

PROBIOTIC CHARACTERIZATION OF *LACTOBACILLUS* ISOLATES ON CANCER CELL
PROLIFERATION AND ANTIMICROBIAL ACTIVITIES



Presented in Partial Fulfillment of the Requirements for the
Master of Science Degree in Molecular Biology
at Srinakharinwirot University

May 2012

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AN ABSTRACT
BY
JUNTANA CHIMCHANG

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Lactic acid bacteria (LAB) are probiotic organism, particularly *Lactobacillus* strains. In this study, LAB isolated from healthy infant feces and fermented foods were investigated for their two main probiotic properties: production of reuterin and anti-proliferative effect against cancer cell lines as well as characterization of selected strains.

Three hundreds *Lactobacillus* isolates were tested for their production of reuterin by spot overlay technique. Six *Lactobacillus* isolates, H8, H9, H122, H123, H210 and H323, produced reuterin and they were tested antimicrobial activity by using agar well diffusion method. Only 1 isolate, H210 strains demonstrated potent antimicrobial activity against 11 indicator strains including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, methicillin resistant *S. aureus* (MRSA) DMST 20651, methicillin resistant *S. aureus* (MRSA) DMST 20654, *Vibrio cholerae* DMST 2873, *Vibrio parahaemolyticus*, *Salmonella* Typhimurium, *Shigella dysenteriae*, *Helicobacter pylori* (H40) and *Candida albicans* ATCC 10231.

Three hundreds *Lactobacillus* isolates were tested for their anti-proliferative effect against human colon carcinoma cell line (Caco-2) and human monocytic leukemic cell line (U937) by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The results demonstrated that 15 strains isolated from healthy infant feces and fermented foods significantly exhibited anti-proliferative effects against Caco-2 cells. However, 11 positive strains showed significant cytotoxic effect on normal cells, African green monkey kidney (Vero). In addition, 32 strains showed significant exhibited anti-proliferative effects against U937 cells, but 13 positive strains showed significant cytotoxic effect on Vero cells.

On the basis of the phenotypic characteristic by API 50 CHL, H210 reuterin-producing strain which showed the most potent antimicrobial activity against indicator strains, were identified as *Lactobacillus plantarum* 76.2% similarity. Seven isolates have shown the strongest anti-proliferative effect on cancer cell lines, 4 strains, H286, H468 and H305, H320 showed inhibitory effect on the cell proliferation in Caco-2 and U937 cells were identified as *Lactobacillus paracasei* which displayed 61.5%, 92.8% and 99.9%, 92.8% similarity. Two strains, H308 and H415 showed inhibitory effect on the cell proliferation in

U937 cells were identified as *Lactobacillus salivarius* 99.9% and *Lactobacillus plantarum* 66.6% similarity. One strain, H323 showed inhibitory effect on the cell proliferation against both Caco-2 and U937 cells were identified as *Lactobacillus pentosus* 99.8% similarity.



การศึกษาคุณลักษณะโพรไบโอติกของสายพันธุ์แลคโตบาซิลลัสต่อการเพิ่มจำนวนของเซลล์มะเร็ง
และการต้านเชื้อจุลินทรีย์



บทคัดย่อ
ของ
จันทนา ฉิมแจ้ง

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษาตาม
หลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาอนุชีววิทยา
พฤษภาคม 2555

จันทนา ฉิมแจ้ง. (2555). การศึกษาคุณลักษณะโพรไบโอติกของสายพันธุ์แลคโตบาซิลลัสต่อการเพิ่มจำนวนของเซลล์มะเร็งและการต้านเชื้อจุลินทรีย์. วิทยานิพนธ์ วท.ม. (อณูชีววิทยา). กรุงเทพฯ: บัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ. คณะกรรมการควบคุม: อาจารย์ ดร. มัลย์ ทวีโชติภักดิ์, รองศาสตราจารย์ ดร. เบญจมาศ วงศ์สัตยพนธ์.

จุลินทรีย์โพรไบโอติก ได้แก่ จุลินทรีย์ในกลุ่มแบคทีเรียกรดแลคติก (Lactic acid bacteria) โดยเฉพาะเชื้อแบคทีเรียกลุ่มแลคโตบาซิลลัส (*Lactobacillus*) ในการศึกษาครั้งนี้ได้คัดเลือกเชื้อแลคโตบาซิลลัสจากอุจจาระของทารกหลังคลอดที่มีสุขภาพดีและอาหารหมัก เพื่อนำมาศึกษาคุณสมบัติของโพรไบโอติกด้านความสามารถในการสร้างสารต้านจุลินทรีย์ชนิดรอยเทอริน และความสามารถในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง ตลอดจนการศึกษาคุณลักษณะของแลคโตบาซิลลัสสายพันธุ์ที่คัดเลือกได้

จากการนำแลคโตบาซิลลัสจำนวน 300 สายพันธุ์ มาทดสอบความสามารถในการสร้างสารต้านจุลินทรีย์ชนิดรอยเทอริน ผลการศึกษาโดยวิธี spot overlay technique พบว่าเชื้อแลคโตบาซิลลัส 6 สายพันธุ์ คือ H8, H9, H122, H123, H210 และ H323 สามารถสร้างสารต้านจุลินทรีย์ชนิดรอยเทอรินได้ เมื่อนำแบคทีเรียจำนวน 6 สายพันธุ์ นี้ มาทดสอบฤทธิ์ต้านการเจริญของจุลินทรีย์ โดยวิธี agar well diffusion พบว่ามีเพียง 1 สายพันธุ์ คือ H210 ที่มีฤทธิ์ต้านการเจริญของเชื้อจุลินทรีย์ทดสอบ 11 ชนิด ประกอบด้วย *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, methicillin resistant *S. aureus* (MRSA) DMST 20651, methicillin resistant *S. aureus* (MRSA) DMST 20654, *Vibrio cholerae* DMST 2873, *Vibrio parahaemolyticus*, *Salmonella* Typhimurium, *Shigella dysenteriae*, *Helicobacter pylori* (H40) and *Candida albicans* ATCC 10231.

เมื่อนำแลคโตบาซิลลัสทั้ง 300 สายพันธุ์ มาทดสอบความสามารถในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง โดยทดสอบกับเซลล์มะเร็งลำไส้ใหญ่ (Caco-2) และเซลล์มะเร็งเม็ดเลือดขาวชนิดโมโนไซต์ (U937) ของคน โดยวิธี MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) พบว่าแลคโตบาซิลลัส 15 สายพันธุ์ ที่แยกได้จากอุจจาระของทารกและอาหารหมักดอง สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งลำไส้ใหญ่ Caco-2 อย่างมีนัยสำคัญ อย่างไรก็ตามในจำนวน 15 สายพันธุ์นี้ พบว่ามี 11 สายพันธุ์ที่มีความเป็นพิษต่อเซลล์ปกติชนิดเซลล์ไตอย่างมีนัยสำคัญ นอกจากนี้พบว่าแลคโตบาซิลลัส จำนวน 32 สายพันธุ์ สามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งเม็ดเลือดขาว (U937) ได้อย่างมีนัยสำคัญ โดยมี 13 สายพันธุ์ที่เป็นพิษต่อเซลล์ Vero อย่างมีนัยสำคัญ

การศึกษาคุณลักษณะทางฟิโนไทป์ของเชื้อโดยใช้ API 50 CHL พบว่าแลคโตบาซิลลัสสายพันธุ์ H210 ที่สามารถสร้างสารต้านจุลินทรีย์ชนิดรอยเทอรินและมีฤทธิ์ต้านการเจริญของเชื้อทดสอบ เป็น *Lactobacillus plantarum* เชื้อ 7 สายพันธุ์ ที่มีความสามารถในการยับยั้งการเพิ่ม

จำนวนของเซลล์มะเร็งได้ดีที่สุด พบว่ามี 4 สายพันธุ์ คือ H286, H468 และ H305, H320 ซึ่งสามารถยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งลำไส้ใหญ่ Caco-2 และเซลล์มะเร็งเม็ดเลือดขาว U937 พบว่ามีความคล้ายคลึงกับเชื้อ *Lactobacillus paracasei* 61.5%, 92.8% และ 99.9%, 92.8% พบว่าจำนวน 2 สายพันธุ์ คือ H308 และ H415 ซึ่งสามารถยับยั้งเซลล์มะเร็งเม็ดเลือดขาว U937 พบว่ามีความคล้ายคลึงกับเชื้อ *Lactobacillus salivarius* 99.9% และ *Lactobacillus plantarum* 66.6% และมีจำนวน 1 สายพันธุ์ คือ H323 ที่สามารถยับยั้งทั้งเซลล์มะเร็ง Caco-2 และ U937 พบว่ามีความคล้ายคลึงกับเชื้อ *Lactobacillus pentosus* 99.8%.



The thesis titled
“Probiotic Characterization of *Lactobacillus* Isolates on Cancer Cell Proliferation and
Antimicrobial Activities”

By
Juntana Chimchang

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

INTRODUCTION

Lactobacillus is a genus of Gram-positive, non-sporulating, non-motile, catalase-negative, and rod-shaped bacteria. The genus *Lactobacillus* currently includes 199 species (www.dsmz.de) that are aerobic or facultatively anaerobic or strictly anaerobic bacteria. *Lactobacillus* species are widely used in fermented food and dairy industrial⁽¹⁻²⁾. They are acid and bile tolerant and possess a strictly fermentative metabolism with lactic acid as the major metabolic end product⁽³⁾. Lactobacilli are normal microbiota of the gastrointestinal tract (GIT) of humans and animals⁽³⁻⁶⁾ and have been isolated from humans⁽⁴⁻⁵⁾, animals⁽⁷⁻⁸⁾ and fermented products⁽⁹⁻¹¹⁾. There is host specificity in colonization by individual species, for example, *L. fermentum*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. johnsonii*, *L. brevis*, *L. delbrueckii*, *L. curvatus* and *L. sakei* are commonly found in human GIT at various levels⁽¹²⁻¹³⁾.

A joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) define of probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host⁽¹⁴⁾ by improving its gastrointestinal microbial balance⁽¹⁵⁾. Most probiotics are bacteria similar to those naturally found in the human GIT. Recently, scientists have been interesting to study various properties of probiotic bacteria for the potential used as probiotics, especially for infectious diseases or abnormalities in the gastrointestinal tract⁽¹⁶⁻¹⁷⁾. The commonly used and the most studies of probiotics are the members of lactic acid bacteria (LAB), particularly *Lactobacillus* spp. and *Bifidobacterium* spp.⁽¹⁸⁾. Lactobacilli are considered to have beneficial effects on the human and animal health. Several species of lactobacilli are used as probiotics⁽¹⁹⁾. Since, they can survive gastric and bile acids in order to reach the intestinal tract⁽²⁰⁾, adhere to intestinal tissues, colonize intestinal tract, produce antimicrobial substances and are relatively harmless⁽²¹⁾. It has been reported that *Lactobacillus* can reduce the symptoms of the gastrointestinal tract infections⁽²²⁾. Some *Lactobacillus* species can inhibit the growth of enteric pathogens and other microbes⁽²³⁻²⁴⁾. Furthermore, some

Lactobacillus species can produce antimicrobial substances such as bacteriocin⁽²⁵⁻²⁶⁾ and reuterin⁽²⁷⁻²⁸⁾.

Reuterin, a 3-hydroxypropionaldehyde (3-HPA) is a product of glycerol fermentation produced by *Lactobacillus reuteri*. The *L. reuteri* was not the only *Lactobacillus* species which produce reuterin but *L. coryniformis*⁽²⁹⁾, *L. collinoides*⁽³⁰⁾, *L. brevis* and *L. buchneri*⁽³¹⁾ also found to be a producer. Reuterin is a broad-spectrum antibiotic which is active against gram-positive, gram-negative bacteria including yeast, fungi, protozoa and viruses^(32, 33). In addition, reuterin also inhibit growth of various intestinal bacteria except of lactobacilli⁽³³⁾. Reuterin is soluble in water, resistant to heat, proteolytic and lipolytic enzymes and stable over a wide range of pH values⁽³⁴⁻³⁵⁾. Antifungal activity was shown against species of *Candida*, *Torulopsis*, *Saccharomyces*, *Aspergillus* and *Fusarium*⁽³²⁾. Recently, *in vitro* colonic fermentation model have shown that the addition of glycerol alone or with *L. reuteri* ATCC 55730 increased the numbers of *Lactobacillus-Enterococcus* groups and decreased *Escherichia coli*⁽³⁶⁾.

A well known group of probiotic *Lactobacillus* spp. such as, *L. paracasei*, *L. plantarum* and *L. brevis* is commonly shown to have health promoting attributes, including anti-proliferative effect of cancer cells^(37, 41). Many reports also suggested that probiotic bacteria and their fermented product have antitumor effects⁽³⁸⁻³⁹⁾. The fermented milk of *L. helveticus*, *Bifidobacterium*, *L. acidophilus*, *Streptococcus thermophilus* and *L. delbrueckii*, are known to reduce the growth rate of colon carcinoma cell line which resulted in a 50% decrease in cell proliferation⁽⁴⁰⁾. The cytoplasmic fraction (CF) of *Lactococcus lactis* ssp. *lactis* and *L. brevis* have strong arginine deiminase (ADI) activity which can induce apoptosis of the stomach adenocarcinoma cells and human acute leukemia cells, respectively^(37, 41). Furthermore, *L. paracasei* ssp. *paracasei* NTU 101 and *L. plantarum* NTU 102 which were isolated from human feces and pickled vegetables, respectively. These two *Lactobacillus* strains gave shown that their strong evidence of heat-killed cells (HKC) and cytoplasmic fractions (CF) possessed the significant antioxidant activities and anti-proliferative activities against breast and colon cancer cell lines *in vitro*⁽⁴²⁾.

Lactobacillus species are considered to have beneficial effects on the human health by production of antimicrobial substances which inhibit gastrointestinal pathogens and able to suppress cancer cell proliferations. Therefore, several species of lactobacilli are used as probiotics for treatment and prevention of gastrointestinal disorders and cancer. However, the inhibitory effect of *Lactobacillus* is variable even within the same or different species and the underlying mechanism remain unclear.

In this study, *Lactobacillus* strains will be isolated from infant feces and fermented food products to investigate for two main probiotic properties. 1) To determine the production of reuterin and to evaluate for antimicrobial activity against gastrointestinal pathogens. 2) To determine the inhibitory effect on the cancer cell proliferation.

Hypothesis

Specific probiotic *Lactobacillus* strains are capable to produce reuterin and play a role in inhibition of cancer cell proliferation

Objectives

1. To isolate *Lactobacillus* bacteria from infant feces and fermented foods
2. To screen for reuterin production of *Lactobacillus* isolates
3. To evaluate the antimicrobial activity of reuterin produced from *Lactobacillus* isolates against pathogenic microorganisms
4. To investigate the anti-proliferative activity of *Lactobacillus* conditioned media against the cancer cell lines
5. To study the acid and bile tolerances of the selected *Lactobacillus* candidates
6. To study the phenotypic characteristic of the selected *Lactobacillus* candidates

Expected Outcome

1. *Lactobacillus* isolates from infants feces and fermented foods with capable to produce reuterin and potential application use as probiotics for antimicrobial
2. *Lactobacillus* isolates from infants feces and fermented foods which inhibited cancer cells proliferation have potential application use as probiotics for adjuncts in cancer therapy
3. Candidated *Lactobacillus* isolates which acid and bile tolerances have potential as a good probiotics properties
4. Carbohydrates fermentation pattern for specific for probiotic *Lactobacillus* strains



CHAPTER II

LITERATURE REVIEW

1. Genus *Lactobacillus*

The genus *Lactobacillus* belongs to the group of lactic acid bacteria (LAB) which shows characteristics of gram-positive, non-motile, non-sporulating, non-catalase producing, rod-shaped bacteria (shown in Figure 1). Genus *Lactobacillus* includes 199 species (www.dsmz.de) that are aerobic or facultatively anaerobic or strictly anaerobic bacteria. Taxonomically, the genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae* ⁽⁴³⁾. Lactobacilli are able to produce lactic acid as main end product of the fermentation of carbohydrates ⁽⁴⁴⁾. Lactobacilli are microbiota of the gastrointestinal tract (GIT) of humans and animals ⁽⁴⁴⁻⁴⁶⁾. *Lactobacillus* species have been isolated from humans ⁽⁴⁵⁻⁴⁶⁾, animals ^(9, 48) and fermented food products ⁽⁴⁹⁻⁵¹⁾. There is host specificity in colonization by individual species, for example, *L. fermentum*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. johnsonii*, *L. brevis*, *L. delbrueckii*, *L. curvatus* and *L. sakei* are commonly found in human GIT at various levels ⁽¹²⁻¹³⁾.



Figure 1 Genus *Lactobacillus*

2. *Lactobacillus* as probiotics

2.1 Definition of probiotics

The word “probiotics” was initially used as an antonym of the word “antibiotic”. It is derived from the Greek meaning “for life” was first used to described substances produced by one microorganism that stimulate the growth of another microorganism⁽⁵²⁾. Fuller defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”⁽⁵³⁾. Salminen et al.⁽⁵⁴⁾ proposed that probiotics be defined as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host. Following recommendations of the FAO/WHO the accepted definition describes probiotics as “live microorganisms that when administered in adequate amounts confer a health benefit on the host”. Probiotics consist of bacteria or yeasts and can be considered functional foods that can re-colonize and restore the microflora symbiosis of the intestinal tract. Most probiotics are bacteria similar to those naturally found in people’s guts, especially in those of breast fed infants because they are known to have natural protection against many diseases. Most often, the probiotic bacteria come from two groups, *Lactobacillus* or *Bifidobacterium*⁽⁵⁵⁾

2.2 Probiotic properties

The criteria which is used for classifying the probiotic microorganism is summarized in Figure 2⁽⁵⁶⁾. Strains of human origin are most suitable because some health promoting benefits may be species specific and microorganisms may perform optimally in the species from which they were isolated⁽⁵⁷⁾. However, it is the specificity of the action, not the source of the microorganism that is recognized as the most important consideration of selecting the candidate probiotic strains for particular applications⁽⁵⁸⁾. All probiotic strains have generally recognized as safe (GRAS) status, moreover they should survive passage through the stomach and small intestine by tolerating the acidic and protease-rich conditions of the stomach. Adherent probiotics strains are desirable because they have a greater chance of establishment in the gastrointestinal tract, thus enhancing their probiotics effect⁽⁵⁸⁾. The production of antimicrobial substances is one of the most important selection

criteria for probiotics. Many probiotic *Lactobacillus* species have been shown to produce them. The ability to modulate immune responses is another important advantage for probiotics in immune dysfunction state. Probiotic microorganisms should also be technologically suitable for incorporation into food products and should be capable of surviving industrial applications⁽⁵⁹⁻⁶⁰⁾.

