	34
CM204	0.303	1.141	CM204	354.78	10.785	27
CM206	0.32	0	CM206	422.82	69.409	13
CM236	0.333	3.439	CM236	315.9	26.133	35
CM261	0.61	12.288	CM261	206.55	141.517	57.5
CM283	0.356	4.859	CM283	104.49	19.596	78.5
CM293	0.409	2.947	CM293	417.96	157.094	14
CM316	0.375	8.106	CM316	510.3	69.981	5
CM319	0.404	7.463	CM319	451.98	0.383	7
CM339	0.551	8.549	CM339	262.44	124.228	46

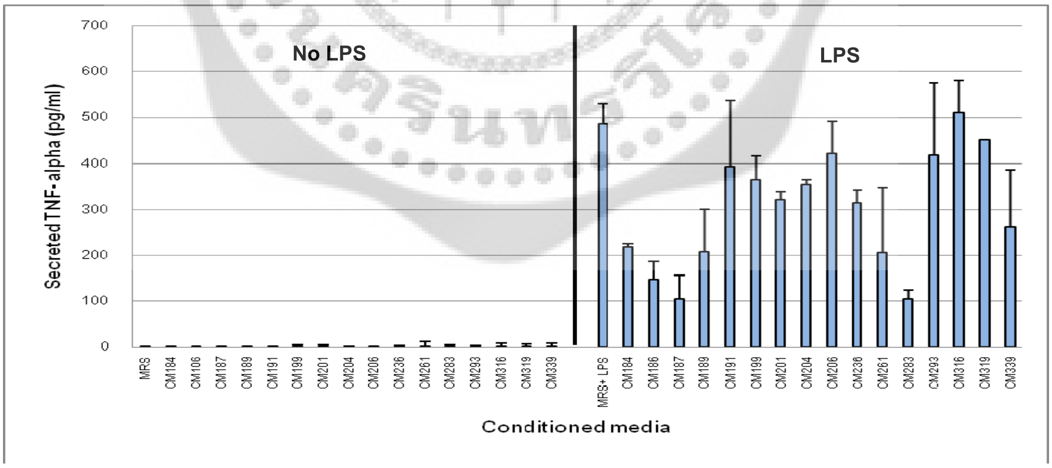


Figure 12 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 13 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	LCM+LPS	TNF-α (pg/ml)	SD	% Inhibition
MRS	0	0	MRS	547	10.415	
F1	0.55	0	F1	27.35	0.064	95
F2	0.46	0.268	F2	54.7	0.244	90
F3	0.487	0	F3	246.15	0.291	55
F4	0.491	0.238	F4	125.81	0.337	77
F5	0.503	0.213	F5	136.75	0.295	75
F6	1.486	0	F6	667.34	0.082	22
F7	0.376	0.258	F7	339.14	0.204	38
F8	0.564	0	F8	317.26	0.733	42
F9	0.363	0	F9	153.16	0.420	72
F10	0.883	0.28	F10	196.92	0.666	64
F11	0.353	1.141	F11	355.55	0.102	35
F12	0.341	25.07	F12	645.46	0.029	18
F13	0.333	26.919	F13	185.98	0.183	66
F14	0.445	8.161	F14	333.67	0.518	39
F15	0.334	1.157	F15	202.39	0.546	63
F16	0.467	17.400	F16	289.91	0.602	47
F17	0.354	1.602	F17	109.4	0.292	80
F18	0.337	19.383	F18	218.8	0.810	60
F19	0.349	27.353	F19	10.94	0.176	98
F20	0.341	7.837	F20	147.69	0.778	73

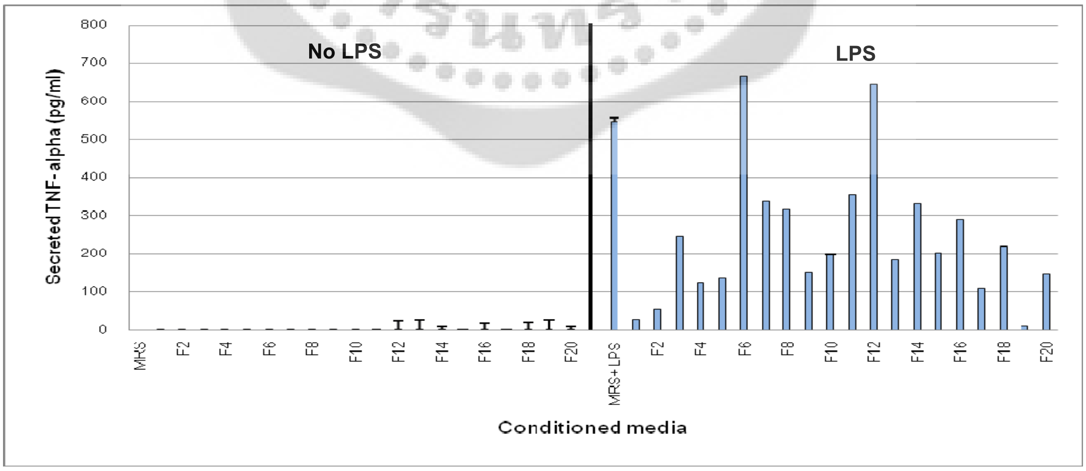


Figure 13 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. LPS, lipopolysaccharide; error bars indicate standard deviations

Table 14 Summary of immunomodulatory effects of 93 *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control

LCM	% Inhibition	LCM+LPS	% Inhibition	LCM+LPS	% Inhibition
MRS	0	CM102	47	CM201	34
CM1	25	CM104	67.5	CM204	27
CM2	36	CM106	41	CM206	13
CM4	30	CM107	40	CM236	35
CM5	20	CM114	58	CM261	57.5
CM6	44	CM118	56	CM283	78.5
CM7	44	CM119	75	CM293	14
CM9	25	CM120	73.5	CM316	5
CM10	62	CM121	75	CM319	7
CM12	60	CM122	75	CM339	46
CM16	25	CM123	67.5	F1	95
CM17	69	CM128	82	F2	90
CM18	45	CM130	80	F3	55
CM21	45	CM134	68	F4	77
CM23	21	CM139	49	F5	75
CM29	29	CM141	68	F6	22
CM30	4	CM146	46.5	F7	38
CM33	37	CM148	37	F8	42
CM36	51	CM152	21	F9	72
CM47	35	CM154	12	F10	64
CM50	35	CM158	11	F11	35
CM53	8	CM167	3	F12	18
CM57	7.9	CM169	9	F13	66
CM61	61	CM173	7	F14	39
CM71	25	CM178	21	F15	63
CM73	42	CM182	2	F16	47
CM76	14	CM184	55	F17	80
CM79	16	CM186	69.5	F18	60
CM89	49	CM187	76	F19	98
CM90	51	CM189	57	F20	73
CM98	74	CM191	19		
CM100	28	CM199	25		

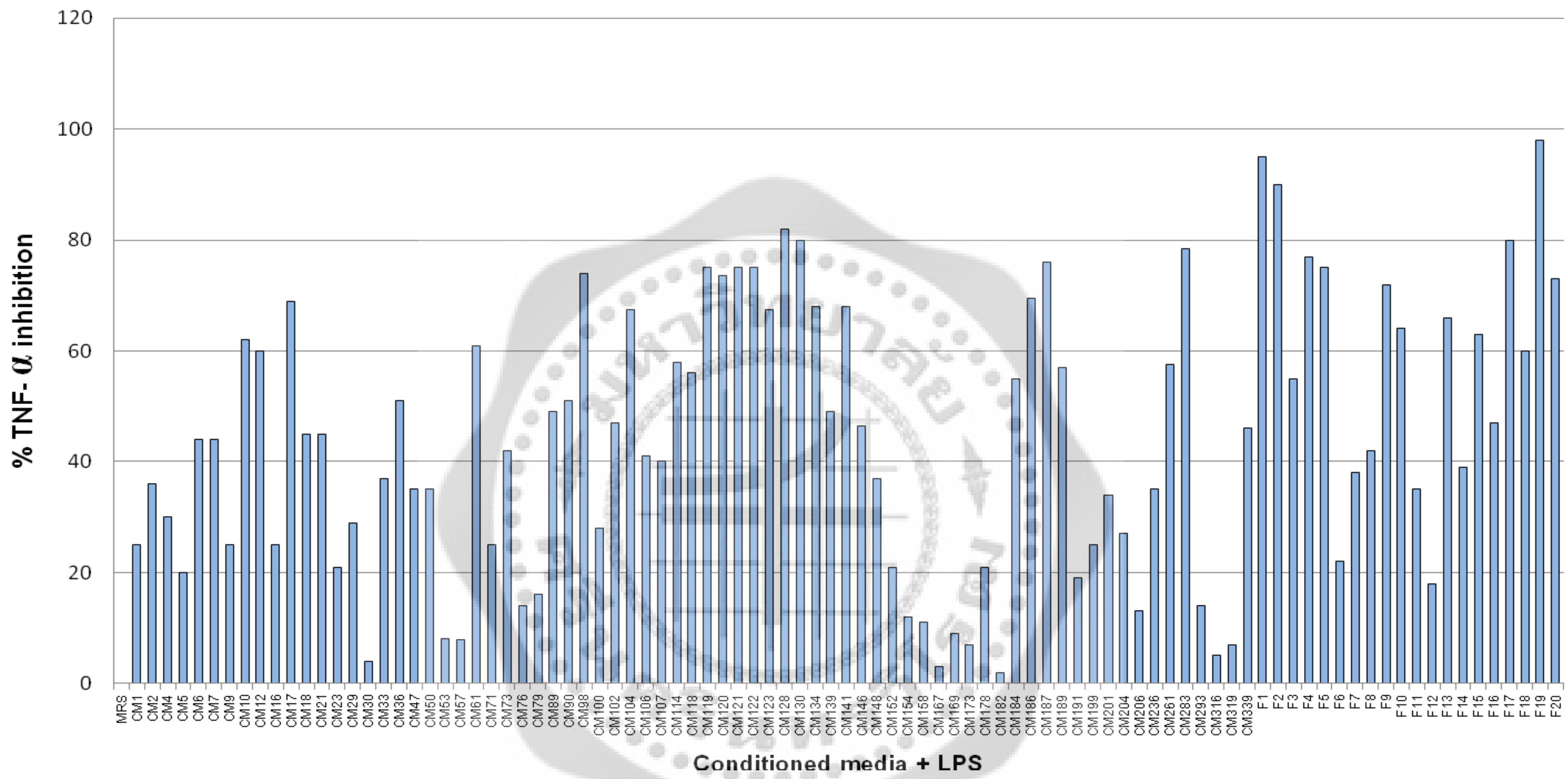


Figure 14 Summary of immunomodulatory effects of 93 *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells.