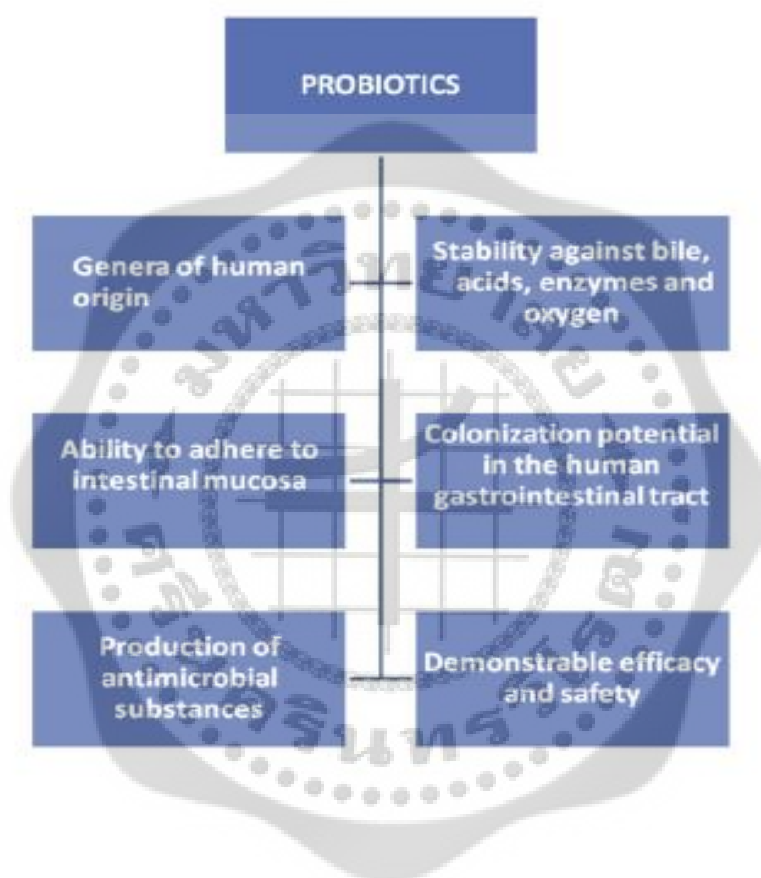


Figure 2 Criteria for classifying a bacterial strain as probiotics⁽⁵⁶⁾

3. Mechanism of action of probiotics

Mechanisms for the benefits of probiotics are not completely understood⁽⁶¹⁻⁶²⁾. Much work is needed to clarify the mechanisms of action of particular probiotics against specific pathogens. In addition, the same probiotics may inhibit different pathogens by different mechanisms⁽⁶³⁾. Listed below is a brief description of mechanisms by which probiotics may protect the host against intestinal disease⁽⁶³⁻⁶⁴⁾.

3.1 Production of antimicrobial substances

Probiotic bacteria are known to produce various antimicrobial substances which can be classified as low-molecular-mass (LMM) compounds such as organic acid (lactic acid, acetic acid and propionic acid), hydrogen peroxide (H_2O_2), reuterin and high-molecular-mass (HMM) compounds like bacteriocins⁽⁶⁵⁾.

3.2 Organic acids

Organic acids, such as acetic acid, citric acid and lactic acid, have been reported to possess a higher bactericidal activity than inorganic acids, such as hydrochloric acid⁽⁶⁶⁻⁶⁷⁾. Acetic and lactic acid are major metabolites of lactic acid bacteria and have been reported to be responsible for their antimicrobial activity against *E. coli* in the intestine⁽⁶⁸⁾. Additionally, acetic and lactic acid have been shown to decrease Shiga-toxin-producing *Escherichia coli* (STEC) virulence gene expression in *E. coli* O157:H7 EDL 933⁽⁶⁹⁾.

3.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a compound with known antimicrobial effect has been reported by many researchers. It is produced in the presence of oxygen. The production of H_2O_2 by lactobacilli is a common feature in the dominant *Lactobacillus* strains isolated from the vagina of healthy women⁽⁷⁰⁻⁷¹⁾. Several *Lactobacillus* strains of vaginal origin have been found to produce H_2O_2 to inhibit gonococci growth in the female genital tract⁽⁷²⁾.

3.4 Reuterin

Reuterin, a low molecular-weight, neutral, water-soluble, non-protein material, is produced by *L. reuteri*, a heterofermentative species and a member of microbiota of gastrointestinal tract of humans and animals. It is formed during the anaerobic growth on a mixture of glucose and glycerol by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin, 3-hydroxypropionaldehyde (3-HPA) ⁽⁷³⁾. In addition, chemical analysis has been shown reuterin to exist in solution as an equimolar mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of 3-hydroxypropionaldehyde (Figure 3) ^(28, 74-75).

Moreover, reuterin can be produced by other *Lactobacillus* species such as *Lactobacillus brevis*, *L. buchneri* ⁽⁷⁶⁾, *L. collinoides* ⁽⁷⁷⁾ and *L. coryniformis* ⁽⁷⁸⁾. Other bacteria including *Clostridium perfringens* ⁽⁷⁹⁾, *C. butyricum* ⁽⁸⁰⁾, *Bacillus amaryllus* ⁽⁸¹⁻⁸²⁾, *Enterobacter agglomerans* ⁽⁸³⁾, *Citrobacter freundii* ⁽⁸³⁻⁸⁴⁾ and *Klebsiella pneumoniae* ⁽⁸⁵⁾ have also been reported to use glycerol via the same pathway and produce intermediate 3-HPA ^(29, 86-87). However, these other bacteria can only produce 3-HPA as a temporarily metabolite, which is immediately further reduced. However *L. reuteri* has shown the unique ability to retain more than the required 3-HPA ^(73, 88).

During log phase, no reuterin is produced since it is reduced by the reducing power from glucose metabolism. However, when cells enter stationary phase, reuterin starts to accumulate ⁽⁸⁹⁾. Reuterin is a very broad spectrum of antimicrobial activity. It was found to have antibacterial, antifungal and anti-protozoal activity. Harmful organisms sensitive to reuterin include species of *Salmonella*, *Shigella*, *Vibrio*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida* and *Trypanosoma* ^(28, 32, 90-91). The addition of glycerol to the growth medium of *Lactobacillus coryniformis* gave an increased antifungal activity ⁽⁹²⁾. Recently, an *in vitro* colonic fermentation model showed that the addition of glycerol alone or with *Lactobacillus reuteri* ATCC 55730 increased numbers of the *Lactobacillus–Enterococcus* group and decreased *Escherichia coli* ⁽⁹³⁾.

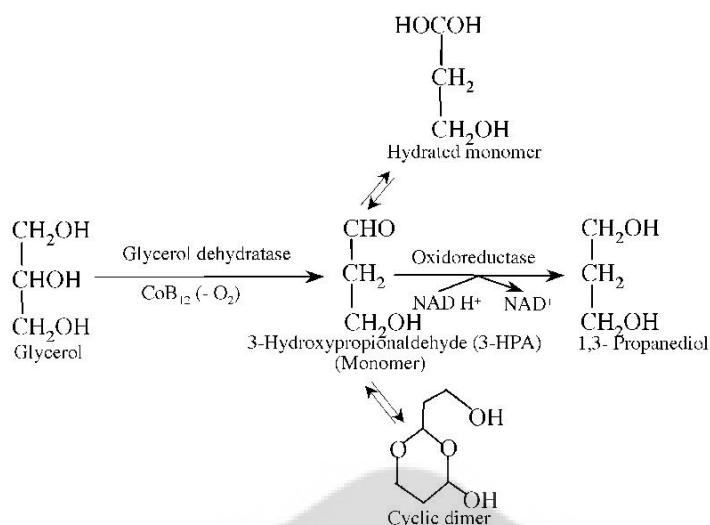


Figure 3 *Lactobacillus reuteri* production of 3-HPA during glycerol fermentation

Three forms of 3-HPA co-exist in equimolar concentration in the aqueous solution forming the reuterin system, and with NAD-dependent oxidoreductase, 3-HPA monomers are further reduced to 1, 3-propanediol⁽⁷⁴⁾

3.5 Bacteriocins

Many lactobacilli are reported to secrete antimicrobial peptides called bacteriocins. Bacteriocins are biologically active, low molecular-weight proteins or peptides. They have been grouped into 4 classes based on their structure and mode of action⁽⁹⁴⁾. Bacteriocins can be regarded as antibiotics, but their mode of action is different from many antibiotics. They have a narrow killing spectrum and thus they are generally able to kill only bacteria closely related to the producing strains⁽⁹⁵⁾. Most of the bacteriocins produced by *Lactobacillus* spp. belong to the Class II bacteriocins that act generally by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria⁽⁹⁶⁾. *L. plantarum* is most often associated with bacteriocin production. A number of bacteriocins have been isolated from various *L. plantarum* strains⁽⁹⁷⁾.

3.6 Competition for pathogen binding sites

The competitive blocking of pathogens binding sites to intestinal epithelial cells has been proposed to represent a potential mechanism for mediating colonization resistance⁽⁹⁸⁻¹⁰⁰⁾. Competitive exclusion of pathogens occur based on binding to the same receptor⁽¹⁰¹⁻¹⁰²⁾. In one study, the collagen-binding protein of *L. fermentum* RC-14 inhibit the adhesion of *Enterococcus faecalis* 1131⁽¹⁰³⁾. In this way, probiotic bacteria may resist peristalsis which would otherwise flush them from the gut. As well as occupying a niche at the expense of potentially harmful organisms, they may specifically block the adherence of enteropathogens^(97, 104).

3.7 Inhibition of virulence factor expression

This mechanism can result from inhibition of toxin expression in pathogens. Ryan et al. have shown that *Lactobacillus salivarius* significantly reduced expression of CAG virulence genes in *H. pylori*. This relates with a *Lactobacillus*-mediated decrease in inflammatory cytokine production by host cells following *H. pylori* infection⁽¹⁰⁵⁾.

3.8 Modulation of host immune system

Several studies investigating probiotics have shown the ability to influence both innate and adaptive immunity. Some strains have been shown to increase phagocytosis and to modify cytokine production in different cell populations⁽¹⁰⁶⁻¹⁰⁹⁾. It has also been reported that lactic acid bacteria (LAB) stimulate, *in vitro*, the production of both pro-inflammatory cytokines (TNF- α , IL-12) and the anti-inflammatory cytokine IL-10 in cells of the innate immune system such as macrophages and natural killer cells⁽¹¹⁰⁻¹¹²⁾.

4. Strains used as probiotics

Many microorganisms have been used or considered for use as probiotics⁽¹¹³⁾. The most commonly used probiotics are lactobacilli and bifidobacterium⁽¹¹⁴⁾. Probiotics are available in different forms, including foods in a fermented state and in the form of pills, mainly as capsules or microencapsulated forms⁽¹¹⁵⁾. The beneficial effects of *Lactobacillus*

and *Bifidobacterium* have been discussed for decades. Bacteria in these two genera are non-pathogenic, resist gastric acid, bile salts, pancreatic enzymes and technological processing. They have the potential to adhere to gut epithelial tissue, colonize the lumen of the GI tract and produce antimicrobial substances ⁽¹¹⁶⁻¹¹⁷⁾. They are considered important components of the gastrointestinal microbiota and are relatively harmless. *Lactobacillus* species are typically used as human probiotics because they are easy to cultivate in bulk and have a long history of safe use in fermented foods ⁽¹¹⁸⁾. As shown in Figure 3, the genus *Lactobacillus*, *Bifidobacterium*, and others are currently being used as probiotics either singly or in combination ⁽¹¹⁹⁾. *Lactobacillus* species have been demonstrated to inhibit the *in vitro* growth of many enteric pathogens including *Escherichia coli*, *Salmonella paratyphi* A, *Shigella sonnei*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Vibrio* spp., *Clostridium difficile* and *Campylobacter jejuni* ⁽¹²⁰⁻¹²²⁾. Therefore, they have been used in both humans and animals to treat a broad range of gastrointestinal disorders ⁽¹²³⁻¹²⁴⁾. Several publications have described the use of probiotics to prevent and treat a variety of gastrointestinal disorders.

<i>Lactobacillus</i> species	Bifidobacterium species	<i>Streptococcus</i> species
<i>L. acidophilus</i>	<i>B. bifidum</i>	<i>S. thermophilus</i>
<i>L. casei</i>	<i>B. breve</i>	Yeasts
<i>L. fermentum</i>	<i>B. lactis</i>	<i>Saccharomyces cerevisiae</i> (boulardii)
<i>L. gasseri</i>	<i>B. longum</i>	Mixtures
<i>L. johnsonii</i>	<i>B. animalis</i>	<i>L. acidophilus</i> + <i>L. casei</i> , <i>L. rhamnosus</i> +
<i>L. lactis</i>	<i>B. infantis</i>	<i>L. reuteri</i> , VSL#3 (mixture of 1 strain of
<i>L. paracasei</i>		<i>Streptococcus thermophilus</i> , four
<i>L. plantarum</i>		<i>Lactobacillus</i> spp., & three <i>Bifidobacterium</i>
<i>L. reuteri</i>		spp. strains), <i>L. acidophilus</i> +
<i>L. rhamnosus</i>		<i>Bifidobacterium bifidum</i> , <i>L. helveticus</i> + <i>L.</i>
<i>L. salivarius</i>		<i>rhamnosus</i> , <i>Bacillus clausii</i> strains

Figure 4 Commercially used probiotic strains ⁽¹¹⁹⁾

5. Role of probiotics in intestinal disorders and infectious diarrhea

5.1 Antibiotic-induced diarrheal disease

Antibiotic-associated diarrhea (AAD) is a common complication of antibiotic treatment ⁽¹²⁵⁾. The pathogenesis of antibiotic-induced diarrhea is not understood but is undoubtedly related to quantitative and qualitative changes in the intestinal microbiota ⁽¹²⁶⁾. Many studies have evaluated a variety of probiotics for their efficacy as a means of treating and preventing antibiotic-associated diarrhoea ⁽¹²⁷⁻¹²⁹⁾.

5.2 *Clostridium difficile*–associated diarrhea

Clostridium difficile is an anaerobic, gram positive, spore-forming bacillus that colonizes the intestinal tract after alteration of normal intestinal flora, usually by antibiotic therapy⁽¹³⁰⁻¹³¹⁾. After antibiotic intake by animals and human, *C. difficile* increases in number and releases two protein exotoxins, toxin A (an enterotoxin) and toxin B (a cytotoxin), leading to diarrhoea. Toxigenic *C. difficile* is the cause of nearly 25% of cases of antibiotic-associated diarrhoea⁽¹³²⁾. The occurrence of *C. difficile* diarrhea can also be significantly reduced by administering probiotics⁽¹³³⁾. *L. paracasei*, *L. plantarum* and *L. salivarius* have been reported to inhibit several *C. difficile* toxin A-producing strains *in vitro*^(121, 134). An *in vivo* study by Castagliuolo and colleagues in animal models indicates that pretreatment with *S. boulardii* can directly inhibit *C. difficile* toxin⁽¹³⁵⁾. Another study showed a decreased of synthesis and/or secretion of both clostridial toxins in the presence of some strains of bifidobacteria or lactobacilli⁽¹³⁶⁾.

5.3 Rotavirus diarrhea

Rotavirus is the most important cause of infantile diarrhea. Rotavirus is a significant cause of infant morbidity and mortality, particularly in developing countries⁽¹³⁷⁻¹³⁸⁾. The rotavirus has several ways of transmission: fecal-oral, through contaminated water supplies, poor hygiene, food and fomites⁽¹³⁹⁻¹⁴⁰⁾. Saavedra et al.⁽¹⁴¹⁾ reported that *Lactobacillus* GG has been observed to decrease the rate of rotavirus associated diarrhea and rotaviral shedding. Moreover the author reported an interesting study which highlights that 90% of children treated with *Lactobacillus* GG had an IgA specific Antibody- Secreting Cells (sASC) response to rotavirus versus 46% for placebo.

5.4 Travellers' diarrhea

Travellers' diarrhea (TD) is a condition usually due to the ingestion of food and beverages contaminated by fecal matters. 80% of the cases are bacteria caused. Bacterial pathogens are the major cause of TD, the most common of which are enterotoxigenic *Escherichia coli* (ETEC), *Campylobacter*, *Salmonella* and *Shigella*, viruses or parasites⁽¹⁴²⁻

¹⁴⁴⁾. Several studies were performed with the use of probiotics including *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Saccharomyces* that have shown beneficial effects as a preventive for traveler's diarrhea ⁽¹⁴⁵⁻¹⁴⁷⁾. Some other probiotics have been tested *in vitro* and using animal models. Studies with human epithelial cell lines have shown that probiotic strains *Lactobacillus helveticus* R0052 and *Lactobacillus rhamnosus* R0011 could block the adhesion of Shiga-toxin-producing *E. coli* and enteropathogenic *E. coli* ⁽⁹⁹⁾. A recent study conducted in a new animal model for ETEC diarrhea, using male Wistar rats, showed that the probiotics preparation FF1 and *S. boulardii*, significantly limited body weight loss, decreased the secretion of pro-inflammatory cytokines, and inhibited the production of diarrhea in treated rats ⁽¹⁴⁸⁾.

5.5 Food borne pathogen-associated gastrointestinal infections

Disruption of the equilibrium of the GI bacterial microflora ecology leads to an imbalance that can allow establishment and growth of transient enteropathogens like *Salmonella*, *Campylobacter*, *E. coli*, *Listeria* and *Shigella* spp. Several *Lactobacillus* species have been examined and exhibited antagonistic to *B. cereus*, *E. coli*, *S. aureus*, *Yersinia enterocolitica* and *Listeria* *in vitro* ⁽¹⁴⁹⁾. In addition *Lactobacillus paracasei* subsp. *paracasei* M5-L, *L. rhamnosus* J10-L, *L. casei* Q8-L and *L. rhamnosus* GG have been shown to inhibit *Shigella sonnei*, *Escherichia coli* and *Salmonella typhimurium* ⁽¹⁵⁰⁾. Moreover, *L. fermentum* isolated from intestinal biopsy samples exerted potent antimicrobial activities against enteroinvasive and foodborne pathogens such as *Escherichia coli*, *Salmonella paratyphi* A, *Shigella sonnei*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Vibrio* spp. ⁽¹⁵¹⁾.

5.6 *Helicobacter pylori* gastroenteritis

Helicobacter pylori (*H. pylori*) is an intestinal pathogen that is the main cause of chronic gastritis, peptic ulcer disease ⁽¹⁵²⁾ and is a risk factor for gastric cancer and other gastric malignancies in humans ⁽¹⁵³⁾. Several *in vitro* studies have shown that various lactobacilli can inhibit *H. pylori* growth. Strains with this ability include *Lactobacillus acidophilus* and *L. acidophilus* strain CRL 639 ⁽¹⁵⁴⁾. Similarly, *L. johnsonii* La1 and *L. gasseri* OLL2716 were also found to reduce *H. pylori* colonization and inflammation ⁽¹⁵⁵⁾. In a

double-blind placebo-controlled study, 40 *H. pylori*-positive subjects were given *Lactobacillus reuteri* ATCC 55730 once a day for 4 weeks. *L. reuteri* reduced *H. pylori* intragastric load *in vivo*, decreases dyspeptic symptoms, and affects eradication rates after conventional treatment. Nevertheless, it does not seem to affect antibiotic therapy outcome. The authors report that a 4-week supplementation with *L. reuteri* is effective in reducing *H. pylori* bacterial load in humans and theoretically may help to control gastric inflammation⁽¹⁵⁶⁾.