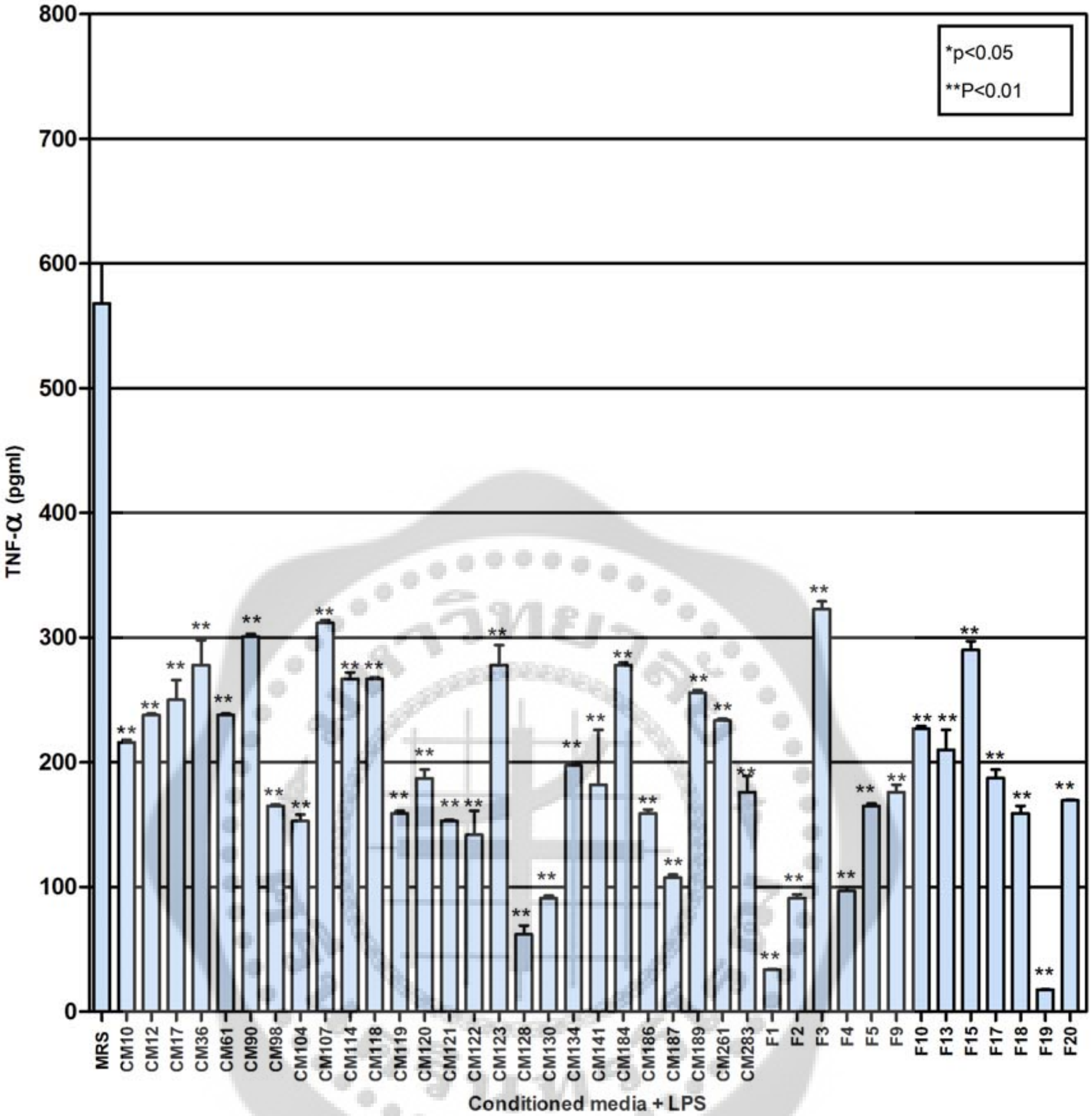
LPS, lipopolysaccharide; MRS, bacterial media control

Table 15 Immunomodulatory effects of selected *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media, control; SD, standard deviation

LCM	TNF-α (pg/ml)	SD	% Inhibition	p-value
MRS	568	45.255		
CM10	216	2.828	62	<0.01
CM12	238	1.414	58	<0.01
CM17	250	18.475	56	<0.01
CM36	278	28.284	51	<0.01
CM61	238	1.414	58	<0.01
CM90	301	2.828	47	<0.01
CM98	165	1.414	71	<0.01
CM104	153	7.071	73	<0.01
CM107	312	2.828	45	<0.01
CM114	267	7.071	53	<0.01
CM118	267	1.414	53	<0.01
CM119	159	2.828	72	<0.01
CM120	187	9.899	67	<0.01
CM121	153	1.414	73	<0.01
CM122	142	26.870	75	<0.01
CM123	278	22.627	51	<0.01
CM128	62	9.899	89	<0.01
CM130	91	2.828	84	<0.01
CM134	197.5	2.121	65	<0.01
CM141	182	62.225	62	<0.01
CM184	278	2.828	51	<0.01
CM186	159	4.243	72	<0.01
CM187	107.5	3.536	81	<0.01
CM189	256	2.828	55	<0.01
CM261	233.5	2.121	59	<0.01
CM283	176	18.385	69	<0.01
F1	33.5	0.707	94	<0.01
F2	91	4.243	84	<0.01
F3	323	8.485	43	<0.01
F4	97	4.243	83	<0.01
F5	165	2.828	71	<0.01
F9	176	8.485	69	<0.01
F10	227	2.828	60	<0.01
F11	415	21.213	27	<0.01
F12	704	2.828	24	<0.01
F13	210	22.627	63	<0.01

Table 15 Immunomodulatory effects of selected *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. LCM, *Lactobacillus* conditioned media; LPS, lipopolysaccharide; MRS, bacterial media control; SD, standard deviation (continued).

LCM	TNF-α (pg/ml)	SD	% Inhibition	p-value
F15	290	9.899	49	<0.01
F17	187.5	9.192	67	<0.01
F18	159	8.485	72	<0.01
F19	17.5	0.707	97	<0.01
F20	169.5	0.707	70	<0.01



Figures 15 Immunomodulatory effects of *Lactobacillus* isolates on TNF-α production in LPS-activated THP-1 monocytic cells. MRS, bacterial media control. * (p<0.05); ** (p<0.01); error bars indicated standard deviations.

Acid and bile tolerance test

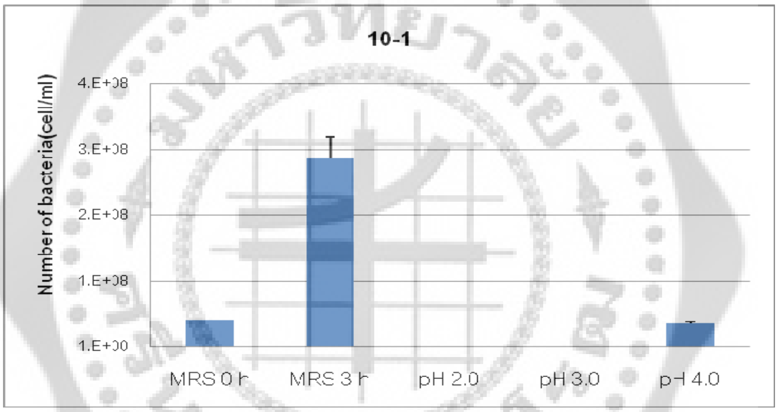
Acid tolerance test

Acid tolerance test for 7 selected *Lactobacillus* strains, 10-1 and 10-2 strains, which showed anti-*H. pylori* H40 activity and CM128, CM130, CM187, F1 and F19 strains, which showed the ability to modulate TNF-α production, were performed by inoculated the *Lactobacillus* into MRS media at pH level 2.0, 3.0 and 4.0 for 3 hours. The result of acid tolerance (survival at various pH values) showed that 10-1 isolates was tolerated at pH 2.0, 3.0 and 4.0 which viable cell decreased at pH 2.0 and pH 3.0 about 3 log values when compared to MRS control but showed more tolerance to the acid at pH 4.0 (Table 16 and Figure 16). 10-2 isolate was tolerated at pH4.0 but cannot resist to pH 2.0 and pH 3.0 when compared to MRS control (Table 17 and Figure 17). Viability of CM128 was changed after incubation for 3 hr at pH 2.0, 3.0, 4.0. The viable counts of pH 2.0 decreased about 2 log values when compared to MRS control, but showed more acid tolerance in the pH 3.0, 4.0 which showed no log difference (Table 18 and Figure 18). CM130 isolate was able to tolerance to the acid at pH 2.0, 3.0 and 4.0 when compared to MRS control (Table 19 and Figure 19). Viability of CM 187 isolate was changed at pH 2.0 and decreased about 4 log values when compared to MRS control but showed more acid tolerance in the pH 3.0, 4.0 (Table 20 and Figure 20). F1 isolate was grown at pH 2.0, 3.0, 4.0 but viable count of pH 2.0 decreased about 4 log values when compared to MRS control (Table 21 and Figure 21). Viability of F19 isolates was changed at pH 2.0 and decreased about 3 log values when compared to MRS control (Table 22 and Figure 22). Summary of the total selected *Lactobacillus* isolates were able to tolerance to the acid at pH 2.0, 3.0 and 4.0 when compared to MRS control, except 10-2 strains was tolerate at pH4.0 only as showed in Figure 23.

Table 16 Survival of *Lactobacillus* 10-1 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	4.08E+07	1.06E+06
MRS 3 h	2.87E+08	3.25E+07
pH 2.0	1.15E+05	1.41E+04
pH 3.0	2.25E+05	3.53E+04
pH 4.0	3.54E+07	2.75E+06

A.



B.

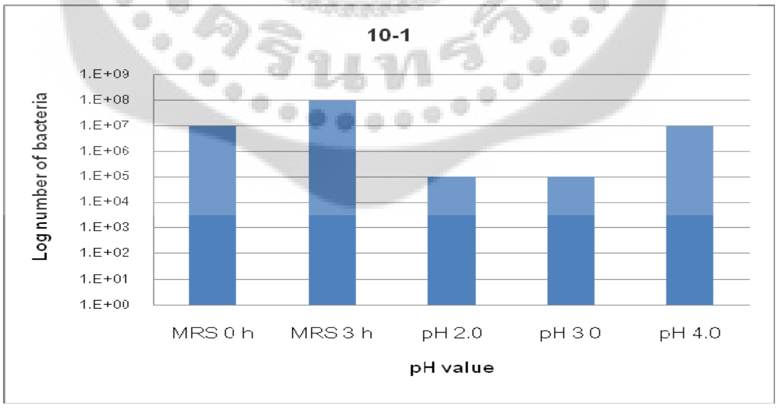


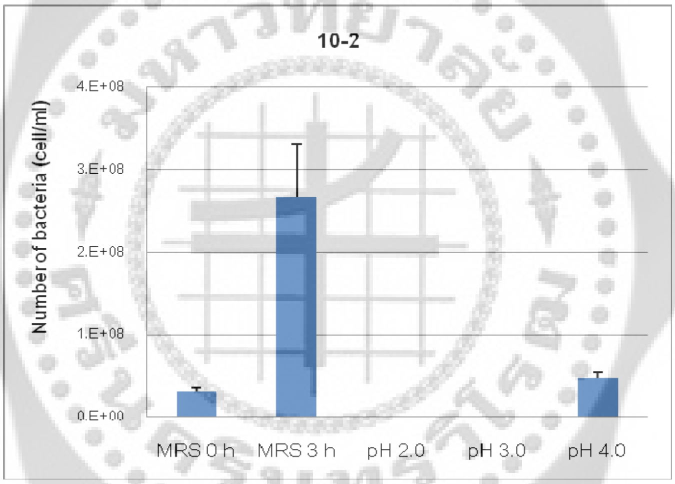
Figure 16 Acid tolerance of *Lactobacillus* 10-1 strain

A: Indicated in cell number

B: Indicated in log number

Table 17 Survival of 10-2 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	3.05E+07	4.95E+06
MRS 3 h	2.67E+08	6.51E+07
pH 2.0	0.00E+00	0.00E+00
pH 3.0	0.00E+00	0.00E+00
pH 4.0	4.76E+07	6.51E+06