6. Role of probiotics in cancer prevention

Cancer is a serious global public health problem. Cancer incidence and mortality have been steadily rising throughout the past century in most places of the world⁽¹⁵⁷⁾. Probiotics microorganisms are commonly known to have health promoting attributes, including anti-proliferative effects and apoptosis against human cancer cell lines and such properties may be useful for cancer prevention and therapy^(41, 158-159). Anti-proliferative and pro-apoptotic effects of probiotic bacteria on various cancer cell lines have been demonstrated⁽¹⁶⁰⁻¹⁶²⁾. Reports also indicated that probiotic strains inhibited liver, bladder and mammary tumours in animal models, highlighting potentially systemic effects of probiotics with anti-neoplastic activities⁽¹⁶³⁻¹⁶⁴⁾. *In vivo* and *in vitro* studies have shown an antitumor activity of lactobacilli in colon cancer, and some epidemiologic studies have indicated a reduced risk of breast cancer in women who consume fermented milk products⁽¹⁶¹⁾. The fermented products of some lactic acid bacteria, such as *Lactobacillus helveticus*, *Bifidobacterium*, *L. acidophilus*, *Streptococcus thermophilus* and *L. delbrueckii*, all have the abilities of reduce growth rate of colon carcinoma cell line which resulted in a 50% decrease in the cell proliferation⁽¹⁶⁵⁾. The cytoplasmic fraction (CF) of *Lactococcus lactis* ssp. *lactis* and *L. brevis* had strong arginine deiminase (ADI) activity which can induce apoptosis of the stomach adenocarcinoma cells and human acute leukemia cells, respectively^(41, 159). Recent studies suggested that daily intake of *L. casei* strain *Shirota* provides a positive effect on the activity of NK cells which seem to exert a key role in protecting the human organism against cancer⁽¹⁶⁶⁾. *L. acidophilus* and *L. casei* strains to

increase the apoptosis of a colorectal cancer cell line in the presence of 5-fluorouracil (5-FU) ⁽¹⁶⁷⁾. Additionally, *L. reuteri* promotes apoptosis in human myeloid cells via modulation of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) signalling ⁽¹⁶⁸⁾.



CHAPTER III

MATERIALS AND METHODS

Materials

1. deMan-Rogosa-Sharpe (MRS) agar and MRS broth (Oxoid, Basingstoke, Hampshire, UK)
2. Brain heart infusion (BHI; Himedia, India)
3. Cary-Blair transport medium (Oxoid, Basingstoke, Hampshire, UK)
4. Amphotericin B (Sigma, USA)
5. Horse serum (Gibco-Invitrogen, USA)
6. Microaerobic and Anaerobic gas package (MGC, Japan)
7. Glycerol (Fisher, USA)
8. 2, 4-dinitrophenylhydrazine (Sigma, USA)
9. Potassium hydroxide (Fisher, USA)
10. L-tryptophan ($C_{11}H_{12}N_2O_2$) (Sigma, Japan)
11. Hydrochloric acid (37% HCl) (Merck, Germany)
12. Bovine bile (Sigma, USA)
13. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrasolium bromide (MTT) (Sigma, Germany)
14. Cell lines: Caco-2 human colon carcinoma cells (ATCC HTB-37)
15. Cell lines: U937 human monocytic leukemic cells (ATCC CRL-2367™)
16. Fetal bovine serum (Gibco-Invitrogen, USA)
17. Penicillin-Streptomycin (Gibco-Invitrogen, USA)
18. Roswell Park Memorial Institute medium number 1640 (RPMI 1640) (Gibco-Invitrogen, USA)
19. Dulbecco Modified Eagle medium (DMEM) (Gibco-Invitrogen, USA)
20. Medium 199 (M199) (Gibco-Invitrogen, USA)
21. 25% Trypsin (Gibco-Invitrogen, USA)
22. Hemocytometer (Hausser Scientific, USA)
23. Counter (Fisher Scientific, USA)
24. Dimethyl sulfoxide (DMSO) (Fisher Scientific, India)
25. 96-well flat-bottomed tissue culture plates (Corning, USA)
26. 0.22 μ m pore size filter Millipore (MA, USA)

27. Syringes (NIPRO, Thailand)
28. Multi-Detection Microplate Reader (BioTek® Synergy™ HT, USA)

Methods

1. Subjects and fermented food samples

1.1 Infant feces

For the isolation of *Lactobacillus* spp. 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 Cary-Blair transport medium (Oxoid, Hampshire, UK).

1.2 Fermented foods

Samples of fermented foods including Naem (fermented sausage made with pork and beef), Koh mak (fermented rice), pickled vegetable-like garlic, pickled tare, pickled cabbage, fermented onions, and Kimchi obtained from fresh-food market were used for isolation of lactobacilli.

2. 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 was spreaded onto deMan-Rogosa-Sharpe (MRS) agar plates (Oxoid, Basingstoke, Hampshire, UK). 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 box (Mitsubishi, Japan). Colonies of different morphologies were isolated on MRS agar plates. After anaerobic incubation, single pure colonies were isolated and sub-cultured for experimental use.

3. Selection of *Lactobacillus* isolates

Each of the *Lactobacillus* isolates was presumptively identified by Gram reaction and catalase test. Catalase test was examined by placing 3% H₂O₂ on the cell of isolates. Select isolates of gram- positive, coccobacilli, short rods or regular rods and catalase-negative were maintained as frozen cultures in MRS broth with 20% glycerol at -80°C for experimental use.

4. Reuterin detection of *Lactobacillus* isolates

Reuterin detection was used to detect reuterin-producing strains of lactobacilli by using the method described by Taweechoatipatr⁽¹¹³⁾. A total of 300 *Lactobacillus* isolates were tested for reuterin production. *L. reuteri* ATCC 55730, a reuterin-producing strain was used as a positive control. *Escherichia coli* ATCC 25922 and MRS were used as a negative control. Each *Lactobacillus* isolate was inoculated into 200 µl MRS broth in each well of the first 96-well plate and incubated anaerobically at 37°C for 24-48 hr. Each culture of *Lactobacillus* was transferred by using Frogger (DAN-KAR CCRP, MA, USA) into 175 µl MRS broths in each well of second 96-well plate. After incubation under anaerobic conditions at 37°C for 24 hr, each culture was inoculated by spotted onto the surface of brain heart infusion agar (BHI; Himedia, India) supplemented with 20 mM glucose. *Lactobacillus* spots were allowed to develop by incubated anaerobic conditions at 37°C for 24 hr. Soft agar (0.9 % agar) solutions were made with 2% glycerol and boiled until agar dissolved, mixed well and kept in a warm water bath at 46-47°C. Subsequently, soft agar was overlaid over the surface of *Lactobacillus* plate and allowed agar to solidify for 5-10 min and then incubated the plates straight up anaerobically at 37°C. After incubation for 3 hr, plates were removed and placed on the laid white paper towel on the bench top. The plates were opened and flood the surface with 2, 4-dinitrophenylhydrazine and waited for 5 minutes. After discarded the 2, 4-dinitrophenylhydrazine in a waste container, KOH solution (potassium hydroxide) was added to the plate and watched for reddish brown color to develop around the reuterin producing strains which indicated reuterin positive strains

appropriately. KOH solution was discarded as a hazardous chemical waste. These experiments were performed three times in duplicate.

5. Confirmation of the reuterin production by colorimetric assay

The confirmation of reuterin production was performed using modified method of Circle et al.⁽¹⁶⁹⁾. Briefly, the cell pellets of selected *Lactobacillus* strains overnight cultures were collected by centrifugation at 4,000 x g for 10 min at 4 °C. The cells were then washed twice with 50 mM sodium phosphate buffer (pH 7.4), centrifuged again, and resuspended in 250 mM glycerol solution, followed by incubation under anaerobic conditions at 37°C for 3 hr. After the cells were pelleted, supernatant containing reuterin were collected. The supernatant was passed through a 0.22 µm pore size syringe filter and stored at 4°C. For reuterin detection, a 20 µl of each supernatant will be mixed with 15 µl of 10 mM L-tryptophan solution and 60 µl of 37% HCl. The mixtures were incubated for 20 min at 37°C. After incubation for 20 min, the mixtures were watched for yellow color indicating the reuterin producing strains.

6. Antagonistic activities of selected reuterin-producing *Lactobacillus*

isolates against pathogenic microorganisms by agar well diffusion method

In this study, reuterin-producing *Lactobacillus* isolates were tested for antagonistic activities against 11 pathogens including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, methicillin resistant *S. aureus* (MRSA) DMST 20651, methicillin resistant *S. aureus* (MRSA) DMST 20654, *Vibrio cholerae* DMST 2873, *Vibrio parahaemolyticus*, *Samonella* Typhimurium and *Shigella dysenteriae*. They were grown on Mueller-Hinton agar (MH: Difco, USA) and were incubated under aerobic condition at 37°C for 24 hr. *Helicobacter pylori* (H40) was grown on brain heart infusion (BHI: Himedia, India) agar supplemented with 10% horse serum (Gibco-Invitrogen, USA) and 0.005 µg/ml amphotericin B (Sigma, USA) and incubated under micro-aerobic condition at 37°C for 72 hr. *Candida albicans* ATCC 10231 was grown on saboraud dextrose agar (SDA: Difco, France) and incubated under aerobic condition at 30°C for 48-72 hr.

Agar well diffusion assay⁽¹⁷⁰⁻¹⁷¹⁾ with some modification to the methods of Circle et al.⁽¹⁶⁹⁾ was used to determine antimicrobial activity of reuterin in supernatants of water glycerol solution. Approximately 1×10^8 cells/ml overnight cultures of *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, methicillin resistant *S. aureus* (MRSA) DMST 20651, methicillin resistant *S. aureus* (MRSA) DMST 20654, *V. cholerae* DMST 2873, *V. parahaemolyticus*, *S. Typhimurium*, *S. dysenteriae* ATCC 10231 in MH and *H. pylori* in BHI with 10% horse serum and 0.005 µg/ml amphotericin B was inoculated at a final concentration of 1×10^9 cells/ml and *C. albicans* in SDA was inoculated at a final concentration of 1×10^8 cells/ml. Each mixture was swabbed onto each plate. Circular wells of 4 mm diameter were made in the agar using sterile cork borer. The wells were filled with reuterin-containing supernatants in water glycerol solution. A solution of water glycerol without reuterin was used as negative control. *L. reuteri* ATCC 55730, a reuterin-producing strain was used as a positive control. The plates were incubated for 24-48 hr at appropriate conditions of pathogens as described above to allow pathogen growth. The inhibition zone, a clear zone around the well showing no growth of pathogens, was measured and recorded in mm from the center of the well. The experiments were performed in duplicate.

7. Screening of inhibitory effects on cancer cell proliferation

7.1 Preparation of conditioned media

Lactobacillus isolates from human fecal samples and fermented foods were selected and recovered from -80°C to test for cell proliferation in Caco-2 colon carcinoma cell line and U937 human monocytic leukemic cell line by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The *Lactobacillus* conditioned media (LCM) was prepared as previously described⁽¹⁷²⁾. 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 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 filter-sterilized using a 0.22 µm pore size filter (Sigma, USA). The conditioned media was 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.

7.2 MTT cell proliferation assay

MTT test ⁽¹⁶⁸⁾ was used to assess cell proliferation based on the capacity for viable cells to metabolize a tetrazolium colorless salt to a blue formazan in mitochondria. Therefore, this procedure was used to evaluate the inhibitory effects of the probiotic lactobacilli on cancer cell line proliferation. For assays, cells were counted with hemacytometer (Hausser Scientific, USA) under an inverted microscope (Nikon eclipse TS100: Nikon, Japan) and diluted to desired cell densities. One hundred seventy µl of Caco-2 cells were seeded in 96-well plates at a final density 5.9×10^3 cells/ml or 1.2×10^4 cells/ml for Vero cells and incubated for 24 hr at 37°C in an atmosphere of 5% CO₂–95% air mixture. For U937 cells, 85 µl were seeded in 96-well plates at a final density 5×10^4 cells/ml. Caco-2 or Vero cells were incubated with 20 µl of *Lactobacillus* conditioned media (10% v/v) or 10 µl for U937 cells and cell culture medium was added to a final volume of 200 µl/well for Caco-2 cells and Vero cells or 100 µl/well for U937 cells. After 24 hr of incubation in the presence of *Lactobacillus* conditioned media, 20 µl or 10 µl of 5 mg/ml solution of thiazolyl blue tetrazolium bromide (MTT) was added to each well. After mixing gently, the cells were incubated for 3 hr at 37°C in a CO₂ incubator. Then, the culture medium was aspirated and 100 µl for Caco-2 cells and Vero cells or 200 µl for U937 of dimethyl sulfoxide (DMSO) was added and mixed. Finally, optical density was measured (595 nm) using a microplate reader (Biotek®, Instrument, Inc., USA). The well contained cells and MRS broth was used as control. The percentage of cell proliferation was calculated by the formula below.

Calculation of the percentage of cell viability

$$\text{The percentage of cell viability} = \frac{\text{absorbance of treated cell}}{\text{absorbance of control cell}} \times 100$$

8. Statistical analysis

The experiments were performed three times in triplicate. Results were reported as means and standard error of the mean (SEM). Statistical analysis was evaluated by one-way ANOVA followed by Dunnett's test using the GraphPad Prism® version 5.01. Significant differences were considered significant at $P < 0.05$.

9. Acid and bile tolerance test

Lactobacillus isolates which displayed reuterin production and showed significantly inhibitory activity against cancer cell lines were tested for acid and bile tolerance in order to evaluate the relative survival ability of candidate probiotics in the gastrointestinal tract.

9.1 Acid tolerance test

MRS broth was adjusted with 1 N HCl to pH 2, 3, and 4 respectively. Unadjusted MRS broth (pH 6.5) was used as control. Overnight cultures of candidate probiotic *Lactobacillus* were diluted to an initial bacterial concentration of 10^8 cells/ml in each 10 ml of adjusted pH MRS broth in duplicates and 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 were 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 log 10 of colonies grown on MRS agar compared to the initial bacterial concentration.

9.2 Bile tolerance test

Candidate probiotics *Lactobacillus* was inoculated in 10 ml MRS broth supplemented with 1%, 2%, 3% and 4% bovine bile (Sigma, USA) and MRS control cultures without bovine and ovine bile. The initial bacterial concentration was 10^8 cells/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 were 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 generated by using an anaerobic GasPak (Mitsubishi, Japan) in an anaerobic jar (Mitsubishi, Japan) and incubated at 37°C for 24-48 hr. Viable bacteria were displayed as cell numbers and the log 10 of colonies grown on MRS agar compared to the initial bacterial concentration.

10. 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 cell pellets of the overnight *Lactobacillus* cultures collected by centrifugation at 2,000 x g for 5 min were 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 mineral oil, and the test strips were incubated at 37°C for 48 hr. 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

1. Isolation of *Lactobacillus* isolates

In all 300 isolates were obtained from infant feces and fermented foods. Genus *Lactobacillus* can be selected by presumptive tests including Gram stain and catalase test. They are all gram-positive (Figure 5A) and catalase-negative. Cell morphology in each isolate varied from long and slender rods, straight rods to bent rods, and sometimes short rods to coccobacilli (Figure 5A). Some isolates exhibit bipolar staining or internal granulations. The most frequently found colonies varied from small to medium colonies (1-2 mm) with white circular, convex and smooth colonial morphologies (Figure 5B).

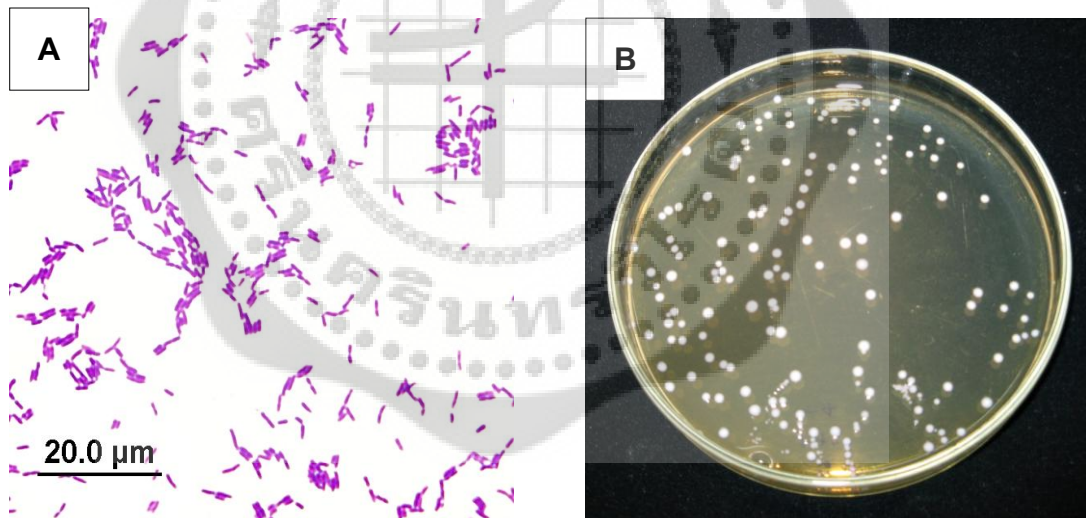


Figure 5 Morphology of *Lactobacillus* isolates

(A) Gram stain morphologies observed under light Microscopic at 1000X

(B) Colony morphologies on MRS agar

2. Reuterin detection of *Lactobacillus* isolates

Three hundred *Lactobacillus* isolates were tested for reuterin production by using a spot overlay method described previously⁽¹¹³⁾. All isolates were grown in MRS broth in each well of 96-well plate and transferring to a plate containing BHI with 20 mM glucose and assayed with indicator solution. In this study, six reuterin-producing isolates, H8, H9, H122, H123, H210 and H323 were identified when compared to *L. reuteri* ATCC 55730, while the positive control (reuterin-producing strain) displayed reddish brown coloration around its spot (Figure 6). They were isolated from different samples of infant feces. They were gram-positive, catalase-negative bacilli as shown in Table 1. Moreover, *Lactobacillus* isolates were isolated from fermented food have no ability to produce reuterin.

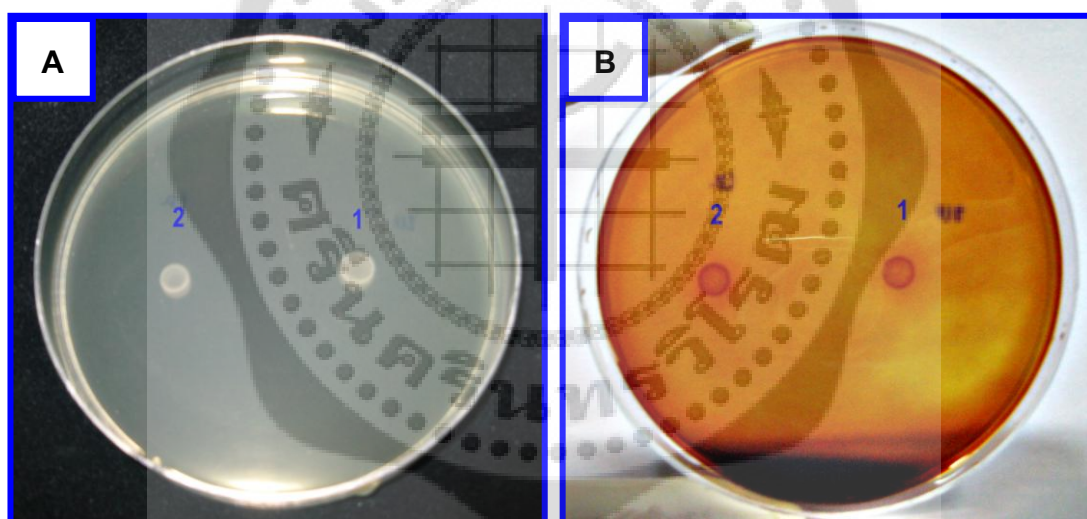


Figure 6 Reuterin screening of *Lactobacillus* isolates using a spot overlay method

1. H210 strain 2. *L. reuteri* ATCC 55730, reuterin-producing strain (positive control)

(A) Spots of *Lactobacillus*, (B) Reuterin detection

Table 1 Cell form, Gram stain and catalase of reuterin-producing strain

<i>Lactobacillus</i> isolates	Gram stain	Cell form	Catalase test
H8	+	Rods	-
H9	+	Rods	-
H122	+	Rods	-
H123	+	Rods	-
H210	+	Rods	-
H323	+	Rods	-

3. Confirmation of the reuterin production by colorimetric assay

The confirmation of the reuterin production was performed using modified method of Circle et al. ⁽¹⁶⁹⁾. Overnight cultures of selected *Lactobacillus* strains were collected by centrifugation. Cells were pelleted, washed twice with potassium phosphate buffer (pH 7.4), and resuspended in 250 mM glycerol solution. Supernatants containing reuterin were collected and mixed with indicator solution. As a positive control, a reuterin-producing *L. reuteri* ATCC 55730 was used and *E. coli* ATCC 25922, MRS broth and glycerol solution was used as a negative control. As shown in Figure 7, the results indicated only 1 isolate H210 of 6 isolated strains showed color change from purple to yellow indicated the presence of reuterin.

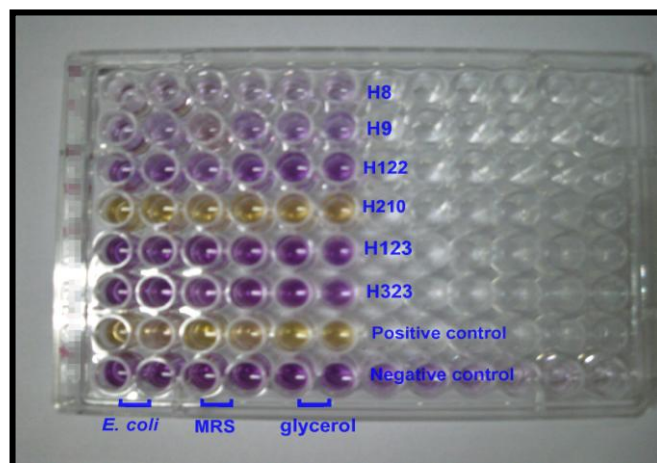


Figure 7 Colorimetric assay of culture supernatants collected from reuterin-producing strains

L. reuteri ATCC 55730 reuterin-producing strain (positive control), *E. coli* ATCC 25922, MRS broth and glycerol solution, no reuterin producing (negative control)

4. Antagonistic activities of selected reuterin-producing *Lactobacillus*

isolates against pathogenic microorganisms by agar well diffusion method

In this study, H210 reuterin-producing strain was tested for antagonistic activities against 11 pathogens including *E. coli* ATCC 25922, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, methicillin resistant *S. aureus* (MRSA) DMST 20651, methicillin resistant *S. aureus* (MRSA) DMST 20654, *V. cholerae* DMST 2873, *V. parahaemolyticus*, *S. Typhimurium*, *S. dysenteriae*, *H. pylori* (H40) and *C. albicans* ATCC 10231. An agar well diffusion method was used to determine the antimicrobial activity of H210 strain. In preliminary studies, H210 strain was cultivated in MRS broth and cell pellets were collected by centrifugation. The cells were then washed twice with potassium phosphate buffer (pH 7.4), and resuspended in 250 mM glycerol solution. Supernatants were collected by centrifugation. The supernatants were tested against pathogens. A clear zone of inhibition around the well as demonstrated in Figure 8 and Figure 9. The results indicated supernatants containing reuterin of H210 strain showed inhibitory activities against all target strains. The results of measurement of antimicrobial activity (clear zone of inhibition) of H210 strain is as shown in Table 2. The results showed very strong inhibitory activities with a

clear zone of 31 ± 2 mm, 24 ± 5 mm and 22 ± 4 mm against *S. Typhimurium*, *S. aureus* (MRSA) DMST 20651 and *S. aureus* ATCC 6538, respectively (Table 2, Figure 8H, 8B, 8A,). Followed by strong inhibitory activities with a clear zone of 21 ± 4 mm, 19 ± 6 mm, 17 ± 5 mm and 16 ± 3 mm against *S. aureus* (MRSA) DMST 20654, *V. parahaemolyticus*, *S. dysenteriae* and *B. subtilis* ATCC 6633, respectively (Table 2, Figure 8C, 8G, 8I, 8D). Moderate to weak inhibitory activities with a clear zone of 14 ± 2 mm, 9 ± 0 mm, 9 ± 1 mm and 5 ± 1 were against *E. coli* ATCC 25922, *V. cholerae* DMST 2873, *H. pylori* (H40) and *C. albicans* ATCC 10231, respectively (Table 2, Figure 8E, 8F, 8J, 8K). The glycerol solution (negative control) as in well number 3 of plate (Figure 8) showed no inhibitory effect on any of the pathogenic strains tested.

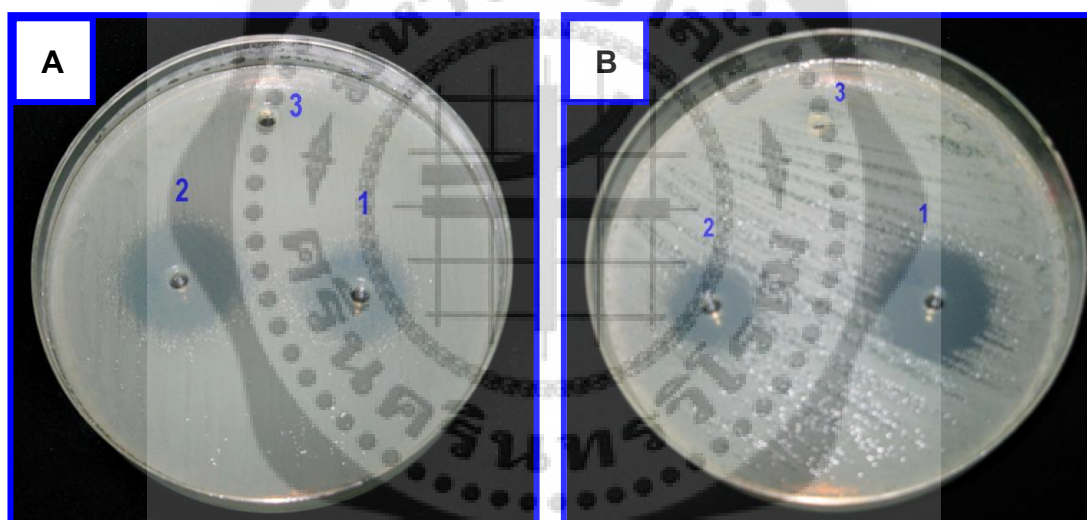


Figure 8 Antimicrobial activities of H210 strain against pathogenic microorganisms

(A) *S. aureus* ATCC 6538, (B) methicillin resistant *S. aureus* (MRSA) DMST 20651. Well 1 = H210 strain, well 2 = *L. reuteri* ATCC 55730 (positive control), glycerol solution (negative control). A minimum well diameter was 4 mm

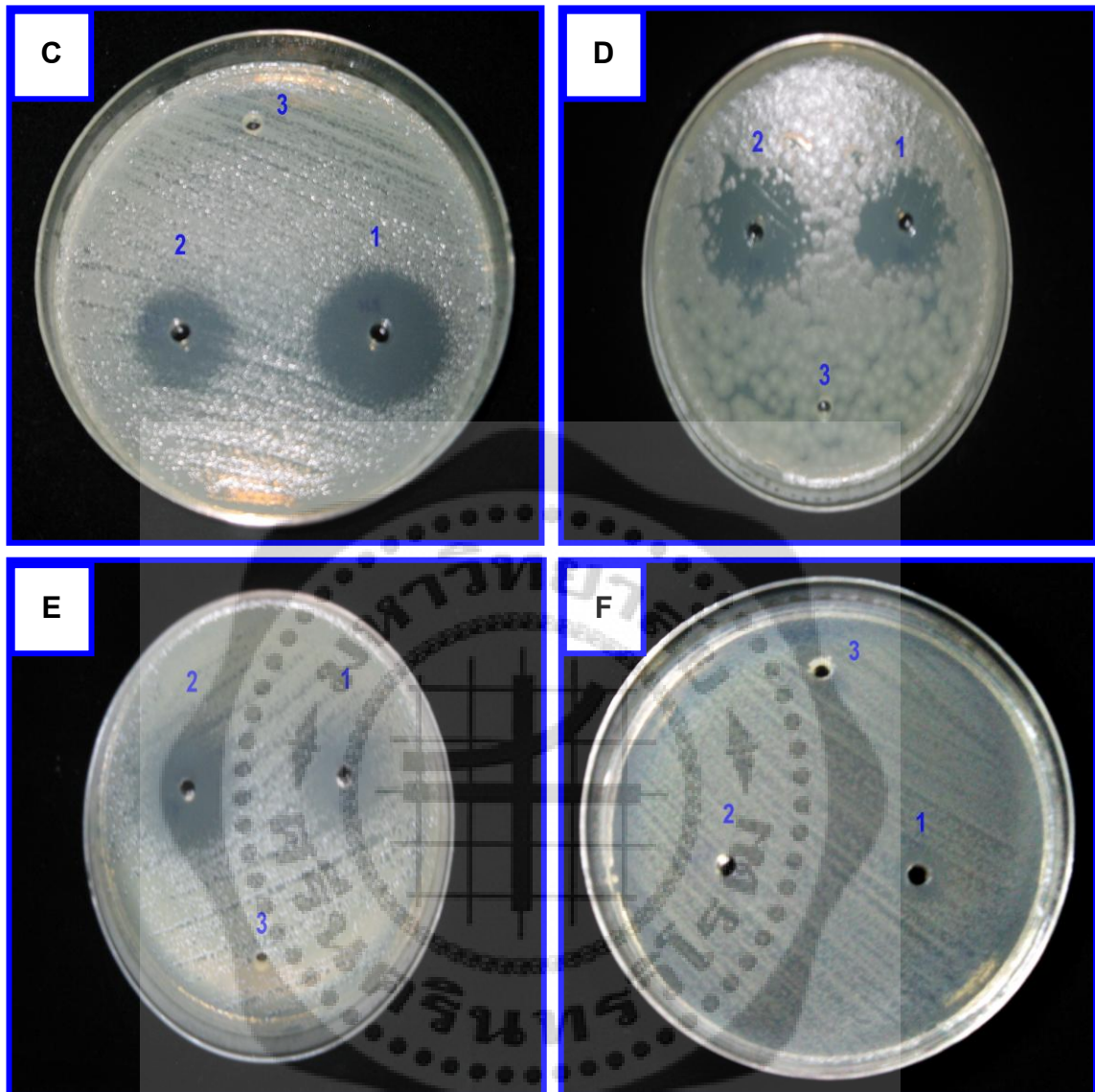


Figure 8 Antimicrobial activities of H210 strain against pathogenic microorganisms

(C) methicillin resistant *S. aureus* (MRSA) DMST 20654, (D) *B. subtilis* ATCC 6633, (E) *E. coli* ATCC 25922, (F) *V. cholerae* DMST 2873. Well 1 = H210 strain, well 2 = *L. reuteri* ATCC 55730 (positive control), glycerol solution (negative control). A minimum well diameter was 4 mm. (continued)

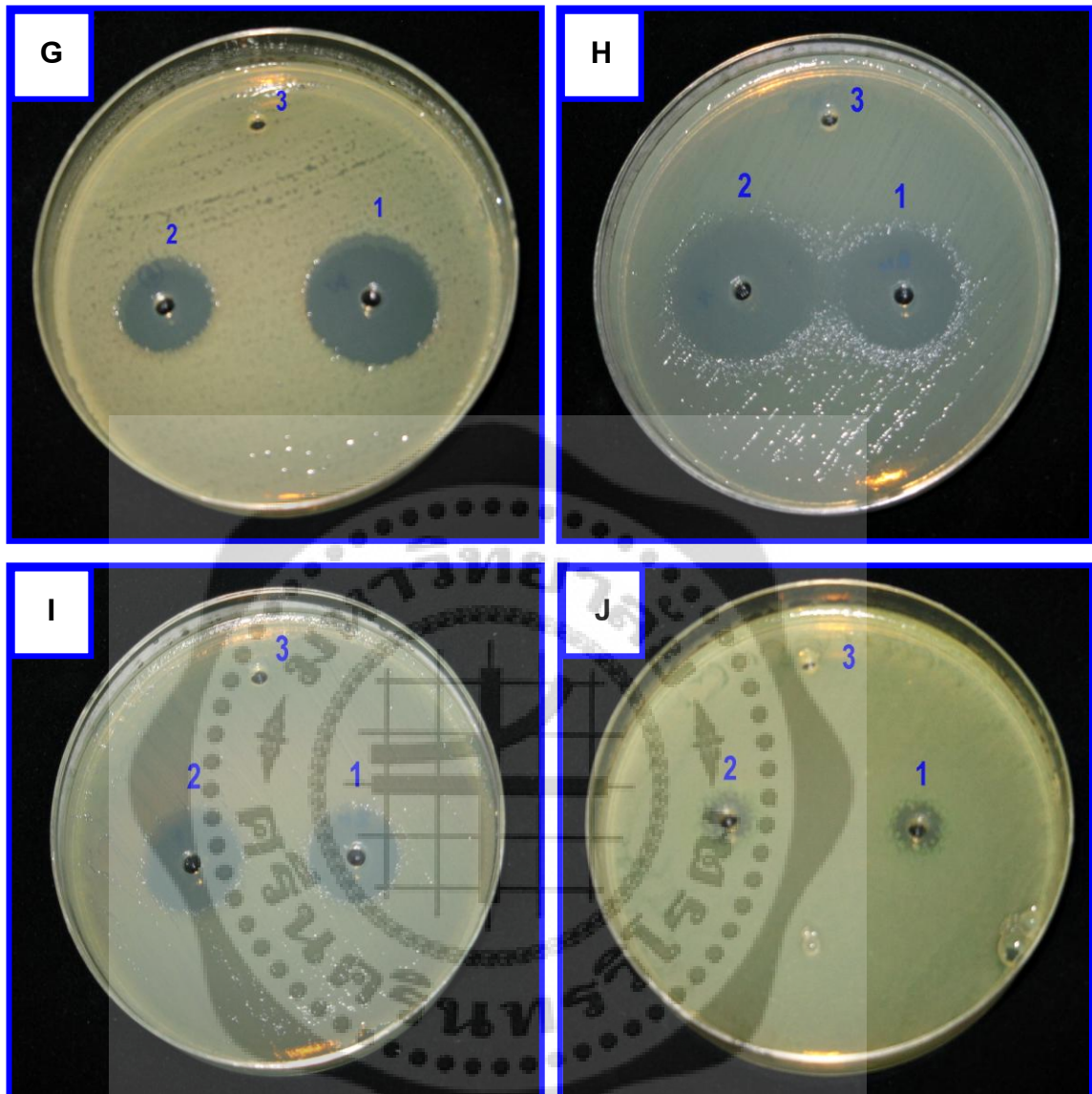


Figure 8 Antimicrobial activities of H210 strain against pathogenic microorganisms

(G) *V. parahaemolyticus*, (H) *S. Typhimurium*, (I) *S. dysenteriae*, (J) *H. pylori* (H40)
 Well 1 = H210 strain, well 2 = *L. reuteri* ATCC 55730 (positive control), glycerol solution
 (negative control). A minimum well diameter was 4 mm. (continued)

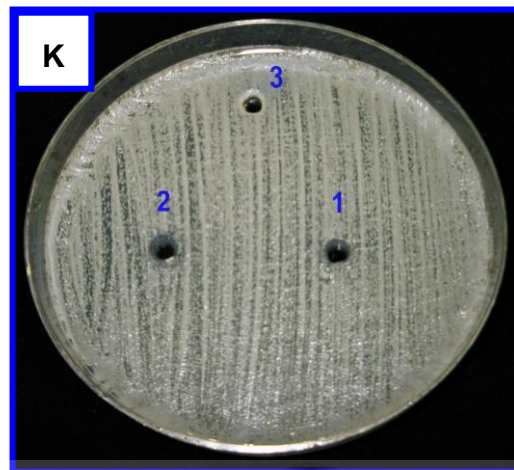


Figure 8 Antimicrobial activities of H210 strain against pathogenic microorganisms

(K) *C. albicans* ATCC 10231 . Well 1 = H210 strain, well 2 = *L. reuteri* ATCC 55730 (positive control), glycerol solution (negative control) (continued)

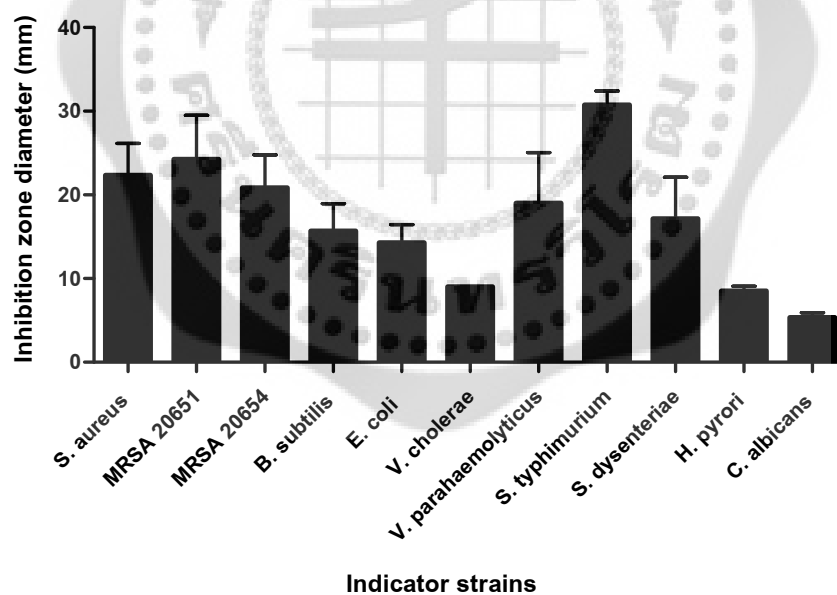


Figure 9 Summary of antimicrobial activities of H210 strain against of pathogenic Microorganisms

The data were expressed as mean $\pm$ SD of three independent experiments, each performed in duplicate

Table 2 Antagonistic activities of H210 strain against pathogenic microorganisms

Group of strains	Indicator strains	Inhibition zone diameter (mm)
Gram positive bacteria	<i>S. aureus</i> ATCC 6538	22±4
	<i>S. aureus</i> (MRSA) DMST 20651	24±5
	<i>S. aureus</i> (MRSA) DMST 20654	21±4
	<i>B. subtilis</i> ATCC 6633	16±3
Gram negative bacteria	<i>E. coli</i> ATCC 25922	14±2
	<i>V. cholerae</i> DMST 2873	9±0
	<i>V. parahaemolyticus</i>	19±6
	<i>S. Typhimurium</i>	31±2
	<i>S. dysenteriae</i>	17±5
	<i>H. pylori</i> (H40)	9±1
Yeast	<i>C. albicans</i> ATCC 10231	5±1

The diameter of the inhibition zone, a clear zone around the well was measured for each pathogen. The data were expressed as mean $\pm$ SD of three independent experiments, each performed in duplicate. Interpretation of zone diameter of inhibition: -, No inhibition zone; +, Weak inhibition zone (<10 mm); ++, Moderate inhibition zone (10-15 mm); +++, Strong inhibition zone (16-21 mm); +++++, Very strong inhibition zone (>22 mm)

5. Inhibitory effects of *Lactobacillus* isolates on cancer cell proliferation

A total of three hundred *Lactobacillus* isolates were tested for cell proliferation in Caco-2 human colon carcinoma cell line and U937 human monocytic leukemic cell line by MTT assay as described previously⁽¹⁶⁸⁾. MTT assay were performed by cell lines incubated with *Lactobacillus* conditioned media. Cell viability was measured directly in the microplate reader. The percentages of cell proliferation were calculated by the formula below.