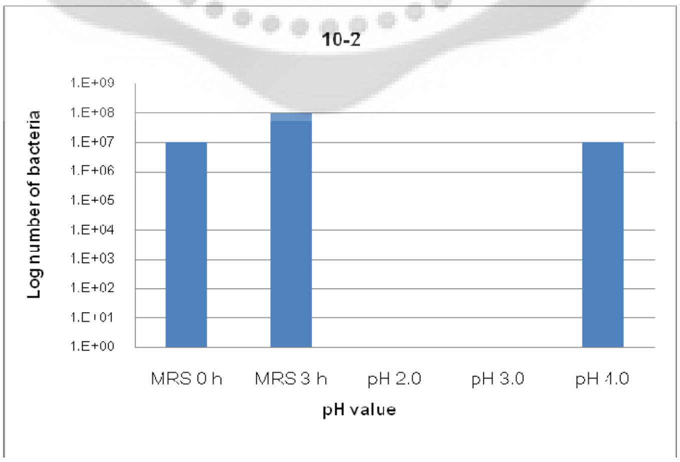


Figure 17 Acid tolerance of 10-2 strain

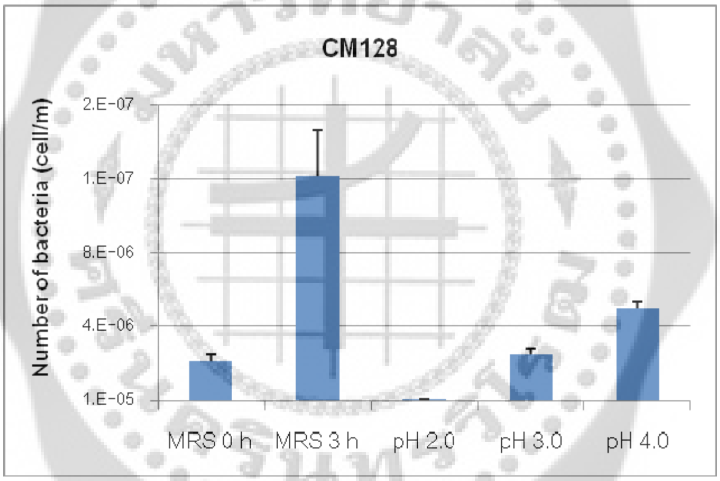
A: Indicated in cell number

B: Indicated in log number

Table 18 Survival of CM128 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	2.25E+06	3.50E+05
MRS 3 h	1.23E+07	2.50E+06
pH 2.0	1.29E+05	1.20E+04
pH 3.0	2.60E+06	2.80E+05
pH 4.0	5.10E+06	3.54E+05

A.



B.

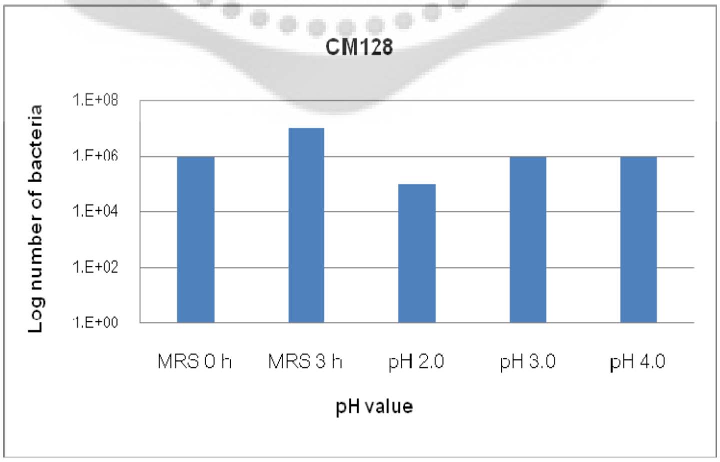


Figure 18 Acid tolerance of CM128 strain

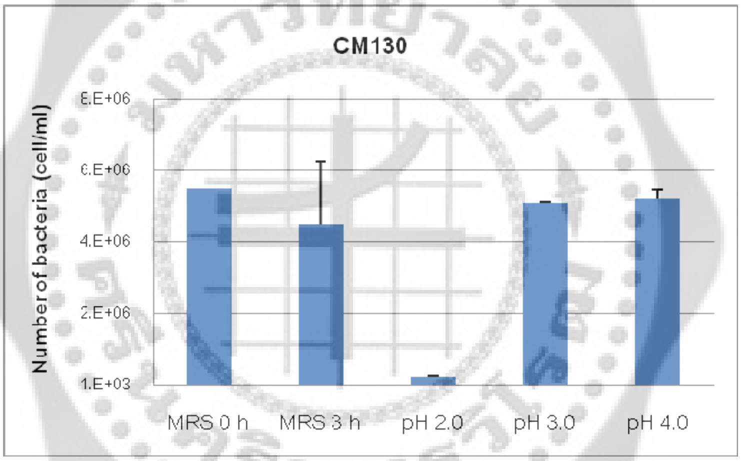
A: Indicated in cell number

B: Indicated in log number

Table 19 Survival of CM130 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	5.50E+06	0.00E+00
MRS 3 h	4.50E+06	1.76E+06
pH 2.0	2.38E+05	1.76E+04
pH 3.0	5.08E+06	3.50E+04
pH 4.0	5.23E+06	2.50E+05

A.



B.

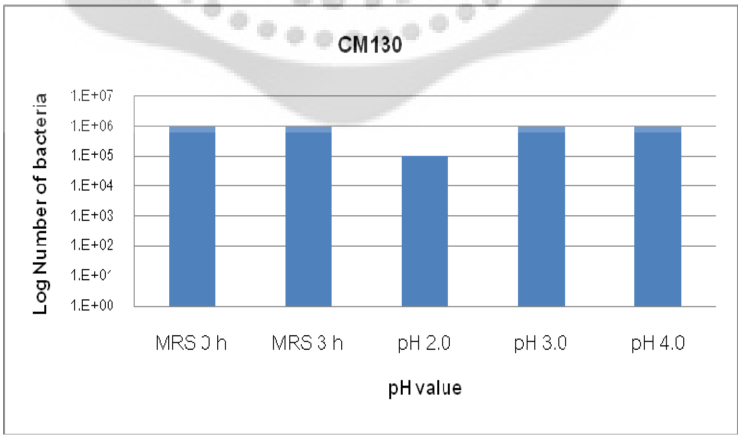


Figure 19 Acid tolerance of CM130 strain

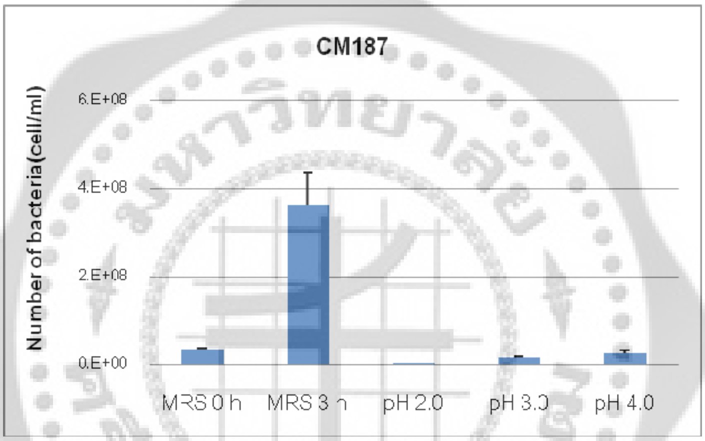
A: Indicated in cell number

B: Indicated in log number

Table 20 Survival of CM187 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	3.18E+07	4.60E+06
MRS 3 h	3.63E+08	7.42E+07
pH 2.0	2.75E+04	3.50E+03
pH 3.0	1.49E+07	3.04E+06
pH 4.0	2.55E+07	6.79E+06

A.



B.

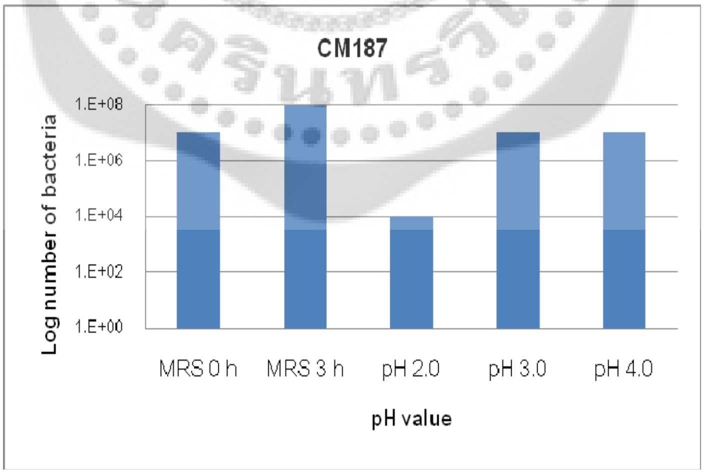


Figure 20 Acid tolerance of CM187 strain

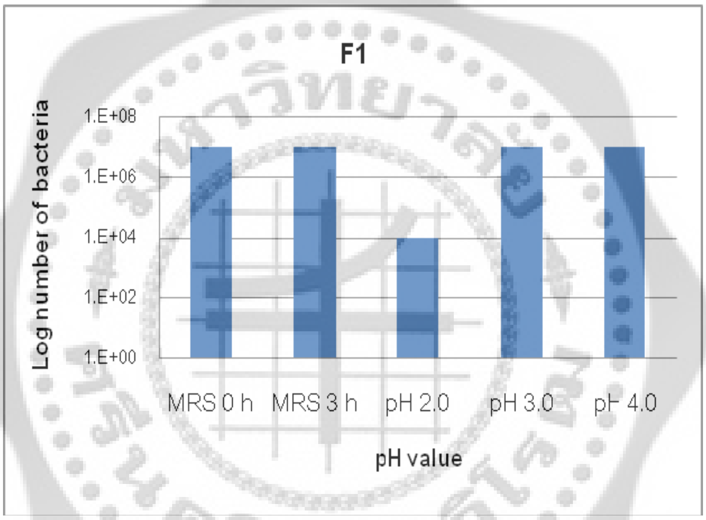
A: Indicated in cell number

B: Indicated in log number

Table 21 Survival of F1 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	2.58E+07	5.30E+06
MRS 3 h	2.58E+07	1.76E+06
pH 2.0	8.50E+04	7.10E+03
pH 3.0	1.87E+07	2.33E+06
pH 4.0	2.45E+07	3.53E+06

A.



B.

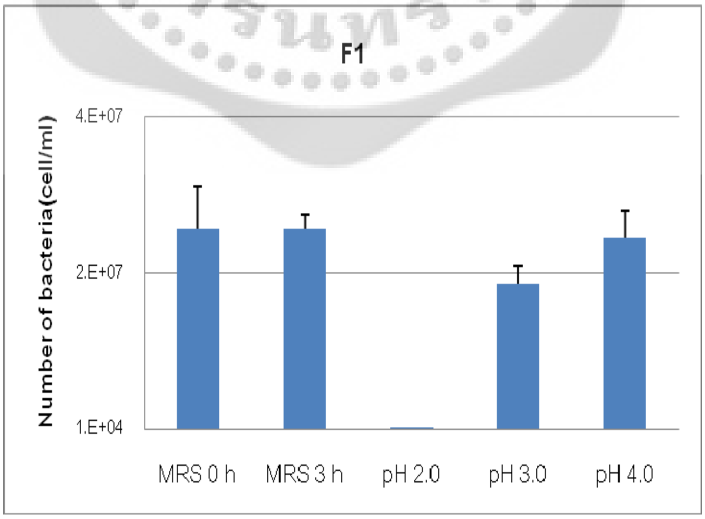


Figure 21 Acid tolerance of F1 strain

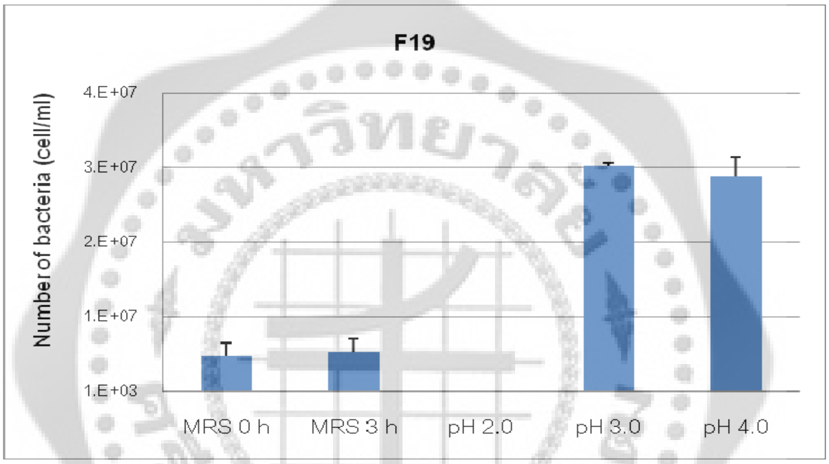
A: Indicated in cell number

B: Indicated in log number

Table 22 Survival of F19 strain after incubated at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 h	4.75E+06	1.76E+06
MRS 3 h	5.25E+06	1.76E+06
pH 2.0	5.00E+03	0
pH 3.0	3.03E+07	3.53E+05
pH 4.0	2.89E+07	2.61E+06

A.