Calculation of the percentage of cell viability

$$\text{The percentage of cell viability} = \frac{\text{absorbance of treated cell}}{\text{absorbance of control cell}} \times 100$$

In our preliminary experiments, concentrations of MRS (bacterial media control) were optimized at 5-20% v/v to assess inhibitory effects to two cancer cell lines and normal cell line. The effect of various concentrations of MRS showed inhibitory effect to all cell lines in a concentration dependent manner. At 15-20% v/v concentration of MRS had significant inhibitory effects on the cell viability in three types of cells as compared control group (Table 3, Figure 10). In addition, at 5-10% v/v concentration of MRS, no significant inhibitory effects on the cell proliferation in those cells (Table 3, Figure 10). Some of the *Lactobacillus* conditioned media at 5-10% v/v were tested to find the most effective concentration on the cell viability. The results showed 5% and 10%v/v no inhibitory effects and had inhibitory effects on the cell proliferation and, respectively (data not shown). In this study, we used at 10% v/v concentration of *Lactobacillus*-conditioned media to determine inhibitory effect on cell proliferation.

Table 3 Summary of the optimum MRS concentration on viability of Vero cells, Caco-2 cells and U937 cells were chosen as optimal concentration for MTT assays

Treatment	Inhibition (%)			Cell viability (%)		
	Caco-2 cells	U937 cells	Vero cells	Caco-2 cells	U937 cells	Vero cells
Media control	-	-	-	100±0.00	100±0.00	100±0.00
MRS 5%	-	-	-	99.34±3.28	104.99±6.40	103.24±3.69
MRS 10%	-	-	-	98.83±2.74	94.20±2.53	93.59±4.37
MRS 15%	+	+	+	76.00±4.24	77.93±4.32	75.44±4.98
MRS 20%	+	+	+	72.22±1.14	79.40±1.42	69.75±3.39

Control, cell culture media control. Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, > 50 %. The data were expressed as mean ± SEM of three independent experiments

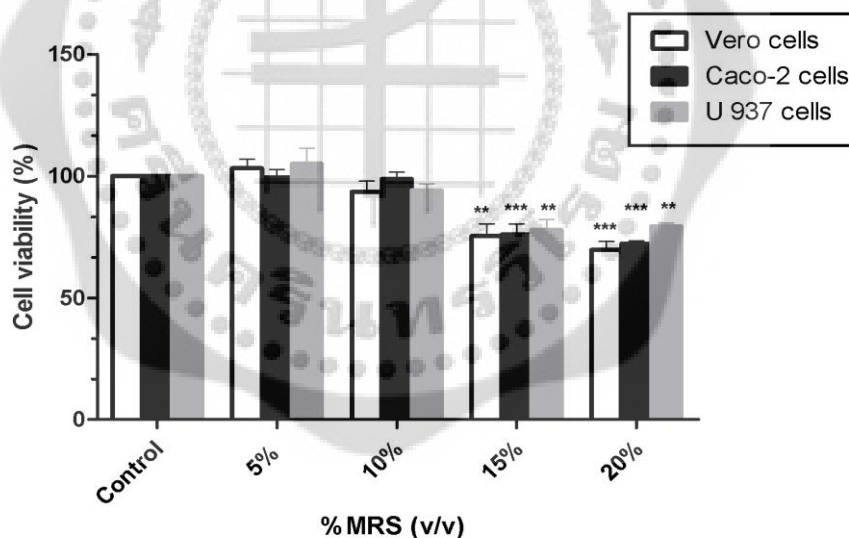


Figure 10 The determination of the optimum MRS concentration on viability of Vero cells, Caco-2 cells and U937 cells were chosen as optimal concentration for MTT assays

Control, cell culture media control. The data were expressed as mean ± SEM of three independent experiments. Asterisks denote significantly different from cell culture media control ** (p<0.01); *** (p<0.001)

In the presence of conditioned media from healthy infant feces on the proliferation of Caco-2 cell as shown in Table 4. One isolates: H370 had a moderate inhibitory effect on the Caco-2 cells with only $53.99 \pm 15.77\%$ viability of cancer cells remaining. Three isolates: H286, H323 and H468 showed strong inhibitory effect by the cell viability to a range of 39.3 ± 11.64 to $28.12 \pm 14.28\%$. Similar results were observed on U937 cell as shown in Table 5. Eight isolates: H61, H114, H115, H121, H267, H268, H450 and H456 have showed weak inhibitory effect by the cell viability to a range of 49.68 ± 14.64 to $41.90 \pm 8.04\%$. Eight isolates: H117, H120, H323, H370, H404, H427, H428 and H461 showed moderate inhibitory effect on cell viability to a range of 39.35 ± 9.13 to $31.28 \pm 4.85\%$. Four isolates: H305, H308, H320 and H415 showed strong inhibitory effect on cell viability to a range of 30.16 ± 4.10 to $26.04 \pm 3.67\%$.

Table 4 Summary of inhibitory effects of 4 *Lactobacillus* isolates from infant feces on growth inhibition (%) and viability of Caco-2 cells and Vero cells

<i>Lactobacillus</i> isolates	Inhibition (%)		Cell viability (%)	
	Caco-2 cells	Vero cells	Caco-2 cells	Vero cells
MRS broth	-	-	96.02 ± 1.30	82.80 ± 1.55
H286	+++	-	39.30 ± 11.64	71.71 ± 19.76
H323	+++	+++	28.12 ± 14.28	31.02 ± 0.88
H370	++	-	53.99 ± 15.77	90.17 ± 5.40
H468	+++	-	30.24 ± 9.17	71.01 ± 14.42

MRS, bacteria media control. Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, > 50 %

The data were expressed as mean $\pm$ SEM of three independent experiments

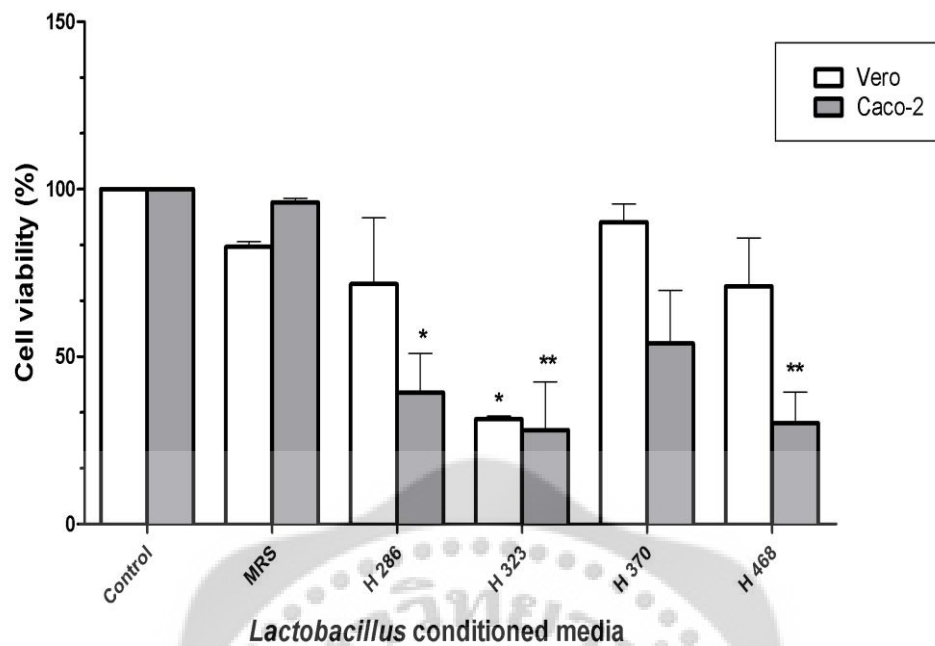


Figure 11 Inhibitory effects of 4 *Lactobacillus* isolates isolated from healthy infant feces on the cell viability of Caco-2 cells and Vero cells

Control, cell culture media control; MRS, bacteria media control. The data were expressed as mean $\pm$ SEM of three independent experiments. Asterisks denote significantly different from MRS bacteria media control * ($p<0.05$); ** ($p<0.01$)

Table 5 Summary of inhibitory effects of 20 *Lactobacillus* isolates isolated from healthy infant feces on growth inhibition (%) and viability of U937 cells and Vero cells

<i>Lactobacillus</i> isolates	Inhibition (%)		Cell viability (%)	
	U937 cells	Vero cells	U937 cells	Vero cells
MRS broth	-	-	80.55±3.83	82.80±1.55
H61	+	-	41.95±7.48	84.79±5.88
H114	+	-	41.90±8.04	68.67±8.77
H115	+	-	49.68±14.64	73.60±5.28
H117	++	-	33.84±4.92	89.87±5.90
H120	++	-	34.82±4.23	89.16±24.17
H121	+	+	48.65±4.52	61.40±17.84
H267	+	-	41.91±11.17	100.86±10.10
H268	+	-	42.71±9.05	64.39±8.16
H305	+++	-	26.04±3.67	105.63±11.58
H308	+++	-	29.49±3.29	68.58±10.56
H320	+++	-	26.30±4.43	102.39±7.41
H323	++	+++	34.23±5.43	31.02±0.88
H370	++	-	34.64±7.47	90.17±5.40
H404	++	-	31.28±4.85	108.50±7.12
H415	+++	-	30.16±4.10	115.61±9.99
H427	++	-	39.35±9.13	96.55±16.23
H428	++	-	36.05±6.18	91.98±11.37
H450	+	-	44.06±10.89	80.53±10.76
H456	+	-	49.38±6.48	93.96±12.81
H461	++	-	35.50±2.44	82.83±10.59

MRS, bacteria media control Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, > 50 %. The data were expressed as mean ± SEM of three independent experiments

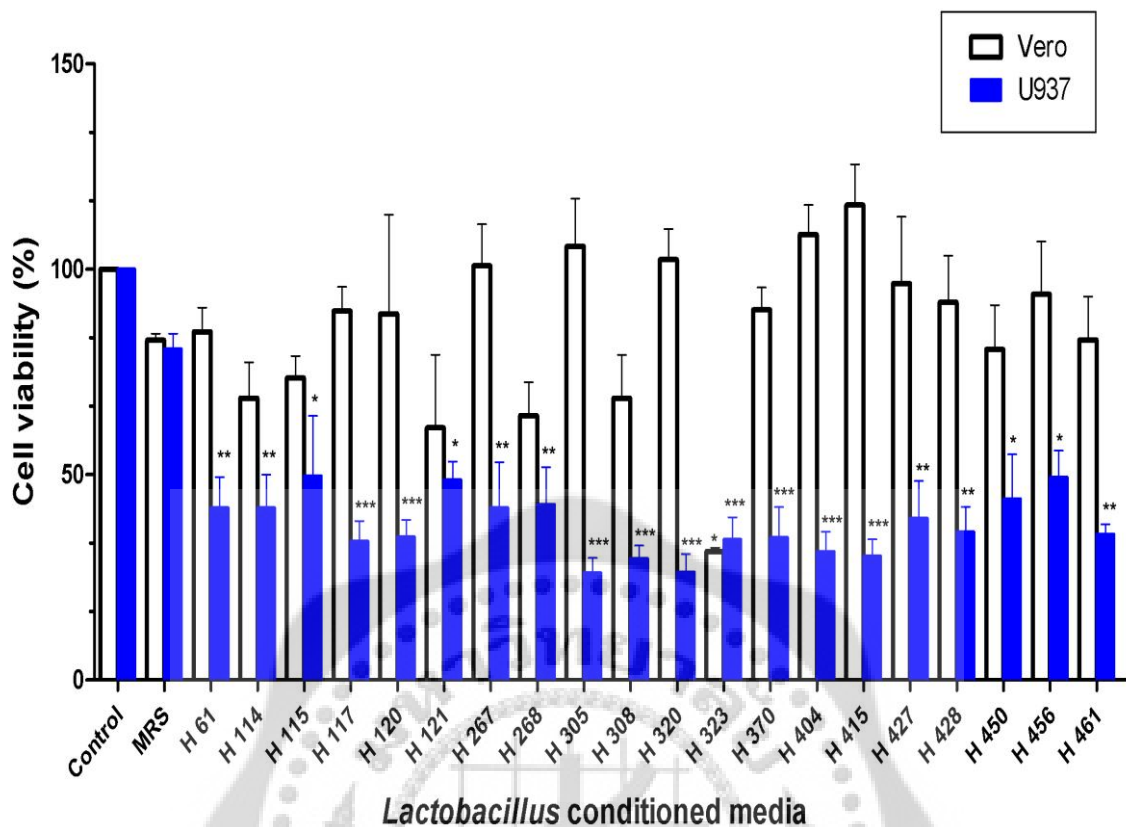


Figure 12 Inhibitory effects of 20 *Lactobacillus* isolates isolated from healthy infant feces on the cell viability of U937 cells and Vero cells

Control, cell culture media control; MRS, bacteria media control. The data were expressed as mean $\pm$ SEM of three independent experiments. Asterisks denote significantly different from MRS bacteria media control * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)

In addition, inhibitory effect of conditioned medium from fermented foods as shown in Table 6. One isolates: F13 had moderate inhibitory effect on Caco-2 cells showing 41.14 ± 20.27 viability of cancer cells. Twelve isolates: F1-F9 and F17-F19 have showed strong inhibitory effect on cell viability to a range of 29.71 ± 11.30 - 17.23 ± 1.19 . Interestingly, studies have shown the strong inhibitory effect on U937 cell of twelve isolates: F1-F9, F12-F13 and F17-F19. As showing cell viability to a range of 48.82 ± 23.64 - 22.09 ± 0.25 . Most characteristics of *Lactobacillus* isolates exert their inhibitory effects on viability of cancer cells. The inhibition strains have various shapes, cocci, cocobacilli and bacilli (Table 7).

Table 6 Summary of inhibitory effects of 12 *Lactobacillus* isolates isolated from fermented foods on grown inhibition (%) and viability of Caco-2 cells and Vero cells

<i>Lactobacillus</i> isolates	Inhibition (%)			Cell viability (%)		
	Caco-2 cells	U937 cells	Vero cells	Caco-2 cells	U937 cells	Vero cells
MRS broth	-	-	-	85.86±3.72	93.24±4.29	88.57±7.75
F1	+++	++	+++	17.23±1.19	49.82±23.64	14.81±3.24
F2	+++	+++	+++	23.65±3.98	26.58±1.23	34.37±16.54
F3	+++	+++	+++	27.96±5.64	24.72±0.57	24.44±7.94
F4	+++	+++	+++	27.05±6.89	28.57±1.92	18.84±8.31
F5	+++	+++	+++	29.71±11.30	22.92±1.99	10.83±0.32
F7	+++	+++	+++	33.82±11.13	22.09±0.25	18.76±3.61
F9	+++	+++	+++	31.40±8.28	23.22±0.39	19.62±10.59
F12	-	+++	-	-	33.87±5.47	96.43±5.85
F13	++	+++	+++	41.14±20.27	24.10±2.86	26.49±7.84
F17	+++	+++	+++	19.63±6.83	25.19±1.54	14.16±2.08
F18	+++	+++	+	24.84±6.51	32.73±3.62	55.45±18.08
F19	+++	+++	+++	21.92±3.60	28.39±2.76	12.34±1.72

MRS, bacteria media control. Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, >50% The data were expressed as mean ± SEM of three independent experiments

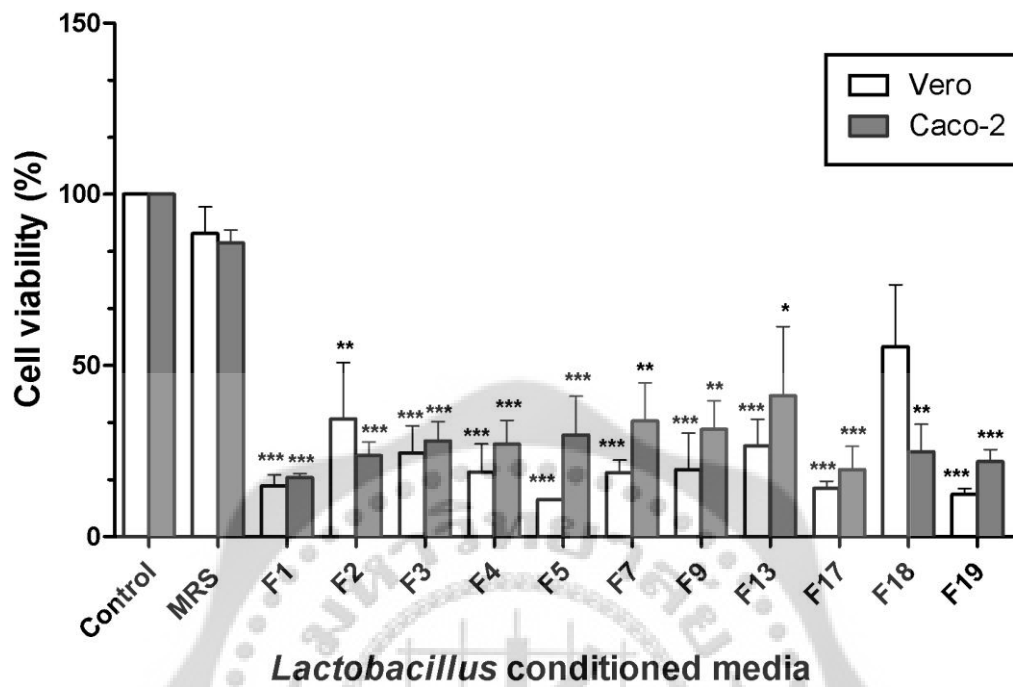


Figure 13 Inhibitory effects of 11 *Lactobacillus* isolates isolated from fermented foods on the cell viability of Caco-2 cells and Vero cells

Control, cell culture media control; MRS, bacteria media control. The data were expressed as mean $\pm$ SEM of three independent experiments. Asterisks denote significant differences from MRS bacteria media control * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)

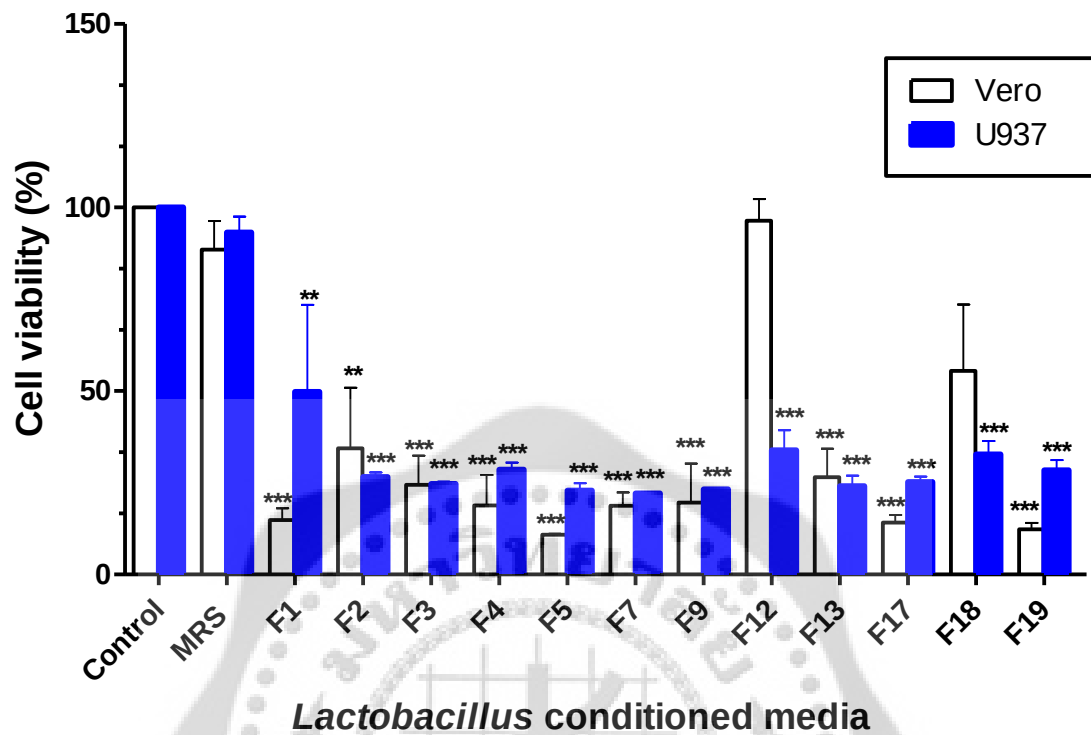


Figure 14 Inhibitory effects of 12 *Lactobacillus* isolates on the cell viability of U937 cells and Vero cells