B.

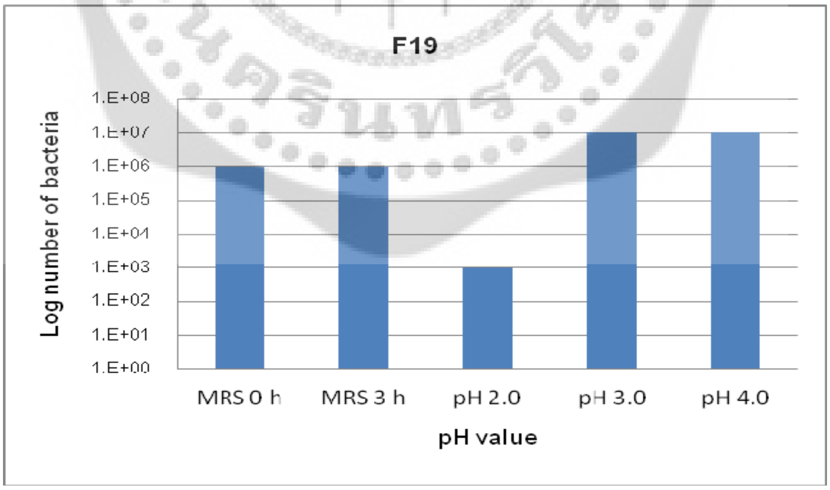


Figure 22 Acid tolerance of F19 strain

A: Indicated in cell number

B: Indicated in log number

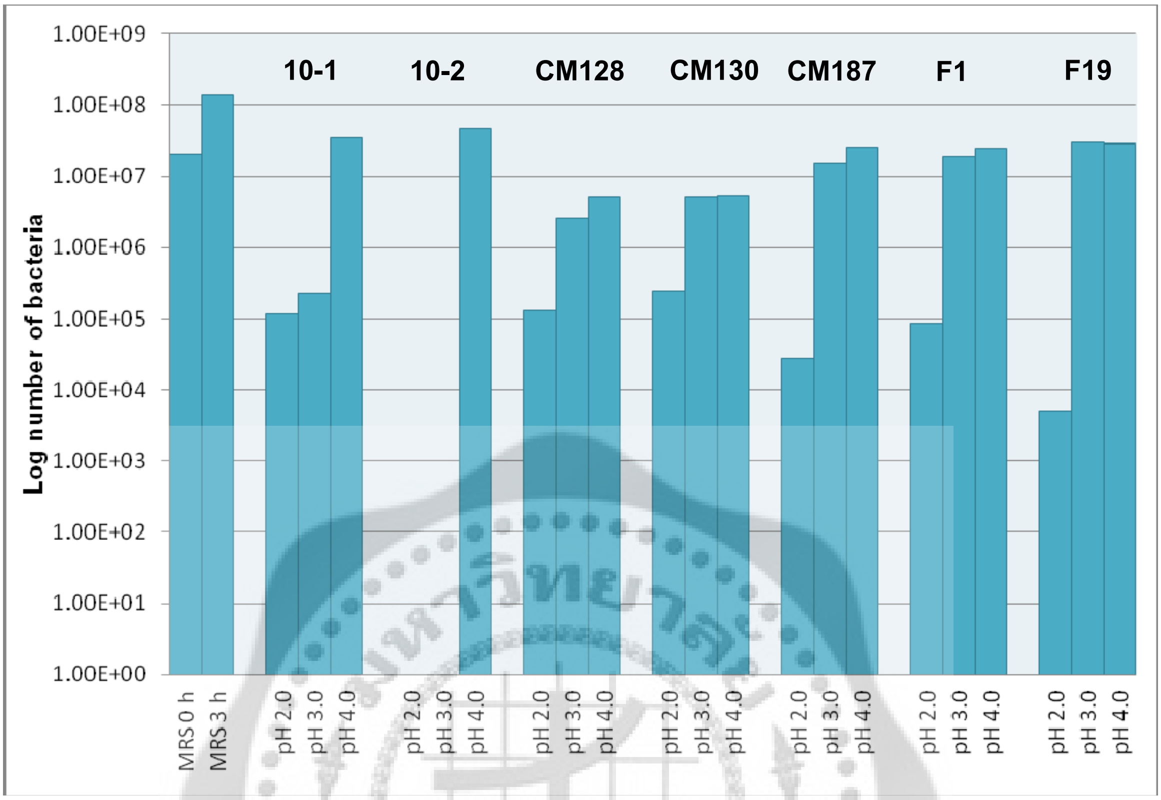


Figure 23 Summary of acid tolerance test of selected *Lactobacillus* isolates after incubated at various pH values, MRS, bacterial media control.

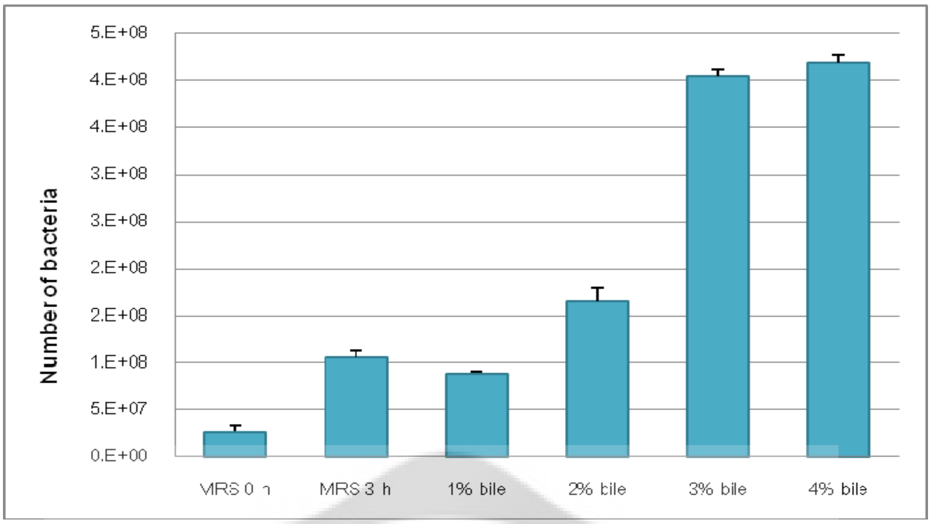
Bile tolerance test

Seven selected *Lactobacillus* strains including 10-1 and 10-2 strains with anti-*H. pylori* H40 activity and CM128, CM130, CM187, F1, F19 strains with modulate TNF-α production property, were cultivated in MRS broth with 1-4% bile for 3 hr. After incubation, viability of 10-1 strain decreased at 1% bile about 1 log value when compared to MRS bacterial media control, which viable count tolerated to bile at 2%, 3%, 4% bile (Table 23 and Figure 24). Viability of 10-2 strain was tolerated to bile at 1%, 2%, 3% and 4%, when compared to MRS bacterial media control (Table 24 and Figure 25). CM128 was tolerate to bile at 1%, 2%, 3% and 4% by viable count decreased of 4% bile about 1 log value when compared to MRS bacterial media control (Table 25 and Figure 26). Viability of CM130 and CM187 strain was tolerated to bile at 1%, 2%, 3% and 4%, when compared to MRS bacterial media control (Table 26 and Figure 27, Table 27 and Figure 28). Viability of F1 isolate was decreased at 3% and 4% about 1 log value but viable count tolerance more bile at 1% and 2% when compared to MRS bacterial media control (Table 28 and Figure 29). Viability of F19 strain was tolerated to bile at 1%, 2%, 3% and 4%, when compared to MRS bacterial media control (Table 29 and Figure 30). Summary of selected *Lactobacillus* isolates were tolerate to bile at 1%, 2%, 3% and 4% when compared to MRS bacterial media control as showed in Figure 31.

Table 23 Survival of 10-1 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	2.70E+07	7.07E+06
MRS 3 h	1.06E+08	7.78E+06
1% Bile	8.90E+07	1.41E+06
2% Bile	1.65E+08	1.41E+07
3% Bile	4.05E+08	7.07E+06
4% Bile	4.20E+08	7.78E+06

A.



B.

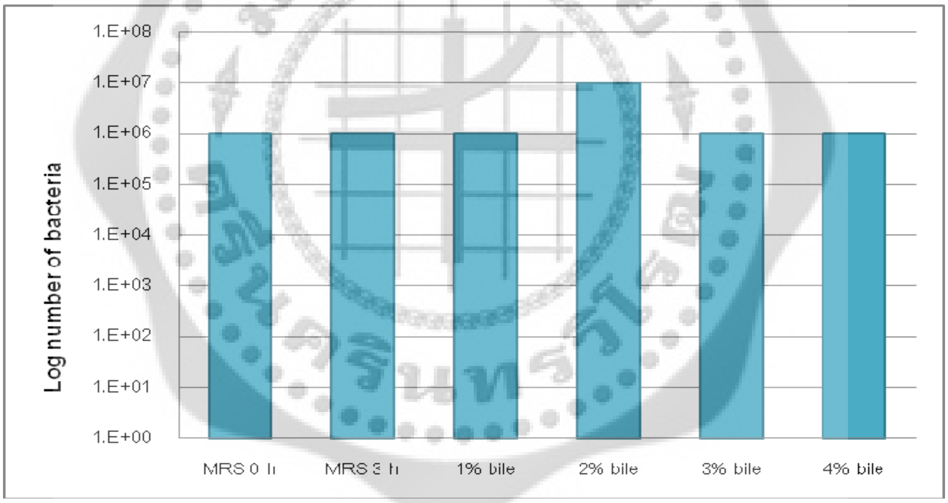


Figure 24 Bile tolerance of 10-1 strain

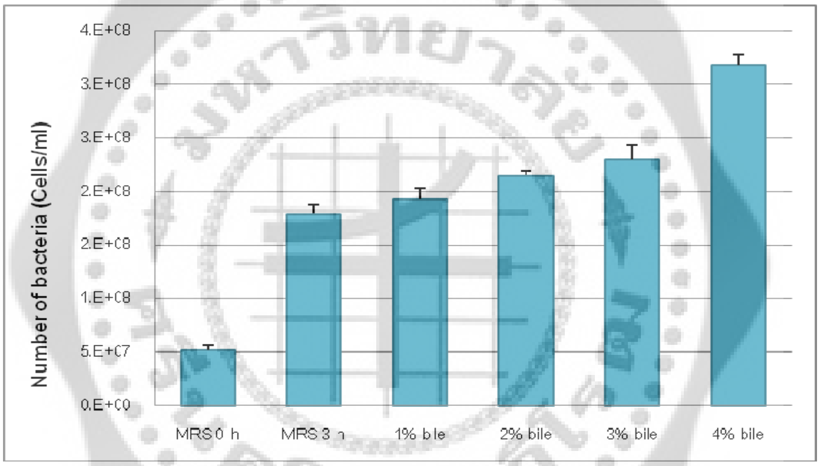
A: Indicated in cell number

B: Indicated in log number

Table 24 Survival of 10-2 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	5.15E+07	4.95E+06
MRS 3 h	1.79E+08	8.49E+06
1% Bile	1.93E+08	9.90E+06
2% Bile	2.15E+08	4.24E+06
3% Bile	2.30E+08	1.41E+07
4% Bile	3.18E+08	1.06E+07

A.