Control, cell culture media control; MRS, bacteria media control. The data were expressed as mean $\pm$ SEM of three independent experiments. Asterisks denote significant differences from MRS bacteria media control * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$)

Table 7 Summary of cell form and inhibitory effects of *Lactobacillus* isolates against Caco-2 cells, U937 cells and Vero cells

<i>Lactobacillus</i> isolates	Cell form	Inhibition (%)		
		Caco-2 cells	U937 cells	Vero cells
H61	Rods	-	+	-
H114	Rods	-	+	-
H115	Rods	-	+	-
H117	Coccobacilli	-	++	-
H120	Rods	-	++	-
H121	Rods	-	+	+
H267	Rods	-	+	-
H268	Cocci	-	+	-
H286	Cocci	+++	-	-
H305	Cocci	-	+++	-
H308	Rods	-	+++	-
H320	Cocci	-	+++	-
H323	Rods	+++	++	+++
H370	Cocci	++	++	-
H404	Rods	-	++	-
H415	Rods	-	+++	-
H427	Rods	-	++	-
H428	Rods	-	++	-
H450	Rods	-	+	-
H456	Rods	-	+	-
H461	Rods	-	++	-
H468	Rods	+++	-	-

Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, > 50 %

Table 7 Summary of cell form and inhibitory effects of *Lactobacillus* isolates against Caco-2 cells, U937 cells and Vero cells (continued)

<i>Lactobacillus</i> isolates	Cell form	Inhibition (%)		
		Caco-2	U937	Vero
F1	Rods	+++	++	+++
F2	Rods	+++	+++	+++
F3	Rods	+++	+++	+++
F4	Rods	+++	+++	+++
F5	Rods	+++	+++	+++
F7	Rods	+++	+++	+++
F9	Rods	+++	+++	+++
F12	Rods	-	+++	-
F13	Rods	++	+++	+++
F17	Rods	+++	+++	+++
F18	Rods	+++	+++	+
F19	Rods	+++	+++	+++

Inhibition (%); -, < 20%; +, 20-40%; ++, 41-50%; +++, > 50 %

The *Lactobacillus*-conditioned media which showed inhibitory effect to two cancer cell lines had some deleterious effect to normal cell line (Vero cells). As the results demonstrate in Figure 10, 12 that results described above, 4, 12 of three hundred isolates isolated from healthy infant feces and fermented foods respectively. All of these isolates showed significant inhibitory effects on the growth of Caco-2 cells. Eleven isolates: H323, F1, F2, F3 F4, F5, F7, F9, F13, F17 and F19 have shown significant inhibitory effects on the growth of Vero cells. For twenty isolates isolated from healthy infant feces and twelve isolates isolated from fermented foods showed significant inhibitory effects on the growth of U937 cells but eleven isolates: H323 (Figure 11), F1, F2, F3, F4, F5, F7, F9, F13, F17 and F19 (Figure 13) have shown exhibited significant inhibitory effects on the growth of Vero cells, when compared to MRS bacterial media control.

6. Acid and bile of selected *Lactobacillus* isolates

6.1 Acid tolerance

One reuterin-producing strain and 7 isolates which showed the strongest inhibitory effect against cancer cells were selected to test for acid tolerance. Testing was performed by inoculated into MRS media at pH level 2, 3, 4. The result of acid tolerance (survival at various pH values) showed that viability of H210 was changed after incubation for 3 hr at pH 2, 3 and 4. The viable counts at pH 2, 3 and 4 decreased by about 4, 1 and 1 log values respectively (Table 8 and Figure 15). H286 viable counts at pH 2 and 3 decreased by about 4 and 3 log values respectively, but showed more acid tolerance at pH 4 which showed no log difference as reported in Table 9 and Figure 16. H305 viable counts at pH 2, 3 and 4 decreased by about 4, 3 and 1 log values respectively (Table 10 and Figure 17). H 308 viable counts at pH 2, 3 and 4 decreased by about 5, 2 and 1 log values respectively (Table 11 and Figure 18). H320 viable counts at pH 2, 3 and 4 decreased by about 6, 4 and 2 log values respectively (Table 12 and Figure 19). H323 viable counts at pH 2 and 3 decreased by about 6 and 3 log values respectively, but showed more acid tolerance at pH 4, which showed no log difference as reported in Table 13 and Figure 20. H415 viable counts at pH 2, 3 and 4 decreased about by 4, 2 and 1 log values respectively (Table 14 and Figure 21). H468 viable counts at pH 2, 3 and 4 decreased by about 5, 2 and 1 log values respectively (Table 15 and Figure 22). All values were compared with MRS bacteria media control.

In this study, all selected *Lactobacillus* strains was tested for acid tolerance, and the results showed in Figure 32 that strain could survive after incubation for 3 hr at various pH values.

Table 8 Survival of H210 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	1.03E+08	1.53E+07
MRS 3 hr	3.13E+08	1.00E+08
pH 2	4.43E+04	5.13E+03
pH 3	1.11E+07	1.01E+06
pH 4	3.50E+07	7.00E+06

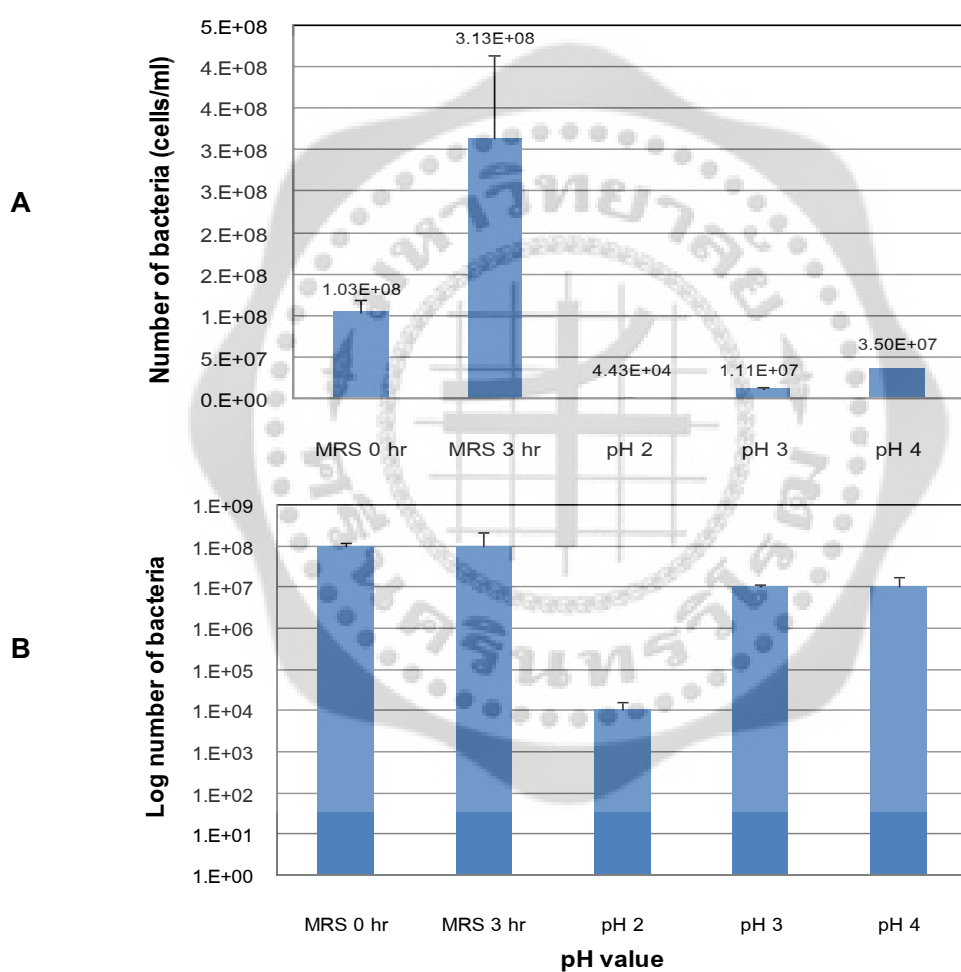


Figure 15 Survival of H210 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 9 Survival of H286 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	1.80E+08	2.00E+07
MRS 3 hr	7.13E+08	2.89E+07
pH 2	6.63E+04	4.04E+03
pH 3	7.03E+05	8.50E+04
pH 4	9.83E+07	1.05E+07

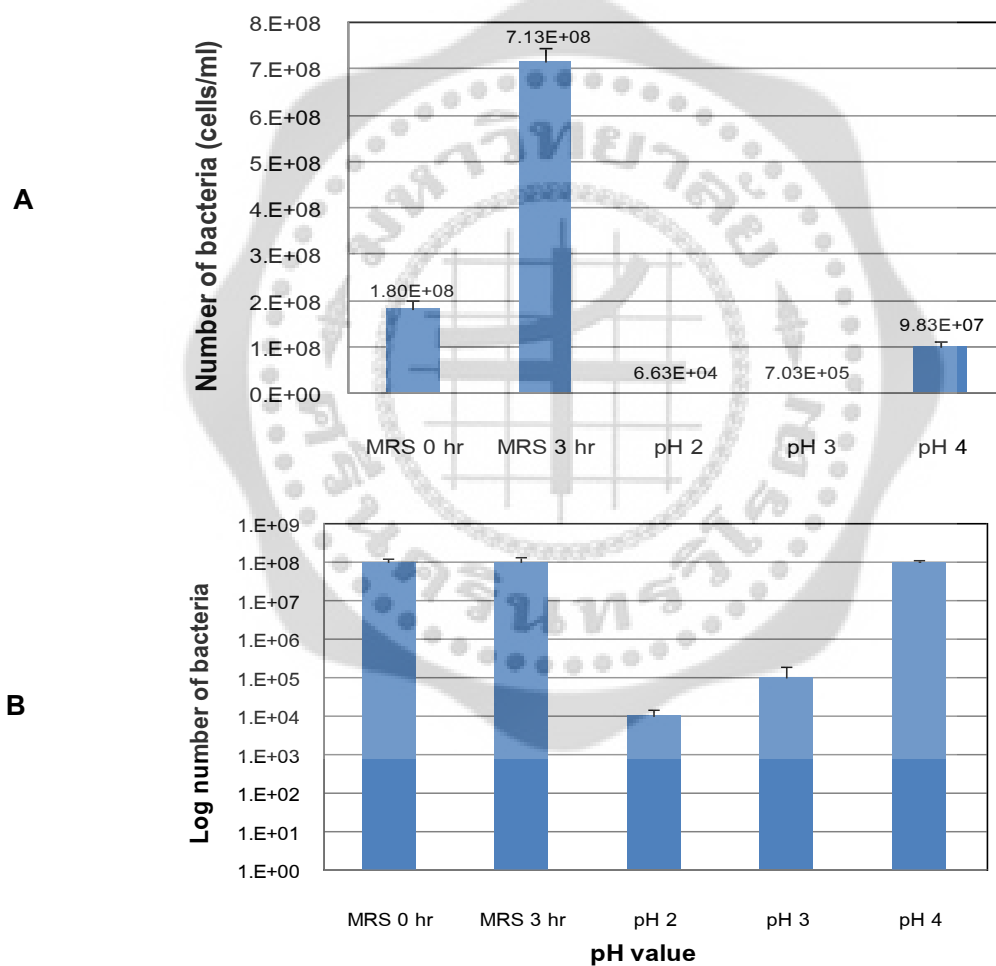


Figure 16 Survival of H286 strain after incubateion at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 10 Survival of H305 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	7.00E+07	1.73E+07
MRS 3 hr	6.23E+08	6.56E+07
pH 2	5.40E+04	5.29E+03
pH 3	1.67E+05	5.86E+04
pH 4	4.97E+07	1.03E+07

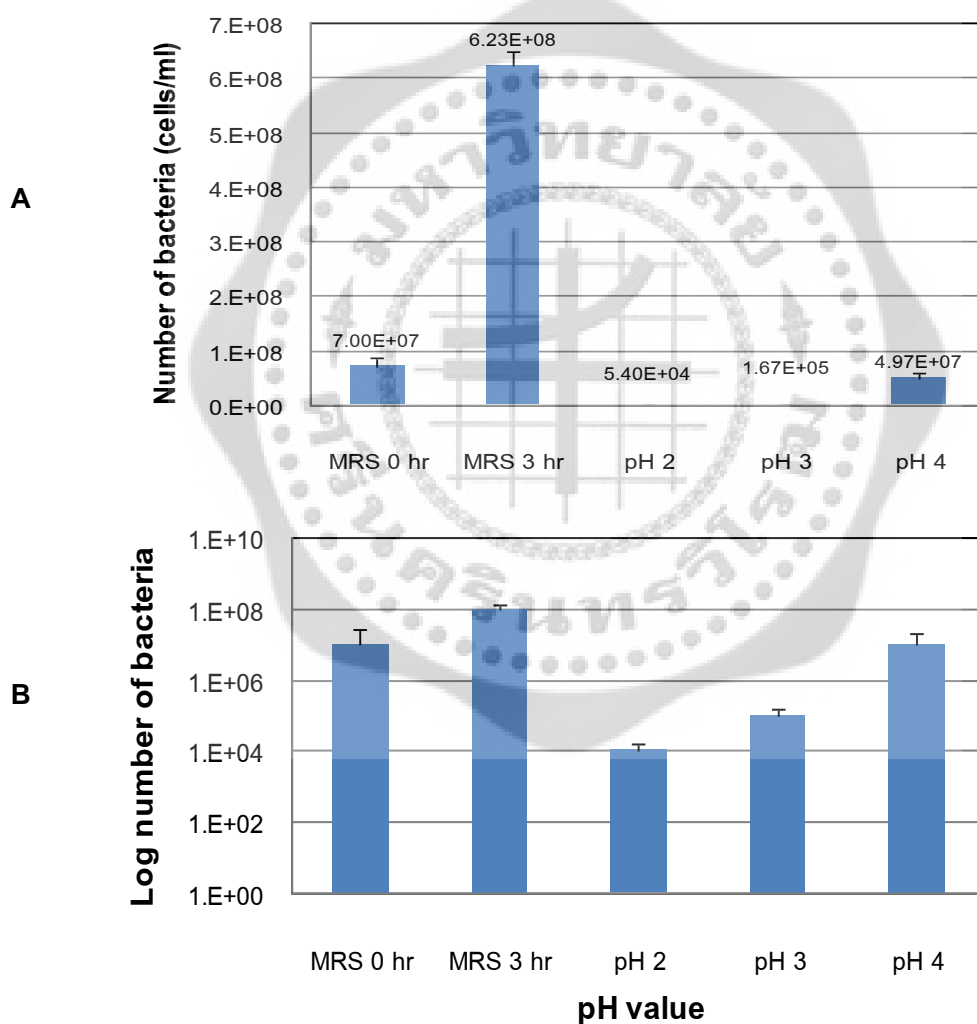


Figure 17 Survival of H305 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 11 Survival of H308 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	7.00E+07	1.73E+07
MRS 3 hr	1.13E+08	1.15E+07
pH 2	9.17E+03	1.32E+03
pH 3	9.20E+06	3.46E+05
pH 4	6.47E+07	4.51E+06

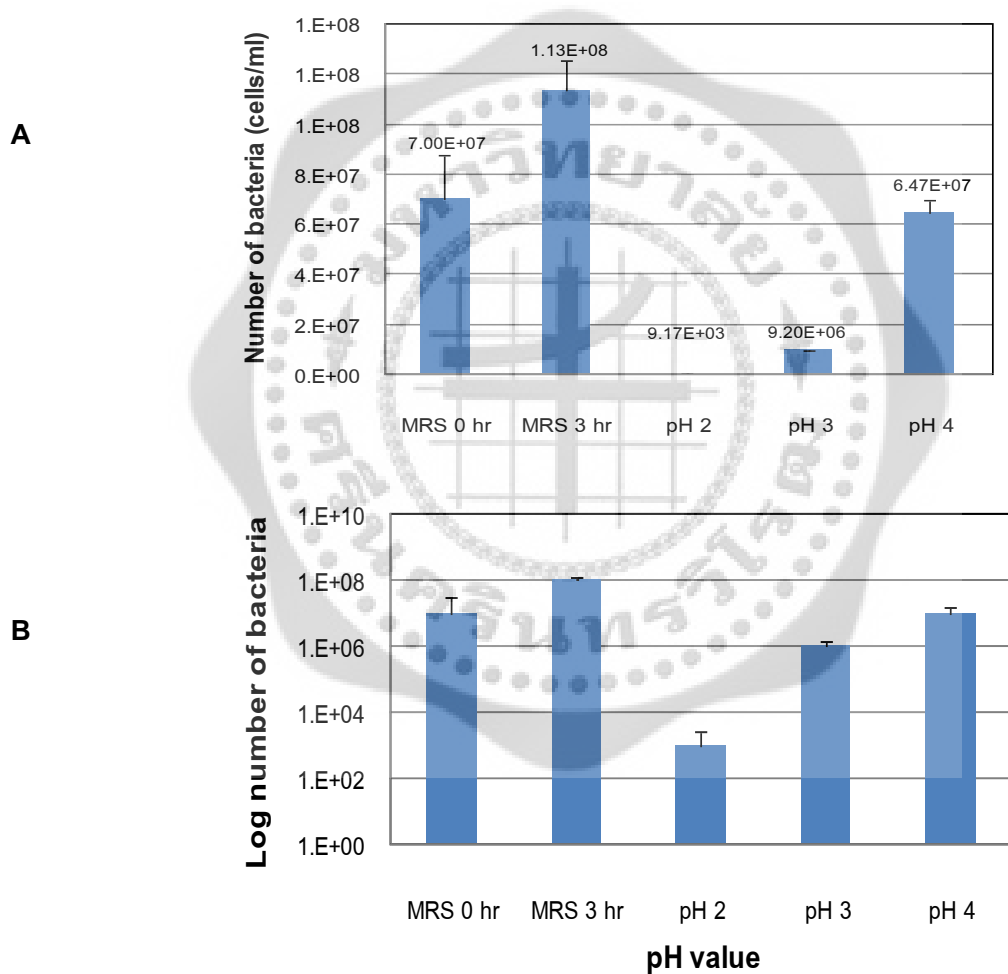


Figure 18 Survival of H308 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 12 Survival of H320 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	2.80E+08	4.00E+07
MRS 3 hr	1.03E+09	6.66E+07
pH 2	1.33E+03	5.77E+02
pH 3	1.63E+05	4.04E+04
pH 4	5.37E+07	9.81E+06

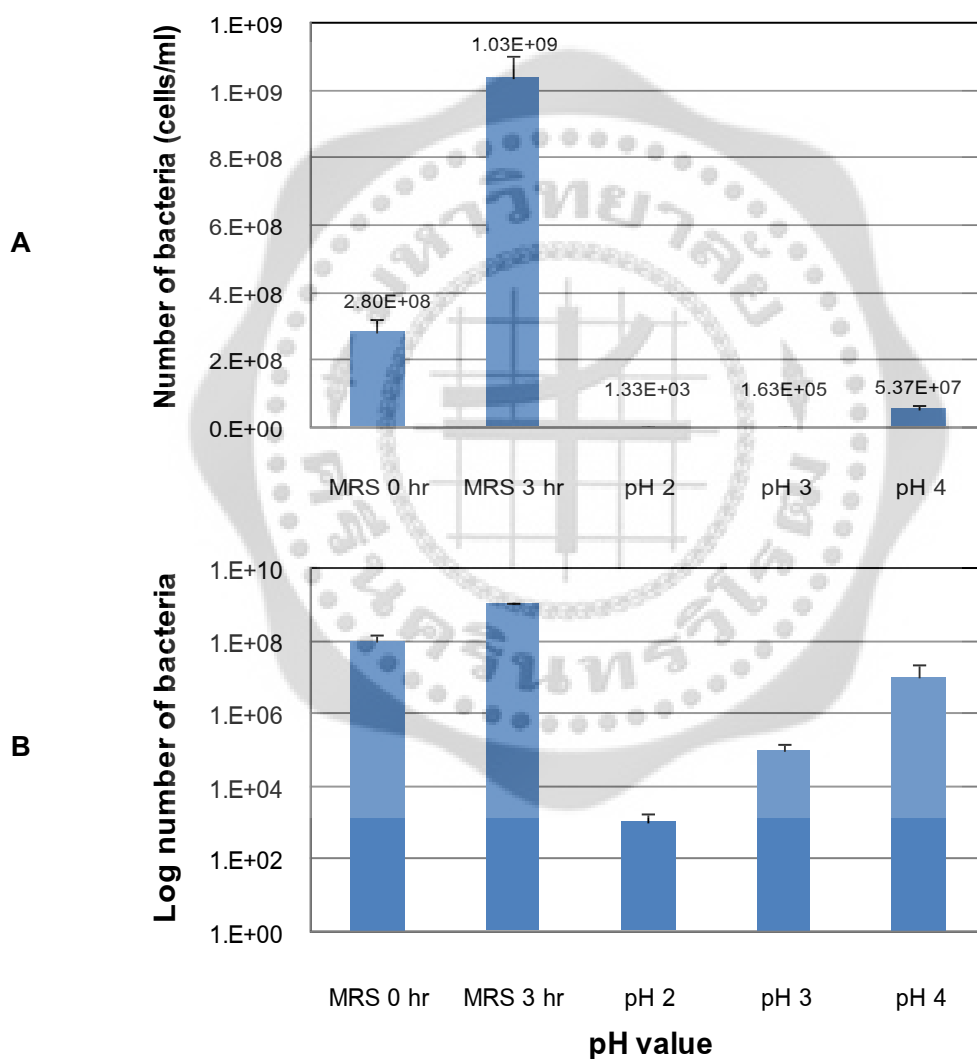


Figure 19 Survival of H320 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 13 Survival of H323 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	1.20E+08	3.61E+07
MRS 3 hr	1.50E+08	2.00E+07
pH 2	1.10E+02	1.73E+01
pH 3	2.40E+05	2.65E+04
pH 4	1.03E+08	1.82E+07

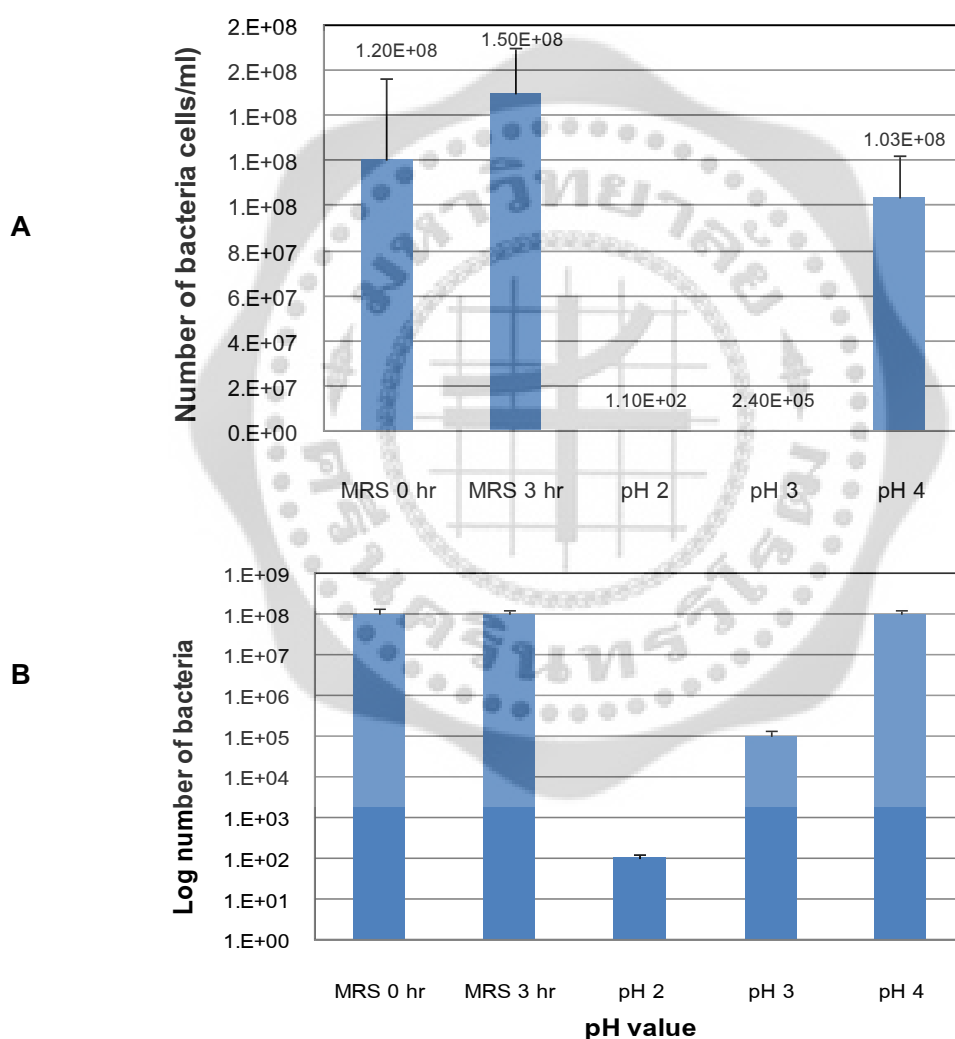


Figure 20 Survival of H323 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 14 Survival of H415 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	9.67E+07	3.51E+07
MRS 3 hr	2.77E+08	2.08E+07
pH 2	2.57E+04	3.79E+03
pH 3	5.70E+06	9.85E+05
pH 4	3.53E+07	2.89E+06

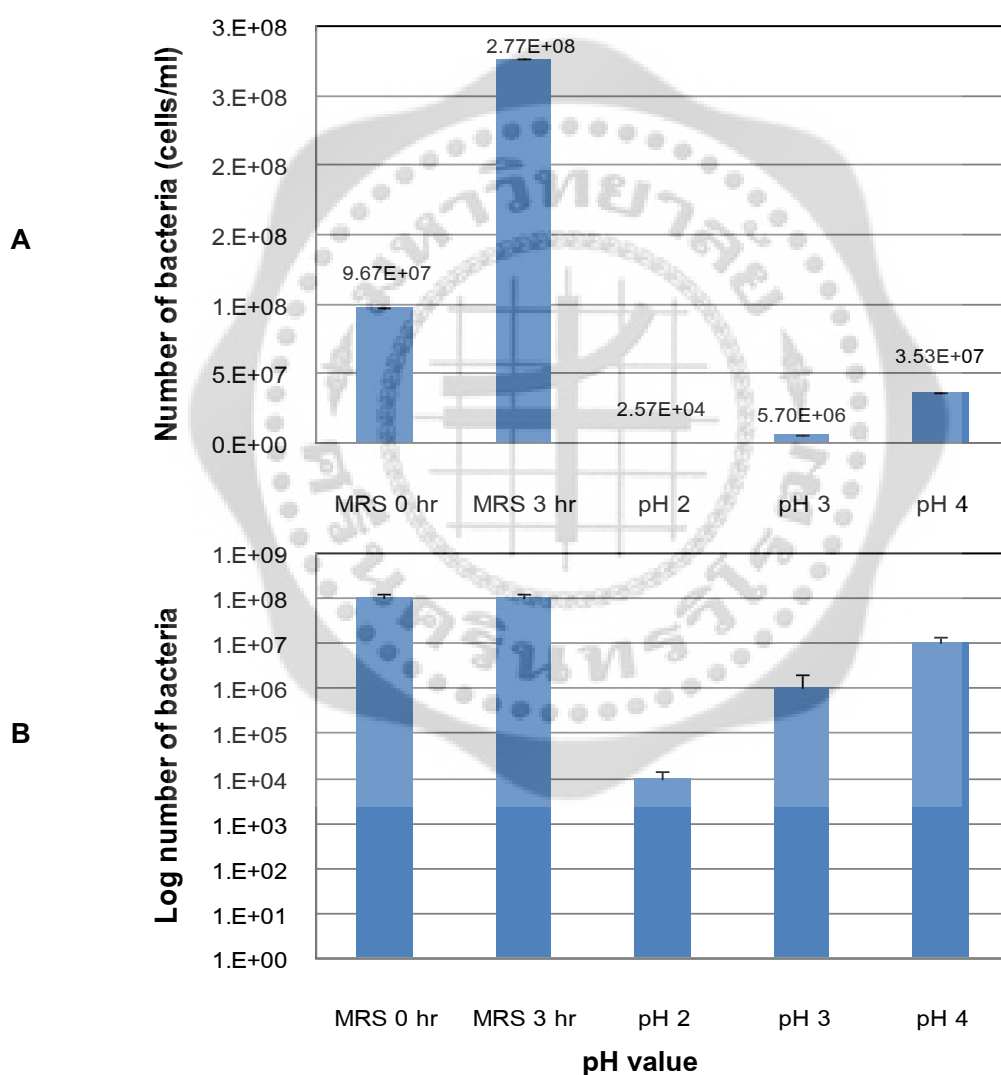


Figure 21 Survival of H415 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 15 Survival of H468 strain after incubation at various pH values

pH value	Number of bacteria (cells/ml)	SD
MRS 0 hr	3.33E+08	4.93E+07
MRS 3 hr	4.43E+08	1.12E+08
pH 2	3.67E+03	3.21E+02
pH 3	3.87E+06	5.51E+05
pH 4	9.33E+07	1.53E+07

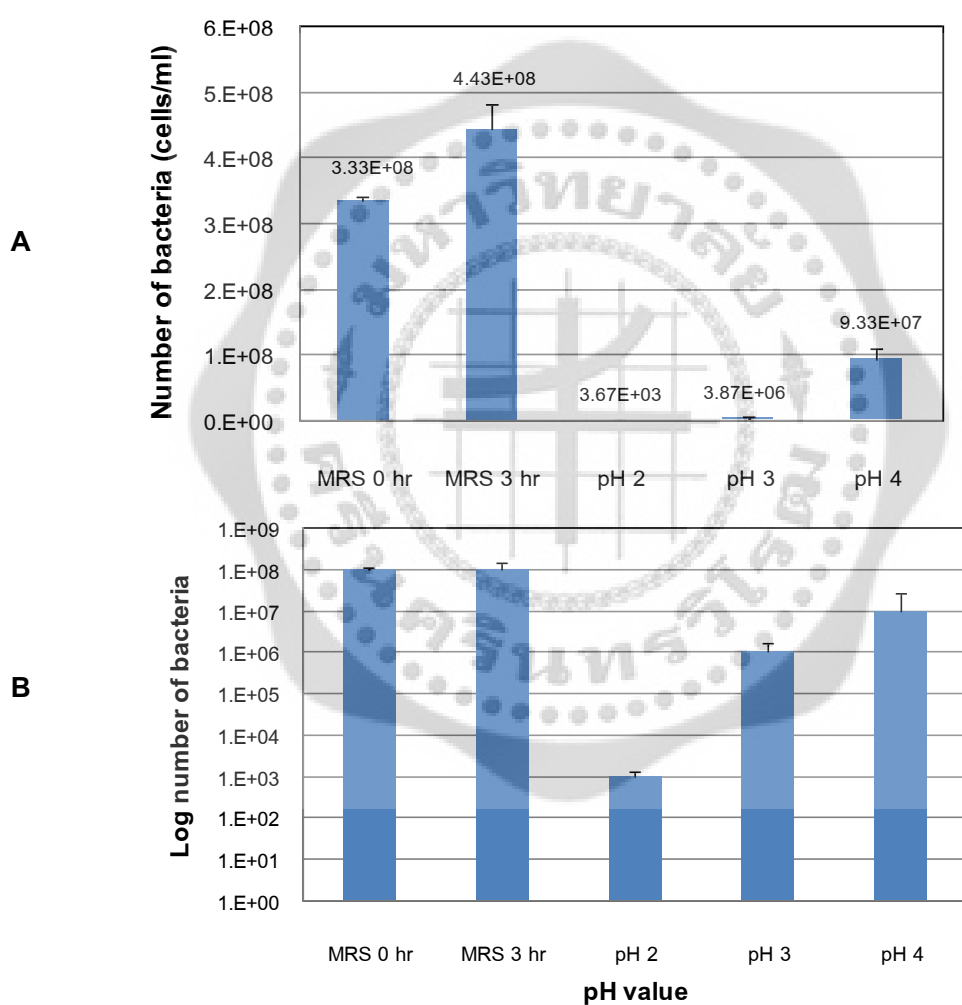


Figure 22 Survival of H468 strain after incubation at various pH values

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

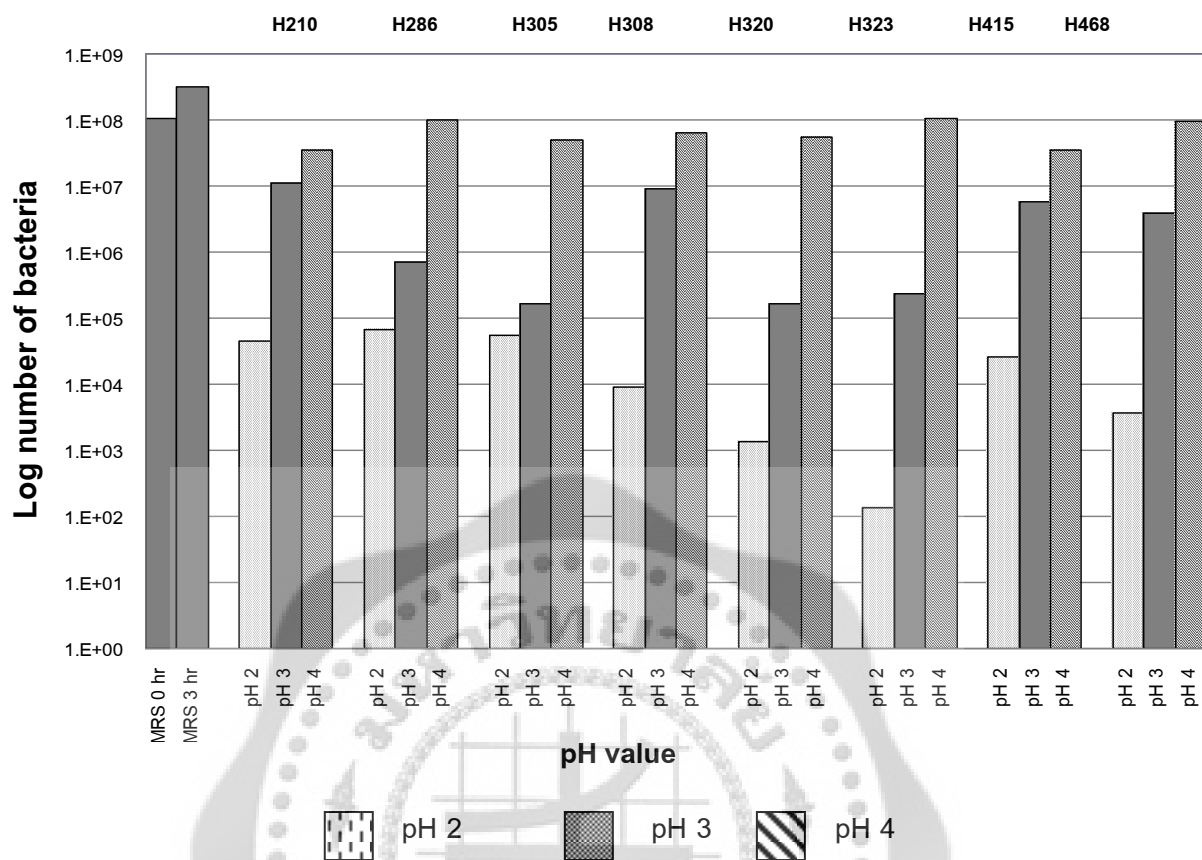


Figure 23 Summary of survival of selected *Lactobacillus* strains after incubation at various pH values. The pH values are numbered on a logarithmic scale

6.2 Bile tolerance

One reuterin-producing strain and 7 isolates which showed the strongest inhibitory effect against cancer cells were selected to test for bile tolerance. In the bile tolerance test selected *Lactobacillus* isolates. Were cultivated in MRS broth with or without various concentrations of bovine bile. The result of bile tolerance (survival at various bile values) showed that viability of H210, H305 and H468 was changed after incubation for 3 hr at 1-4% bile. The viable counts at 1%, 2% and 3% bile, were increased by about 1 log values, but showed more bile tolerance in 4% bile, which showed no log difference as reported in Table 16, 17, 18 and Figure 24, 25, 26. H286, viable counts at 1-4% bile showed no log difference as reported in Table 19 and Figure 27. H308, viable counts of 1% bile showed was increased by about 1 log values, but showed more bile tolerance in 2-3% bile, which showed no log difference. No growth occurred after incubated at 4% bile (Table 20 and Figure 28). H320, viable counts at 1% bile showed no log difference, but showed more bile tolerance in the 2-4% bile which showed increases about 1 log values (Table 21 and Figure 29). H323, viable counts of 1-4% bile showed increased about 1 log values as reported in Table 22 and Figure 30. H415 viable counts at 1-2% bile showed increases about 1 log values, but showed more bile tolerance in the 3-4% bile which showed no log difference as reported in Table 23 and Figure 31. All values were compared with MRS bacteria media control.

In this study, selected *Lactobacillus* strains was tested for bile tolerance, and the results showed in Figure 23 that strain could survive after incubation for 3 hr at various bile concentration. Except H308 strain no growth occurred after incubated at 4% bile.