B.

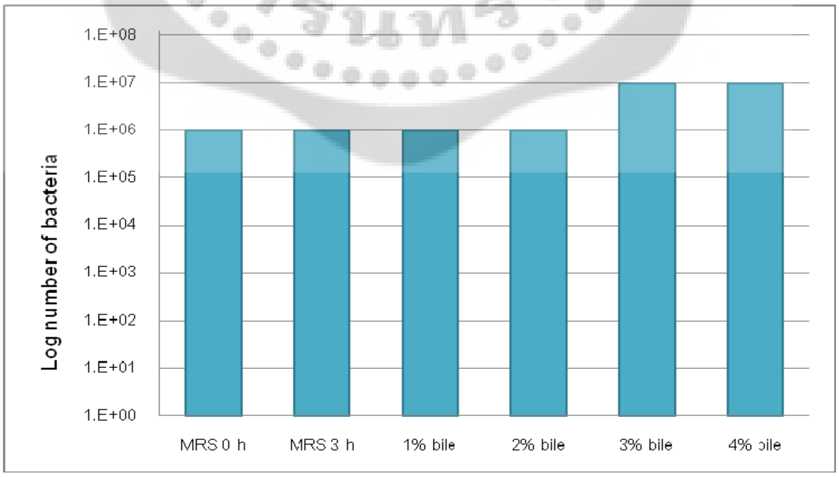


Figure 25 Bile tolerance of 10-2 strain

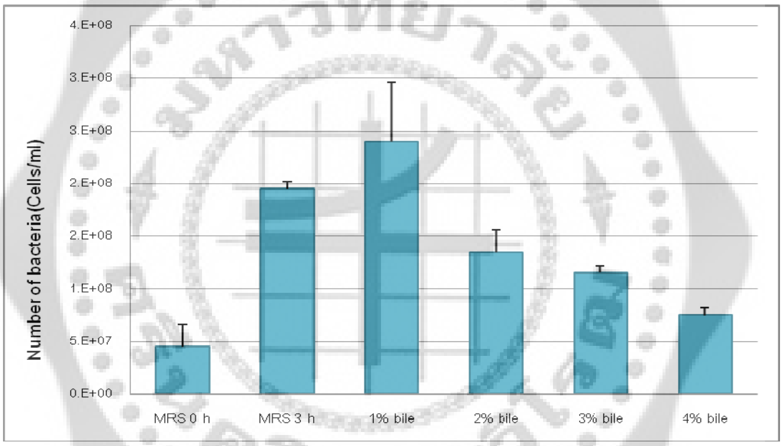
A: Indicated in cell number

B: Indicated in log number

Table 25 Survival of CM128 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	4.50E+07	2.12E+07
MRS 3 h	1.95E+08	7.07E+06
1% Bile	2.40E+08	5.66E+07
2% Bile	1.35E+08	2.12E+07
3% Bile	1.15E+08	7.07E+06
4% Bile	7.50E+07	7.00E+06

A.



B.

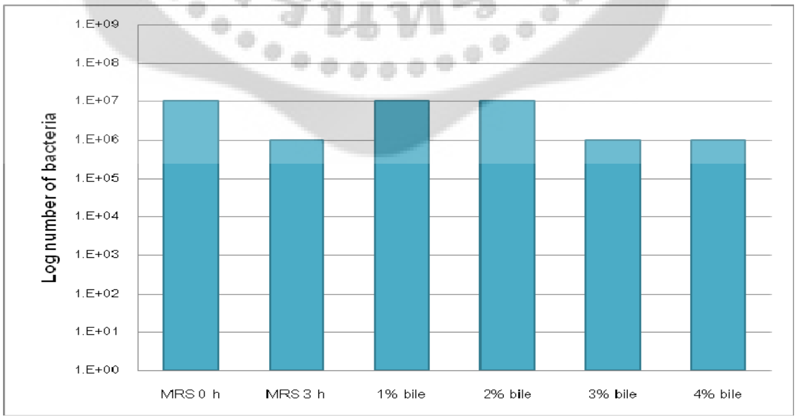


Figure 26 Bile tolerance of CM128 strain

A: Indicated in cell number

B: Indicated in log number

Table 26 Survival of CM130 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	9.50E+07	7.07E+06
MRS 3 h	7.50E+07	7.07E+06
1% Bile	5.00E+08	1.41E+07
2% Bile	3.25E+08	3.54E+07
3% Bile	2.30E+08	2.83E+07
4% Bile	1.95E+08	7.07E+06

A.

B.

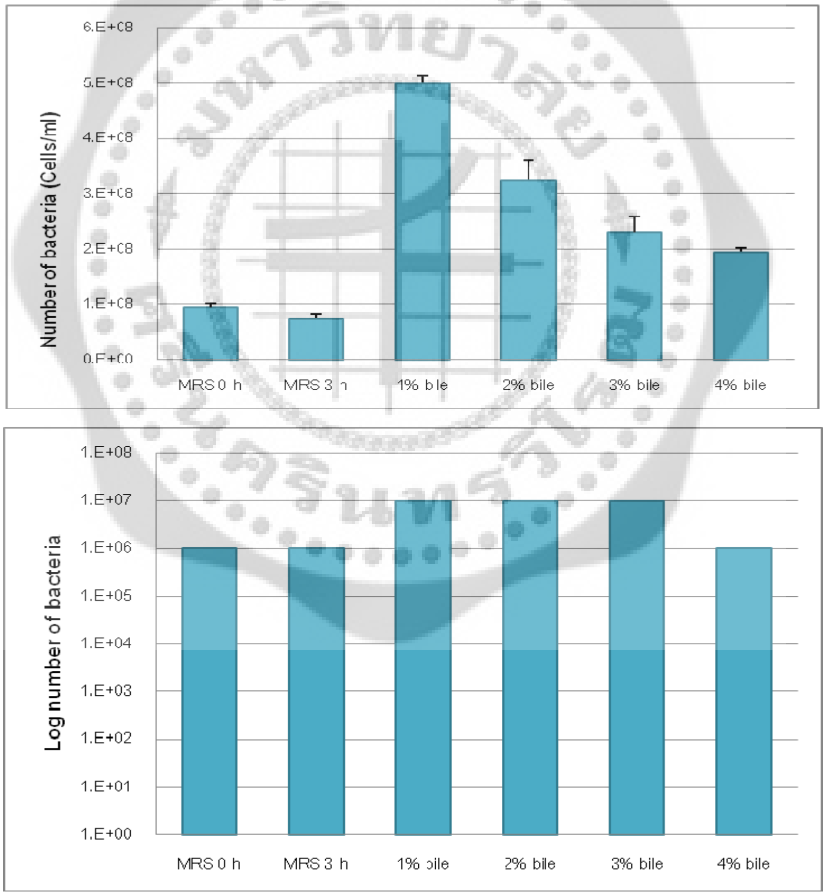


Figure 27 Bile tolerance of CM130 strain

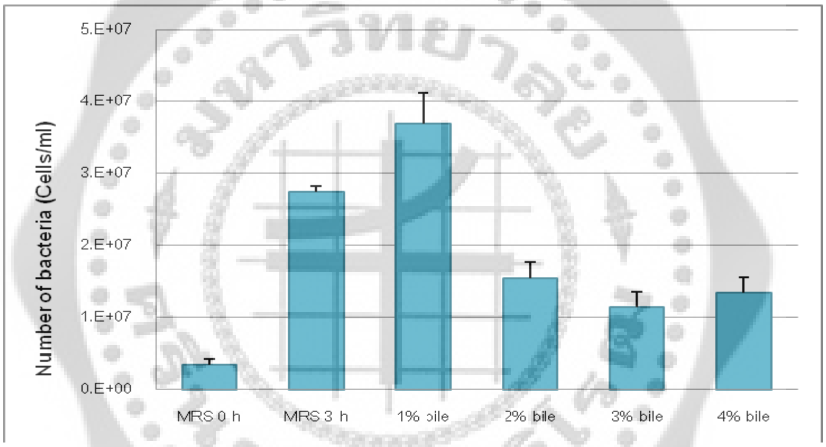
A: Indicated in cell number

B: Indicated in log number

Table 27 Survival of CM187 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	3.50E+06	7.07E+05
MRS 3 h	2.75E+07	7.07E+05
1% Bile	3.70E+07	4.24E+06
2% Bile	1.55E+07	2.12E+06
3% Bile	1.15E+07	2.12E+06
4% Bile	1.35E+07	2.12E+06

A.



B.

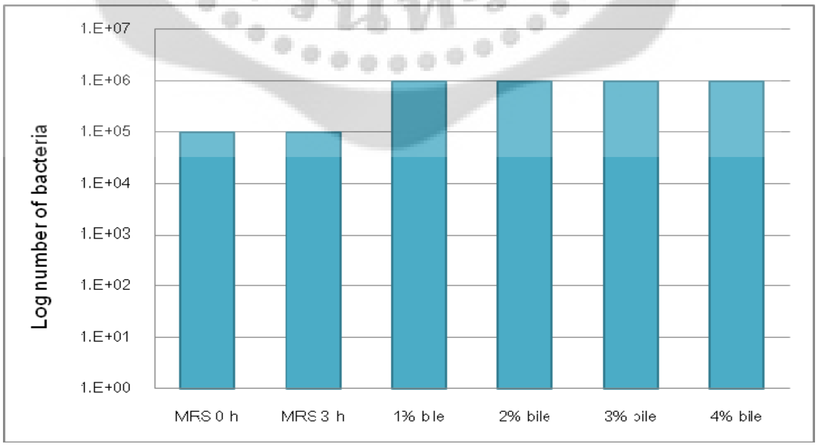


Figure 28 Bile tolerance of CM187 strain

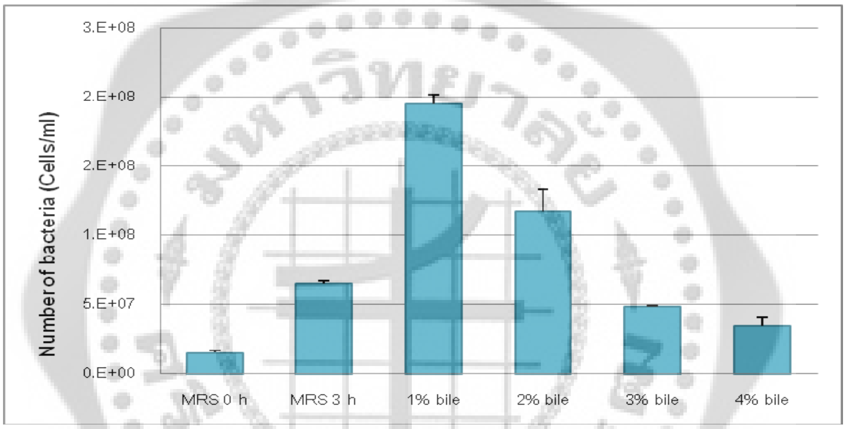
A: Indicated in cell number

B: Indicated in log number

Table 28 Survival of F1 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	1.50E+07	1.41E+06
MRS 3 h	6.55E+07	2.12E+06
1% Bile	1.95E+08	7.07E+06
2% Bile	1.18E+08	1.63E+07
3% Bile	4.85E+07	7.07E+05
4% Bile	3.45E+07	6.36E+06

A.



B.

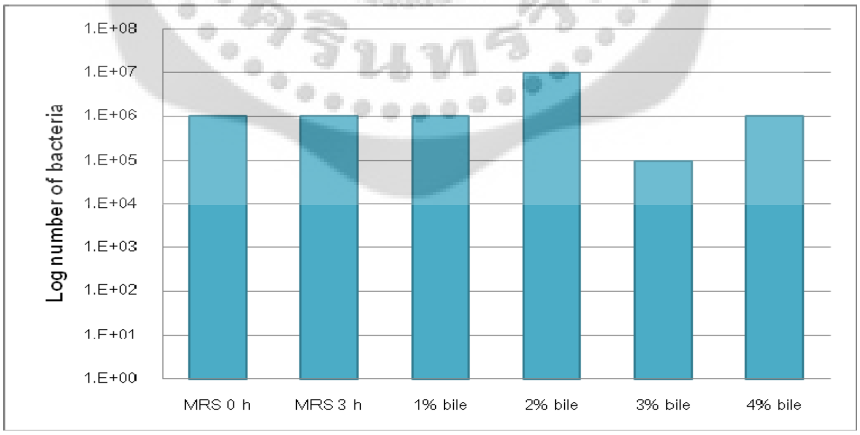


Figure 29 Bile tolerance of F1 strain

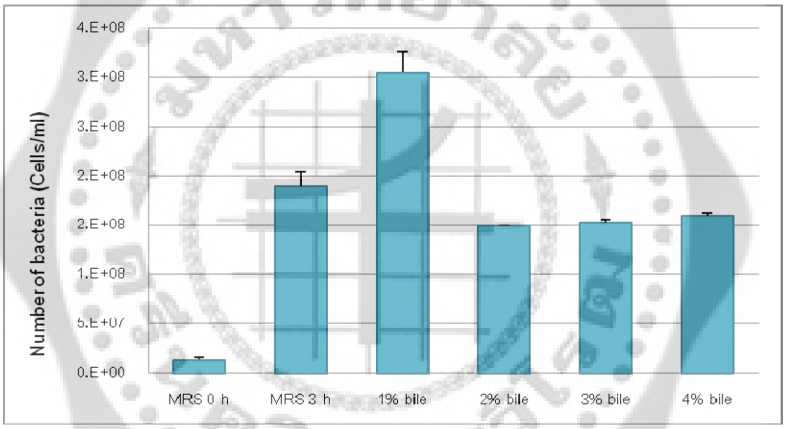
A: Indicated in cell number

B: Indicated in log number

Table 29 Survival of F19 strain after incubated in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 h	1.35E+07	2.12E+06
MRS 3 h	1.90E+08	1.41E+07
1% Bile	3.05E+08	2.12E+07
2% Bile	1.50E+08	7.07E+05
3% Bile	1.53E+08	2.83E+06
4% Bile	1.60E+08	2.83E+06

A.



B.

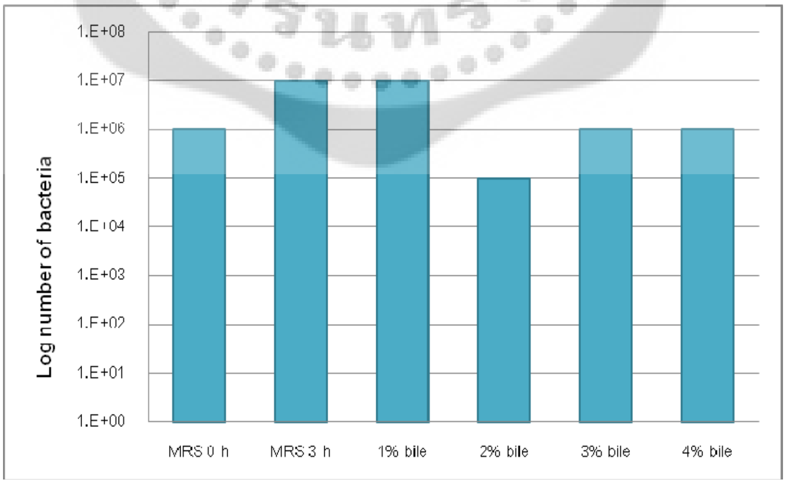


Figure 30 Bile tolerance of F19 strain

A: Indicated in cell number

B: Indicated in log number

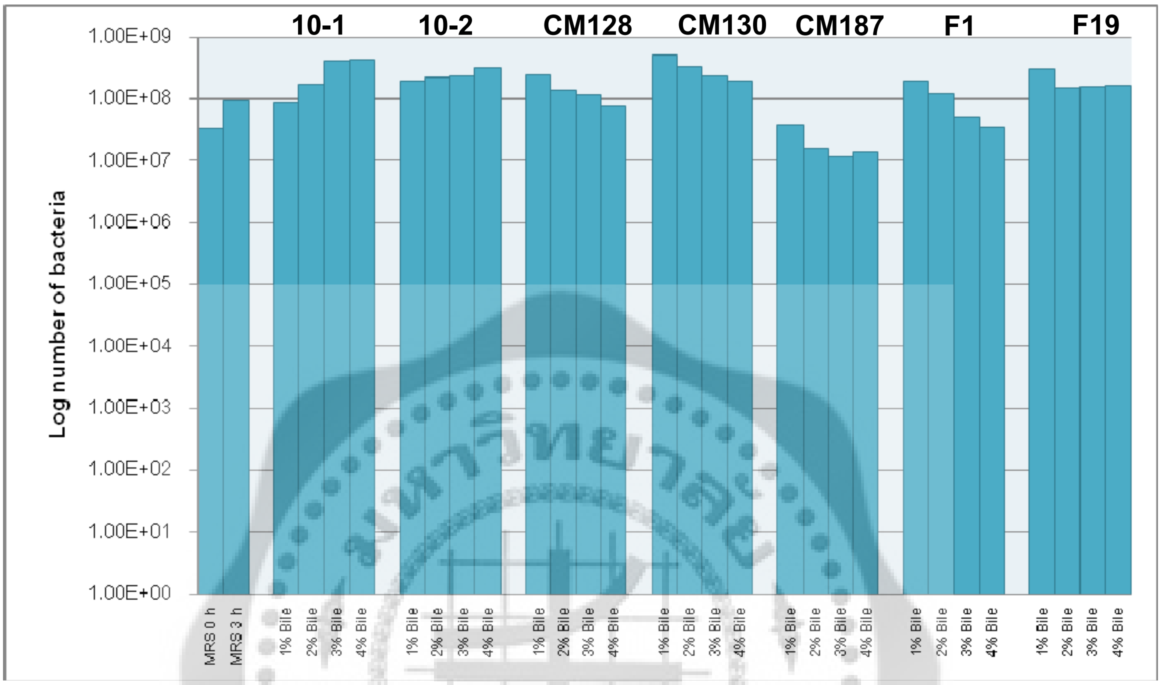


Figure 31 Summary of bile tolerance test of selected *Lactobacillus* isolates after incubated in various concentration of bile, MRS, bacterial media control.

Phenotypic characterization of selected *Lactobacillus* isolates by API 50CHL

Candidated *Lactobacillus* strains with anti-*Helicobacter pylori* H40 activity and with suppression of TNF-α production were characterized by API 50 CHL (Table 30). The carbohydrate fermentation patterns were used to determine the species of selected *Lactobacillus* isolates and analyzed by API database, API 50 CHL V5.1 at https://apiweb.biomerieux.com/servlet/Identify. Anti- *H. pylori* H40 activity strains including 10-1 and 10-2 obtained by spot test were able to utilize glycerol, ribose, galactose, glucose, fructose, mannose, mannitol, sorbital, N-acethyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, gentiobiose, D-tagatose and gluconate. These two strains were showed 92.8% identity to *L. paracasei ssp paracasei* 1 and 6.5 % identity to *L. paracasei ssp paracasei* 2.

Candidated *Lactobacillus* with suppression of TNF-α production strains including CM128, CM130, CM187, F1 and F19 were able to utilize carbohydrates profiles similar to the two *Lactobacillus* strains with anti- *H. pylori* H40 activity as described above, however, there were some fermented sugars differ as indicated in Tables 30. CM128 and CM130 were also identified as *L. paracasei ssp paracasei* in varying identity of 96.8 % and 95.5 %, respectively (Figure 32A). CM187 strain was identitical to *L. fermentum* with 94.1% (Figure 32B). F1 and F19 strains were identitical to *L. plantarum* in varying identity of 96.5 % and 99.1 %, respectively (Table 30).

Table 30 Carbohydrate fermentation of selected *Lactobacillus* strain* (continued)

Test	Carbohydrate	Strain 10-1	Strain 10-2	Strain CM128	Strain CM130	Strain CM187	Strain F1	Strain F19
1	Glycerol	+	+	-	-	-	-	-
2	Erythritol	-	-	-	-	-	-	-
3	D-arabinose	-	-	-	-	-	-	-
4	L-arabinose	-	-	-	+	-	+	+
5	Ribose	+	+	+	+	-	+	+
6	D-Xylose	-	-	-	-	+	-	-
7	L-Xylose	-	-	-	-	-	-	-
8	Adonitol	-	-	-	-	-	-	-
9	β – methyl-D-xyloside	-	-	-	-	-	-	-
10	Galactose	+	+	+	+	+	+	+
11	Glucose	+	+	+	+	+	+	+
12	Fructose	+	+	+	+	+	+	+
13	Mannose	+	+	+	+	+	+	+
14	Sorbose	-	-	+	+	-	-	-
15	Rhamnose	-	-	-	-	-	-	-
16	Dulcitol	-	-	-	-	-	-	-
17	Inositol	-	-	-	-	-	-	-
18	Mannitol	+	+	+	+	-	+	+
19	Sorbital	+	+	+	+	-	+	+
20	Methyl-D-mannoside	-	-	-	-	-	-	-
21	Methyl-D-glucoside	-	-	-	-	-	-	-
22	N-acethyl-glucosamine	+	+	+	+	-	+	+
23	Amygdalin	+	+	-	-	-	+	+
24	Arbutin	+	+	+	+	-	+	+
25	Esculin	+	+	+	+	+	+	+

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

Table 30 Carbohydrate fermentation of selected *Lactobacillus* strain* (continued)

Test	Carbohydrate	Strain 10-1	Strain 10-2	Strain CM128	Strain CM130	Strain CM187	Strain F1	Strain F19
26	Salicin	+	+	+	+	-	+	+
27	Cellobiose	+	+	+	+	+	+	+
28	Maltose	+	+	-	+	+	+	+
29	Lactose	-	-	+	+	+	+	+
30	Melibiose	-	-	-	-	+	+	+
31	Sucrose	+	+	+	+	+	+	+
32	Trehalose	+	+	+	+	-	+	+
33	Inuline	-	-	+	+	-	+	-
34	Melezitose	+	+	+	+	-	+	+
35	Raffinose	-	-	-	-	+	+	+
36	Starch	-	-	-	-	-	-	-
37	Glycogen	-	-	-	-	-	-	-
38	Xylitol	-	-	-	-	-	-	-
39	Gentiobiose	+	+	-	+	-	+	+
40	D-turanose	-	-	+	+	-	+	+
41	D-lyxose	-	-	-	-	-	-	-
42	D-tagatose	+	+	+	+	-	-	-
43	D-fucose	-	-	-	-	-	-	-
44	L-fucose	-	-	-	-	-	-	-
45	D-arabitol	-	-	-	-	-	-	-
46	L-arabitol	-	-	-	-	-	-	-
47	Gluconate	+	+	-	-	-	-	-
48	2-keto-gluconate	-	-	-	-	-	-	-
49	5-keto-gluconate	-	-	-	-	-	-	-

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

A. CM 128 strains

B. CM187 strains

Figure 32 Carbohydrate utilization patterns of TNF-α inhibitory strain CM128 and CM187 by API 50 CHL

CHAPTER V

Discussion

The human gut is a habitat for microflora communities which play an essential in the protection intestinal epithelium and development of immune system [75]. Genus *Lactobacillus* is microflora in the oral cavity, gastrointestinal tract and vagina, as well as fermented food [1]. It has been reported that the most common *Lactobacillus* strains were detected in fecal samples. Some of these *Lactobacillus* strains were able to protect against selected food borne pathogens [60]. Human *Lactobacillus* strains isolated from fecal samples and fermented foods have been reported in many studies [4, 6, 43]. Previous studies demonstrated that *Lactobacillus* isolated from stool samples can modulate TNF-α secretion *in vitro* [9].

Lactobacillus strains: 10-1, 10-2 isolated from infant feces showed strong inhibitory *Helicobacter pylori* H40 activities when compared with *Escherichia coli, Pseudomonas*, the negative control. Similar results were seen in earlier study, *Lactobacillus* species isolated from human gastric biopsy exhibited significantly anti-*H. pylori* in both *in vitro* and *in vivo* [27]. Previous studies described *L. plantarum* isolated from sauerkraut displayed growth inhibition of *H. pylori* [76]. It has been reported that *L. salivarius* was modulated IL-8 induction and downregulated virulence factor gene expression in *H. pylori* [77], while recent studies showed that ingestion of yogurt containing *L. acidophilus* was improved gastric inflammation in *H. pylori*-infected patients through the inactivation of the Smad7 and NF-κB mediated pathways [78]. In this study, some *Lactobacillus* strains from fermented food showed weak inhibitory *H. pylori* H40 activities. These *Lactobacillus* strains probably slightly produced anti-microbial substance in inhibition of *H. pylori*. Thus, different *Lactobacillus* strains isolated from different site may exhibit different inhibition of *H. pylori* activity and these depend on strain specific [79]. Previously studies demonstrated that ingestion of yogurt containing *Lactobacillus*- and *Bifidobacterium* effectively suppressed *H. pylori* infection in humans [16]. Similar result reported that oral administration of *L. casei* from fermented milk was inhibited *H. pylori* activity and was reduced levels of *H. pylori* colonization in infected *H.*

pylori mice [80]. In addition, *L. gasseri* OLL2716 strain antogonized *H. pylori* by inducing the conversion of *H. pylori* morphology form spiral shape to coccoid form, resulting in inhibition of growth activity [81]. *In vitro* and *in vivo* studies indicated that *L. johnsonii* La1 were inhibited motility, adherence and reduced colonization of *H. pylori* [82]. Furthermore randomized prospective study showed that, probiotics *Lactobacillus* species and lactoferrin plus standard triple therapy (esomeprazole + clarithromycin + amoxicillin) significantly improved the efficacy of the standard triple therapy in patient infected *H. pylori* and reduced side effects of antibiotics therapy [83].

Tumor necrosis-α is pro-inflammatory cytokine, induce pathogenesis of the mucosal inflammation in inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis. These cytokine produced by activated macrophages and T lymphocytes and stimulate other pro-inflammatory including interleukin (IL) -1, IL-6. TNF-α play a key role in IBD pathogenesis and recruited neutrophil to local sites of inflammation. Increasing of TNF-α production was detected in intestinal biopsy from IBD patients [84].

In this study, *Lactobacillus* strains from infant feces and fermented foods showed TNF-α inhibition property by inhibitory activities varied from 3 to 98 % inhibition in lipopolysaccharide (LPS)-activated THP-1 monocytic cells. Twenty-four *Lactobacillus* isolates showed weak inhibitory TNF-α activities. Twenty four *Lactobacillus* isolates showed moderate inhibitory TNF-α activities. Thirty nine *Lactobacillus* isolates showed strong inhibitory TNF-α activities. Interestingly, CM128 and F19 exhibited the most potent TNF-α inhibition by 82% and 98%. The data demonstrated that 39 *Lactobacillus* isolates significantly ($p<0.01$) inhibited TNF-α production in LPS-activated THP-1 monocytic cells when compared to MRS bacterial media control. Similarly, results were seen in animal model. *L. rhamnosus* GG decreased TNF-α secretion in LPS-activated murine macrophages [31]. Similarly, *L. reuteri* was suppressed TNF-α production in LPS-activated monocytoid cells [35]. In patient with ulcerative colitis, oral ministration of *L. delbruekii and L. fermentum* decreased TNF-α secretion [34]. It has been reported that probiotic preparation VSL# 3 modulated ulcerative colitis and reduces active inflammation in patient ulcerative colitis [73].

To survive during passage through the human gut, *Lactobacillus* species must tolerate to gastric acid and bile salt conditions and able to grow in the presence of bile and acids. It has been reported that native lactobacilli isolated from fecal samples tolerated to acid and bile [(85)]. In this study, 7 candidate probiotics isolated from feces and fermented foods demonstrated that 10-1 strain showed acid tolerance in the pH 3.0, 4.0. Strain of 10-2 showed resist at pH4.0. Strain of CM128 showed resist at pH 3.0 and 4.0. CM130 and CM187 strain were survived at pH 3.0 and 4.0. Similarly, F1 strain showed strong resist at pH 3.0 and 4.0. Together, our result indicated that candidate *Lactobacillus* strains almost resistance at pH 3.0 and 4.0, which were similar to novel *Lactobacillus* species. The *L. thermotolerans* isolated from chicken feces tolerated to acid at pH 3.0 [(86)].

Probiotic *Lactobacillus* was resist to approximately 0.3% bile in colonization in human intestine [(87)]. In this study, 7 candidate probiotics isolated from feces and fermented foods showed viability in bile. Viability of 10-1 strain tolerated to bile at 2%, 3%, 4% bile. Viability of 10-2 was tolerated to bile at 1%, 2%, 3% and 4% when compared to MRS bacterial media control. CM128 was tolerated to bile at 1%, 2%, 3% and 4% when compared to MRS bacterial media control. Viability of CM130 was resist to 1%, 2%, 3% and 4% bovine bile when compared to MRS bacterial media control. CM187 was tolerated to 1%, 2%, 3% and 4% bile when compared to MRS bacterial media control. Viability of F1 isolate was decreased at 3% and 4% about 1 log value but viable count tolerance more bile at 1% and 2% when compared to MRS bacterial media control. F19 was tolerated to 1%, 2%, 3% and 4% when compared to MRS bacterial media control. In this study, almost all candidate probiotics were able to resist at 1-4% bile, resulting in survival in gastrointestinal tract of human. Similar studies showed that probotic *L. acidophilus* NIT isolated from infant feces was resistant to 1-3% bile [(88)]. Likewise, *Lactobacillus* sp. isolated from raw cow milk was resistant to 0.2% bile [(89)].

In this study, a total of 2 anti-*H. pylori* H40 activity and 5 immunomodulatory *Lactobacillus* strains were subjected to characterize by API 50 CHL. The highest identity closely related to 100% was used for species identification.

In our study, 2 anti-*H. pylori* H40 strains 10-1 and 10-2 were showed 92.8% identity to *L. paracasei ssp paracasei*.

In immunomodulatory strains, two of 5 strains, CM128 and CM130 were also identified as *L. paracasei ssp paracasei* in varying identity of 96.8 %, 95.5 %, respectively. CM187 was identitical to *L. fermentum* with 94.1% identity. F1 and F19 strains were identitical to *L. plantarum* in varying identity of 96.5 %, 99.1 %, respectively.

Base on phenotypic characterization, anti-pathogenic isolates were identified as *L. paracasei ssp paracasei* whereas, consumption of the synbiotic containing anti-oxidative *L. fermentum* ME-3, *L. paracasei* 8700:2 and *Bifidobacterium longum* 46 with prebiotic were reduced systemic anti-oxidative activity in *H. pylori*-colonized asymptomatic subjects [(90)].

In immunomodulatory strains were identified as *L. paracasei ssp paracasei*, *L. fermentum* and *L. plantarum*. Similarly, it has been reported that *L. paracasei* able to immunomodulatory in IBD [(91)]. *L. fermentum* decreased TNF-α production in patient with ulcerative colitis [(34)]. *L. plantarum* inhibited IL-8 secretion which induced by TNF-α [(92)]. Recently studies demonstrated that lipoteichoic acid Isolated from *L. plantarum* inhibited TNF-α production [(93)].

Conclusions, *Lactobacillus* strains isolated from infant feces and fermented food products were investigated for inhibition of *H. pylori* H40 growth and the suppression of TNF-α production. About 400 isolates were tested for their antagonistic function against *H. pylori* H40 and the results revealed that 2 isolates exhibited strong inhibitory effect against *H. pylori* H40 by agar spot method. The inhibitory activity whether it was due to acid production or some bacteriocins production was unclear. Immunomodulation on TNF-α production in LPS-activated THP-1 cells, *Lactobacillus* isolates were investigated and the results revealed that 39 isolates significantly inhibited TNF-α production. Interestingly, CM128 and F19 strains exhibited the most potent TNF-α inhibition by 82% and 98%.

The result revealed that the two main probiotic properties, candidated *Lactobacillus* isolates of anti- *H. pylori* H40 activity and anti-inflammatory strains were almost resisted to acid at pH 3.0, 4.0 and 1-4% bile. *Lactobacillus* strains tolerated to acid and bile resulting in survival in the gastrointestinal tract condition. These candidated *Lactobacillus* isolates probably directly produced anti-microbial substance or other immunomodulation.

The results of present study indicated that most anti- *H. pylori* H40 activity and anti-inflammatory strains belonged to the *L. paracasei ssp paracasei* so that it may be considered to be an important species in human health which was able to inhibit pathogen and suppress TNF-α production. The other immunomodulatory strains were identify as *L. fermentum* and *L. plantarum* which these two strains have been repoted to be essential for the inhibition of TNF-α production.

However, the anti-*H. pylori* H40 activity and immunomodulatory activity by candidated *Lactobacillus* should be addressed for further investigations to understanding the mechanism of *H. pylori* inhibition as well as the supression of TNF-α production, such as the study of cytokine profiles, in order to apply these finding in human physical therapy.

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APPENDIX

APPENDIX A

MATERIALS AND EQUIPMENTS

1 Materials

1 Gas package (BBL, Becton Dickinson, USA)

2 Conical tube: 15, 50 ml (Corning, USA)

3 Sterile serological pipettes: 1, 2, 5, 10, 25 ml (VWR, USA)

4 Filter paper 0.22 μm (Millipore, USA)

5 Tissue culture flash: 25, 75 cm^2 (Corning,USA)

6 Tissue culture plate: 12, 96-well plate (Falcon BD, USA)

7 Cryovial (Nalgene, USA)

8 ELISA plate: 96- well plate: Immunolon 2 HB (Thermo, USA)

9 API 50 CHL medium (BioMerieux, France)

10 API 50 CH rapid fermentation test strips (BioMerieux, France)

11 Ovine bile (Sigma, USA)

12 96-well microtiter plates (Corning, USA)

13 Cary-Blair transport medium (Oxoid, Hampshire, UK)

14 Trypan-blue (Gibco-Invitrogen, USA)

15 Bovine serum album (BSA: Sigma, USA)

16 Anaerobic GasPak (*Mitsubishi*, *Japan*)

2 Equipments

1 Microcentrifuge (Eppendorf, USA)

2 Refrigerated centrifuge (Sorval, USA)

3 Centrifuge (IEC, USA)

4 -20°C Freezer (VWR, USA)

5 -80°C Freezer (Revco, USA)

6 Light microscope (Nikon, Japan)

7 Inverted microscope: Nikon TMS No. 300679 (Nikon, Japan)

8 Pipettes: P-2, P-10, P-200, P-1000 (Gilson, France)

9 Frogger (DAN-KAR CCRP, USA)

10 Multi-channel (Rainan, USA)

11 CO_2 humidified incubator (VWR, USA)

12 Safety cabinet (The baker company, USA)

13 Water bath 37°C (Fisher Scientific, USA)

14 Speed vac DNA (Savant Instruments, USA)

15 Spectrophotometer: SmartSpec 3000 (BioRad, USA)

16 Water bath (VWR,USA)

17 Anaerobic jar (Mitsubishi, Japan)

18 37°C Incubator (VWR, USA)

3 Software and program

GraphPad Prism version 5.01 using Student's *t*-test with one-tailed distribution

API 50 CH version 5.1 database (http://apiweb.biomerieux.com

APPENDIX B

FLOW CHART OF PROTOCOL

1 Isolation of Lactobacilli from Human Feces

Fecal samples in a Cary-Blair transport medium

↓

Suspend in 9 ml NSS and serial 10-fold dilution

↓

Each dilution of $1{:}10^4$ – $1{:}10^6$ spread on MRS agar plate

↓

Incubate anaerobically at 37° C for 48 -72 hr

↓

Peak isolated colonies and streak on new MRS agar plate

↓

Incubate anaerobically at 37° C for 48 -72 hr

↓

Subculture single pure colony for experimental use

2 Presumptive Test of *Lactobacillus*

Single pure colony

Gram-stain

Catalase test

Drop 3% H_2O_2 in bacterial cells

Observation of bubble formation in bacterial cells

Record bubble formation as positive

3 Assessment of Antagonistic Activity by Spot Method

Inoculate each *Lactobacillus* strain in 200 μl MRS broth

in each well of 96-well plate, incubate anaerobically at 37°C for 24-48 hr

↓

Spot of each culture into 175 μl MRS broth

in each well of 96-well plate, incubate anaerobically at 37°C for 24 hr

↓

Spot each culture onto BHI 20 mM glucose in 150 mm plate

incubate anaerobically at 37°C for 24 hr

↓

Add 8 ml of *Helicobacter pylori* 10^8 cells/ml in BHI 0.8 %soft agar

over the plate, incubate anaerobically at 37°C for 72 hr

↓

Measurement of clear zone around the spotted and record in mm

4 Preparation of *Lactobacillus* Conditioned Media (LCM)

Inoculate 1×10^{8} cells/ml *Lactobacillus* isolate in MRS broth

incubate anaerobically at 37°C for 24 or 48 hr

↓

Collect *Lactobacillus* cell-free conditioned media

by centrifugation at 4,000 rpm for 10 min

↓

Filter-sterilized by using a 0.22 μm pore size filter

↓

Drying by speed-vacuum

↓

Re-suspend pellet in equal volume of cell culture media

↓

Store *Lactobacillus*-conditioned media at -20°C until use

5 Bioassay for modulation of TNF-α production

Plate 200 μl of $2.5x10^5$ cells/ml THP-1 cells into each well of a 96-well plate

↓

Add 10 μl (5% v/v) of *Lactobacillus* conditioned media into appropriate wells

↓

Add 5 μl (final conc.100 ng/ml) of *E. coli* LPS (serotype O127:B8, Sigma) into appropriate wells

↓

Incubate at 37°C, CO_2 incubator for 3.5 hours

↓

Collect culture and centrifuge to collect supernatants

↓

Test for cytokine secretion by TNF- α sandwich ELISA

APPENDIX C

Phenotypic characterization of selected *Lactobacillus* isolates by API 50CHL

Table 31 Carbohydrate fermentation of 10-1 and 10-2 *Lactobacillus* strains*

Test	Carbohydrate	Strains 10-1, 10-2	Test	Carbohydrate	Strains 10-1, 10-2
1	Glycerol	+	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	+
4	L-arabinose	-	29	Lactose	-
5	Ribose	+	30	Melibiose	-
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	-
9	β – methyl-D-xyloside	-	34	Melezitose	+
10	Galactose	+	35	Raffinose	-
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	-	39	Gentiobiose	+
15	Rhamnose	-	40	D-turanose	-
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	+
18	Mannitol	+	43	D-fucose	-
19	Sorbital	+	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	+	47	Gluconate	+
23	Amygdalin	+	48	2-keto-gluconate	-
24	Arbutin	+	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production; -, no acid production

API database indicated 92.8% identity to *L. paracasei ssp paracasei* 1 and 6.5 % identity to *L. paracasei ssp paracasei* 2.

Table 32 Carbohydrate fermentation of CM128 or 39-1 *Lactobacillus* strain*

Test	Carbohydrate	Strain CM128	Test	Carbohydrate	Strain CM128
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	-
4	L-arabinose	-	29	Lactose	+
5	Ribose	+	30	Melibiose	-
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	+
9	β – methyl-D-xyloside	-	34	Melezitose	+
10	Galactose	+	35	Raffinose	-
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	+	39	Gentiobiose	-
15	Rhamnose	-	40	D-turanose	+
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	+
18	Mannitol	+	43	D-fucose	-
19	Sorbital	+	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	+	47	Gluconate	-
23	Amygdalin	-	48	2-keto-gluconate	-
24	Arbutin	+	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

API database indicated 96.8% identity to *L. paracasei ssp paracasei* 1 and 1.8 % identity to *L. paracasei ssp paracasei* 3.

Table 33 Carbohydrate fermentation of CM130 *Lactobacillus* strain*

Test	Carbohydrate	Strain CM130	Test	Carbohydrate	Strain CM130
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	+
4	L-arabinose	+	29	Lactose	+
5	Ribose	+	30	Melibiose	-
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	+
9	β – methyl-D-xyloside	-	34	Melezitose	+
10	Galactose	+	35	Raffinose	-
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	+	39	Gentiobiose	+
15	Rhamnose	-	40	D-turanose	+
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	+
18	Mannitol	+	43	D-fucose	-
19	Sorbital	+	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	+	47	Gluconate	-
23	Amygdalin	-	48	2-keto-gluconate	-
24	Arbutin	+	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

API database indicated 95.5% identity to *L. paracasei ssp paracasei* 1 and 3.6 % identity to *L. paracasei ssp paracasei* 3.

Table 34 Carbohydrate fermentation of CM187 *Lactobacillus* strain*

Test	Carbohydrate	Strain CM187	Test	Carbohydrate	Strain CM187
1	Glycerol	-	26	Salicin	-
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	+
4	L-arabinose	-	29	Lactose	+
5	Ribose	-	30	Melibiose	+
6	D-xylose	+	31	Sucrose	+
7	L-xylose	-	32	Trehalose	-
8	Adonitol	-	33	Inuline	-
9	β – methyl-D-xyloside	-	34	Melezitose	-
10	Galactose	+	35	Raffinose	+
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	-	39	Gentiobiose	-
15	Rhamnose	-	40	D-turanose	-
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	-
18	Mannitol	-	43	D-fucose	-
19	Sorbital	-	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	-	47	Gluconate	-
23	Amygdalin	-	48	2-keto-gluconate	-
24	Arbutin	-	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

API database indicated 94.1% identity to *L. fermentum* 1and 3 % identity to *L. fermentum* 3.

Table 35 Carbohydrate fermentation of F1 *Lactobacillus* strain*

Test	Carbohydrate	Strain F1	Test	Carbohydrate	Strain F1
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	+
4	L-arabinose	+	29	Lactose	+
5	Ribose	+	30	Melibiose	+
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	+
9	β – methyl-D-xyloside	-	34	Melezitose	+
10	Galactose	+	35	Raffinose	+
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	-	39	Gentiobiose	+
15	Rhamnose	-	40	D-turanose	+
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	-
18	Mannitol	+	43	D-fucose	-
19	Sorbital	+	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	+	47	Gluconate	-
23	Amygdalin	+	48	2-keto-gluconate	-
24	Arbutin	+	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production;   -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

API database indicated 96.5 % identity to *L. plantarum* and 2.6 % identity to *L. brevis*

Table 36 Carbohydrate fermentation of F19 *Lactobacillus* strain*

Test	Carbohydrate	Strain F19	Test	Carbohydrate	Strain F19
1	Glycerol	-	26	Salicin	+
2	Erythritol	-	27	Cellobiose	+
3	D-arabinose	-	28	Maltose	+
4	L-arabinose	+	29	Lactose	+
5	Ribose	+	30	Melibiose	+
6	D-xylose	-	31	Sucrose	+
7	L-xylose	-	32	Trehalose	+
8	Adonitol	-	33	Inuline	-
9	β – methyl-D-xyloside	-	34	Melezitose	+
10	Galactose	+	35	Raffinose	+
11	Glucose	+	36	Starch	-
12	Fructose	+	37	Glycogen	-
13	Mannose	+	38	Xylitol	-
14	Sorbose	-	39	Gentiobiose	+
15	Rhamnose	-	40	D-turanose	+
16	Dulcitol	-	41	D-lyxose	-
17	Inositol	-	42	D-tagatose	-
18	Mannitol	+	43	D-fucose	-
19	Sorbital	+	44	L-fucose	-
20	Methyl-D-mannoside	-	45	D-arabitol	-
21	Methyl-D-glucoside	-	46	L-arabitol	-
22	N-acethyl-glucosamine	+	47	Gluconate	-
23	Amygdalin	+	48	2-keto-gluconate	-
24	Arbutin	+	49	5-keto-gluconate	-
25	Esculin	+			

+, acid production; -, no acid production

* The patterns were analyzed by API 50 CHL V 5.1

at https://apiweb.biomerieux.com/servlet/Identify

API database indicated 99.1 % identity to *L. plantarum* and 0.7 % identity to *L. pentosus*

VITAE

VITAE

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List of Presentation

Poster presentation

1 Ladda B, Chimchang J, Triviroj T, Tanasupawat S, Taweechotipatr M. Probiotic effects of lactic acid bacteria in the suppression of tumor necrosis factor-α production. Quality of Care and Patient Safety. 17-18, 1, 2012. Faculty of medicine. Srinakharinwirot University. Nakhonnayok. Thailand.

2 Chimchang J, Ladda B, Triviroj T, Wongsatayanon B, Taweechotipatr M. Screening for anti-proliferative effects of lactic acid bacteria against human cancer cell lines. Quality of Care and Patient Safety. 17-18, 1, 2012. Faculty of Medicine Srinakharinwirot University. Nakhonnayok. Thailand.

3 Ladda B, Chimchang J, Triviroj T and Taweechotipatr M. Anti-inflammatory activities of lactobacilli isolated from infant feces on tumor necrosis factor-α production. International Symposium of Probiotics & Prebiotics in Pediatrics 2012. 24-26, 2, 2012. Renaissance Polate hotel. Istanbul. Turkey.

4 Chimchang J, Ladda B, Wongsatayanon B, Taweechotipatr M. Effects of lactic acid bacteria isolated from infant feces on cancer cell proliferation. International Symposium of Probiotics and Prebiotics in Pediatrics 2012. 24-26, 2, 2012. Renaissance Polate hotel. Istanbul. Turkey.

5 บุญรัตน์ ลัดดาและมาลัย ทวีโชติภัทร์. คุณสมบัติของแบคทีเรียกรดแลคติกในการต่อต้านเชื้อ *Helicobacter pylori*. การประชุมวิชาการ “ศรีนครินทรวิโรฒวิชาการ” ครั้งที่ 6 29 – 30 พฤษภาคม 2555. มหาวิทยาลัยศรีนครินทรวิโรฒ. กรุงเทพมหานคร.