Table 16 Survival of H210 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	9.7E+07	5.77E+06
MRS 3 hr	1.47E+08	2.52E+07
1%	1.90E+09	2.65E+08
2%	1.60E+09	4.00E+08
3%	1.03E+09	5.77E+07
4%	4.33E+08	5.77E+07

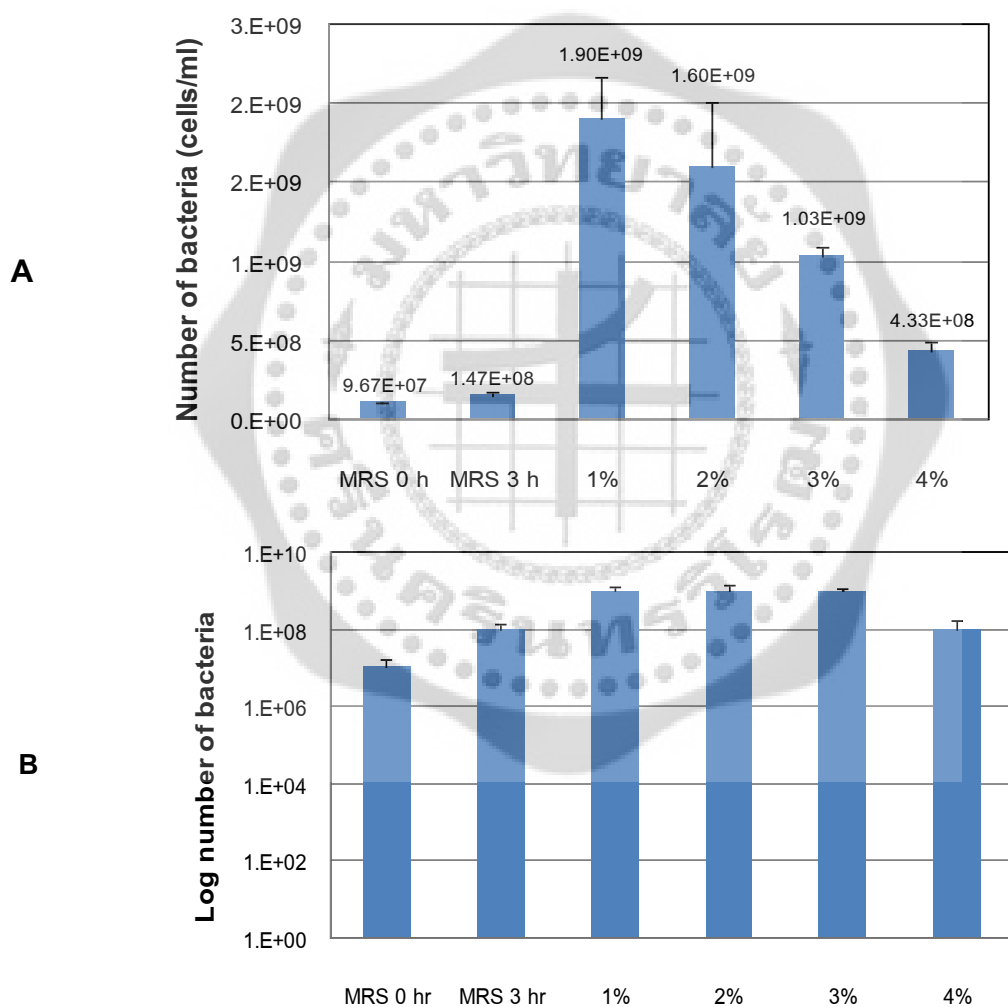


Figure 24 Bile tolerance of H210

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 17 Survival of H305 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	2.4E+08	1.53E+07
MRS 3 hr	3.93E+08	1.15E+07
1%	2.40E+09	1.00E+08
2%	1.63E+09	1.53E+08
3%	1.10E+09	1.00E+08
4%	5.33E+08	1.15E+08

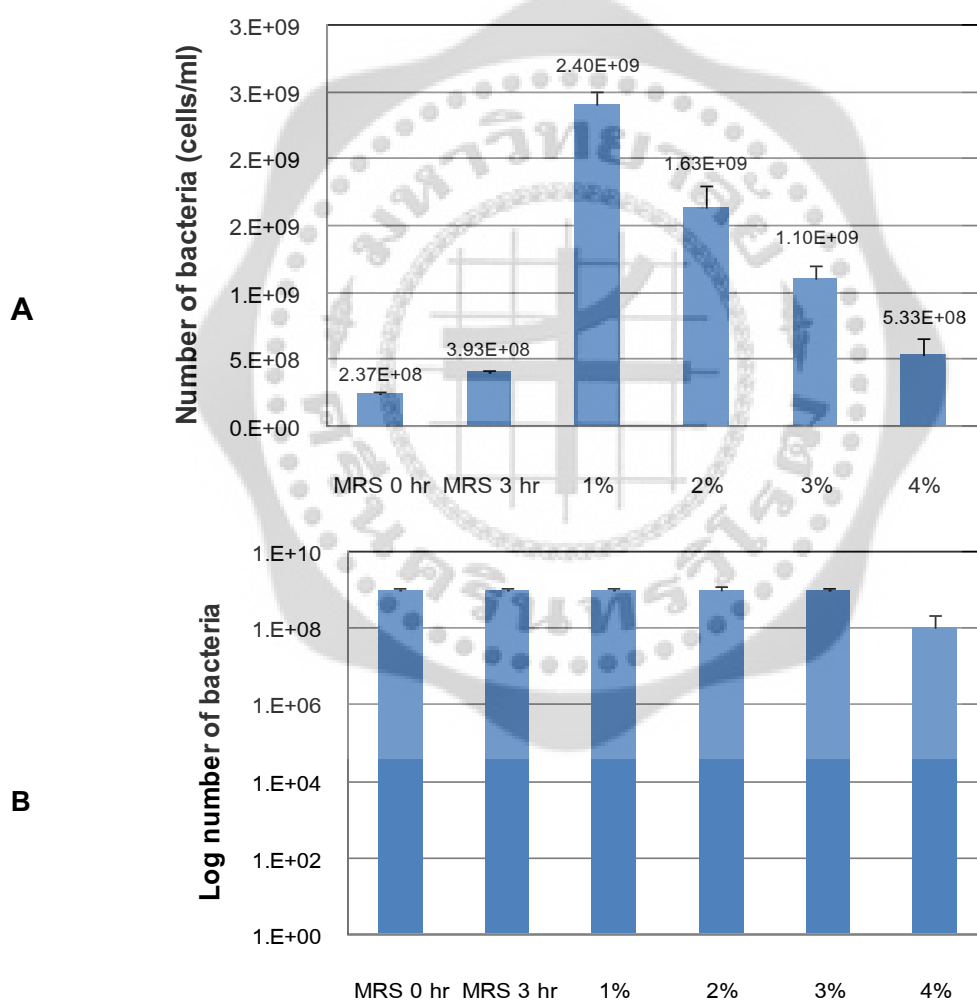


Figure 25 Bile tolerance of H305

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 18 Survival of H468 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	3.3E+08	3.61E+07
MRS 3 hr	4.33E+08	5.86E+07
1%	2.60E+09	5.29E+08
2%	2.20E+09	3.00E+08
3%	1.33E+09	3.51E+08
4%	7.67E+08	1.53E+08

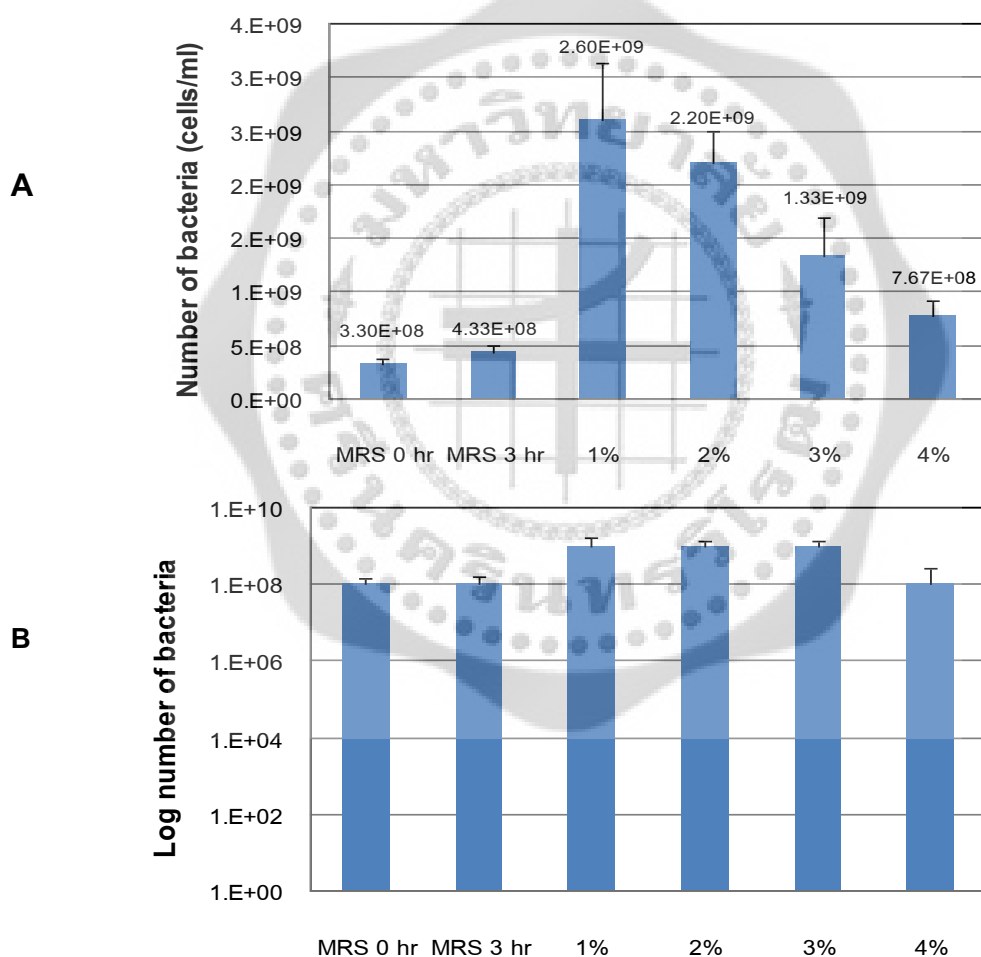


Figure 26 Bile tolerance of H468

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 19 Survival of H286 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	8.1E+08	3.21E+07
MRS 3 hr	1.04E+09	5.29E+07
1%	5.47E+09	4.16E+08
2%	3.23E+09	7.23E+08
3%	2.40E+09	5.00E+08
4%	1.43E+09	4.51E+08

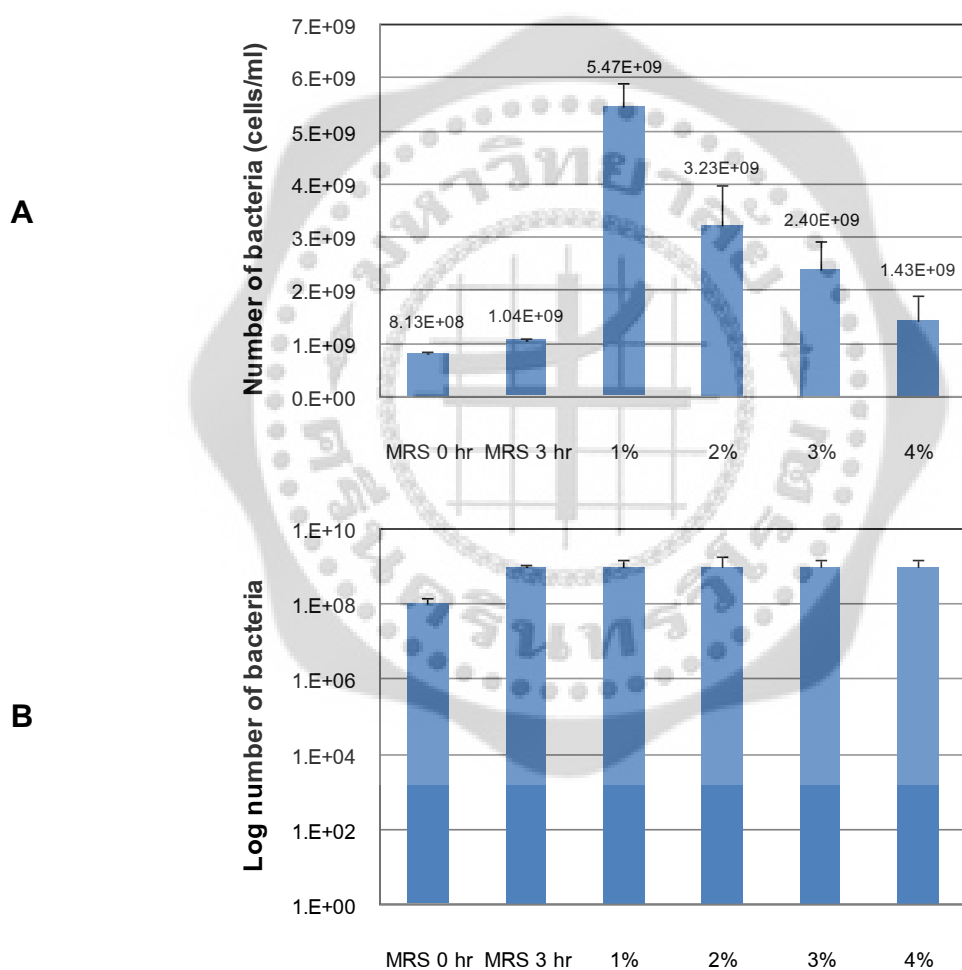


Figure 27 Bile tolerance of H286

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 20 Survival of H308 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	2.8E+08	7.64E+07
MRS 3 hr	5.03E+08	1.53E+07
1%	1.23E+09	4.93E+08
2%	5.67E+08	2.08E+08
3%	1.67E+08	5.77E+07
4%	0.00E+00	0.00E+00

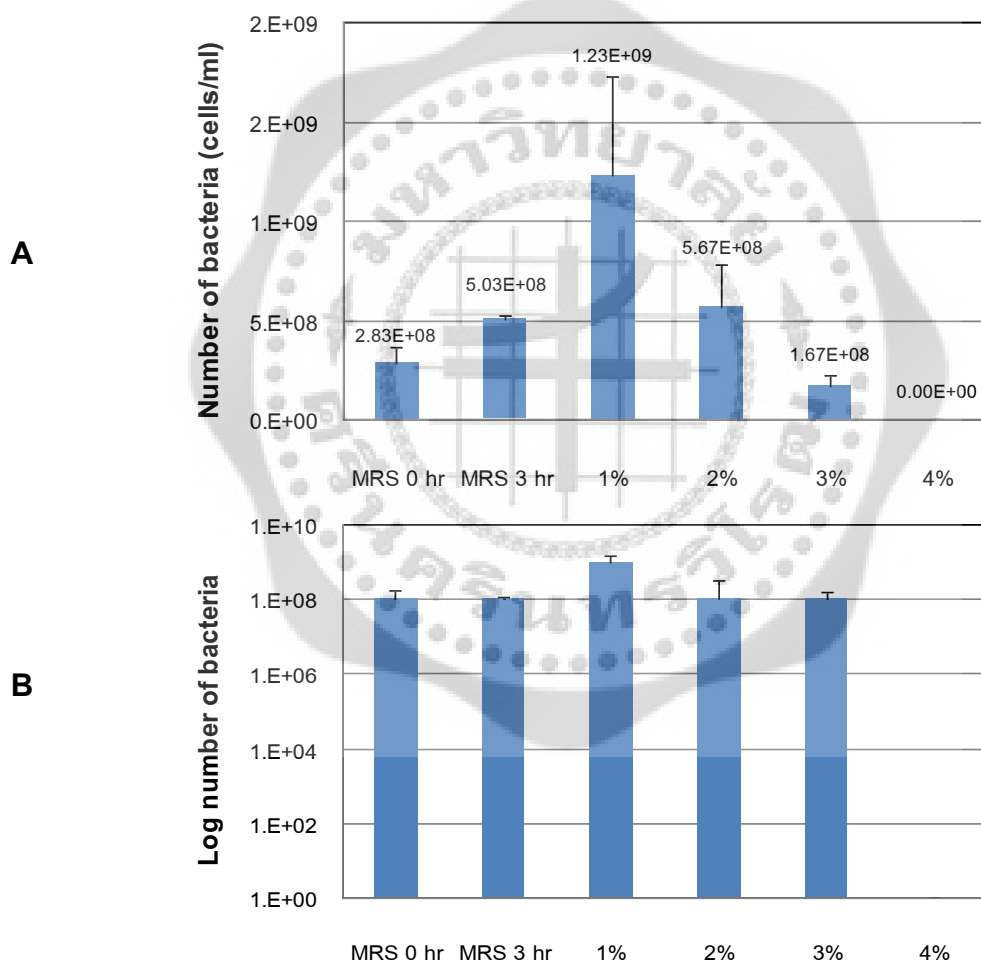


Figure 28 Bile tolerance of H308

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 21 Survival of H320 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	3.7E+07	1.15E+07
MRS 3 hr	1.80E+08	3.00E+07
1%	8.67E+08	1.53E+08
2%	1.40E+09	3.00E+08
3%	2.77E+09	8.02E+08
4%	3.47E+09	4.51E+08

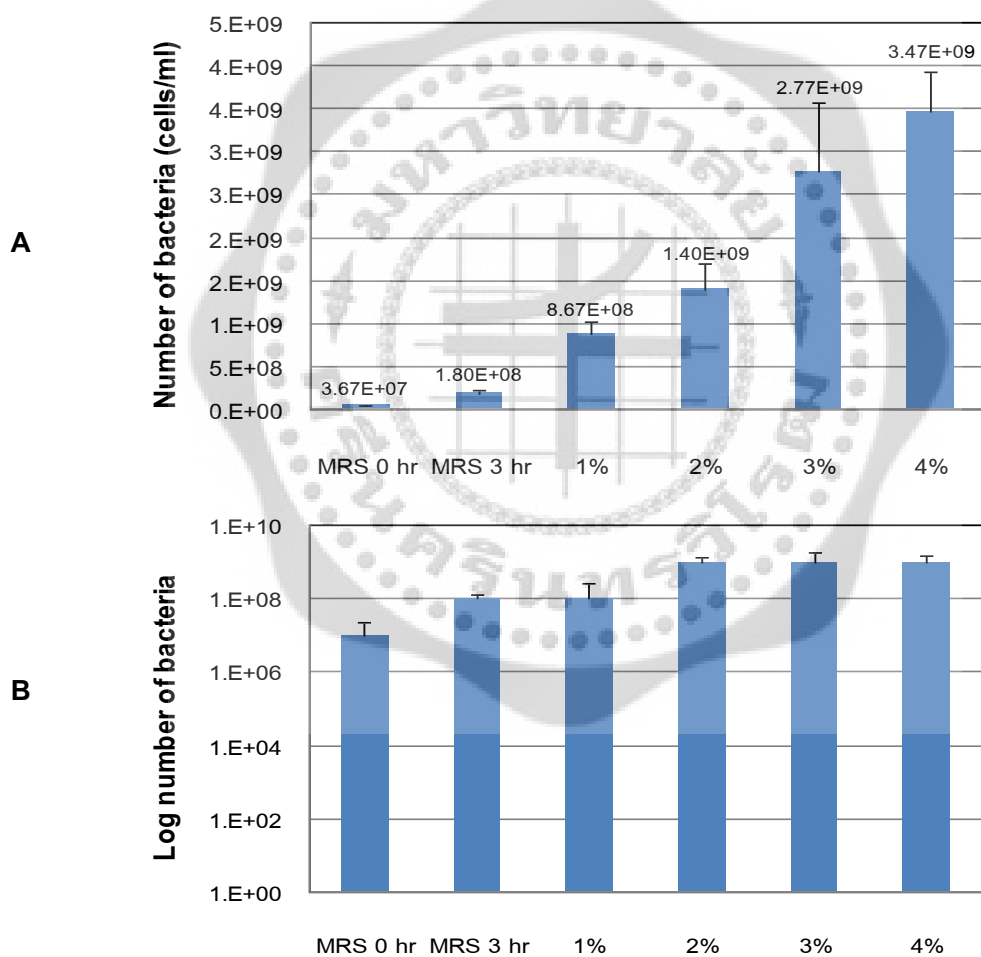


Figure 29 Bile tolerance of H320

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 22 Survival of H323 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	4.4E+08	4.04E+07
MRS 3 hr	5.50E+08	3.00E+07
1%	1.13E+09	1.53E+08
2%	2.23E+09	1.53E+08
3%	3.20E+09	2.00E+08
4%	4.73E+09	3.06E+08

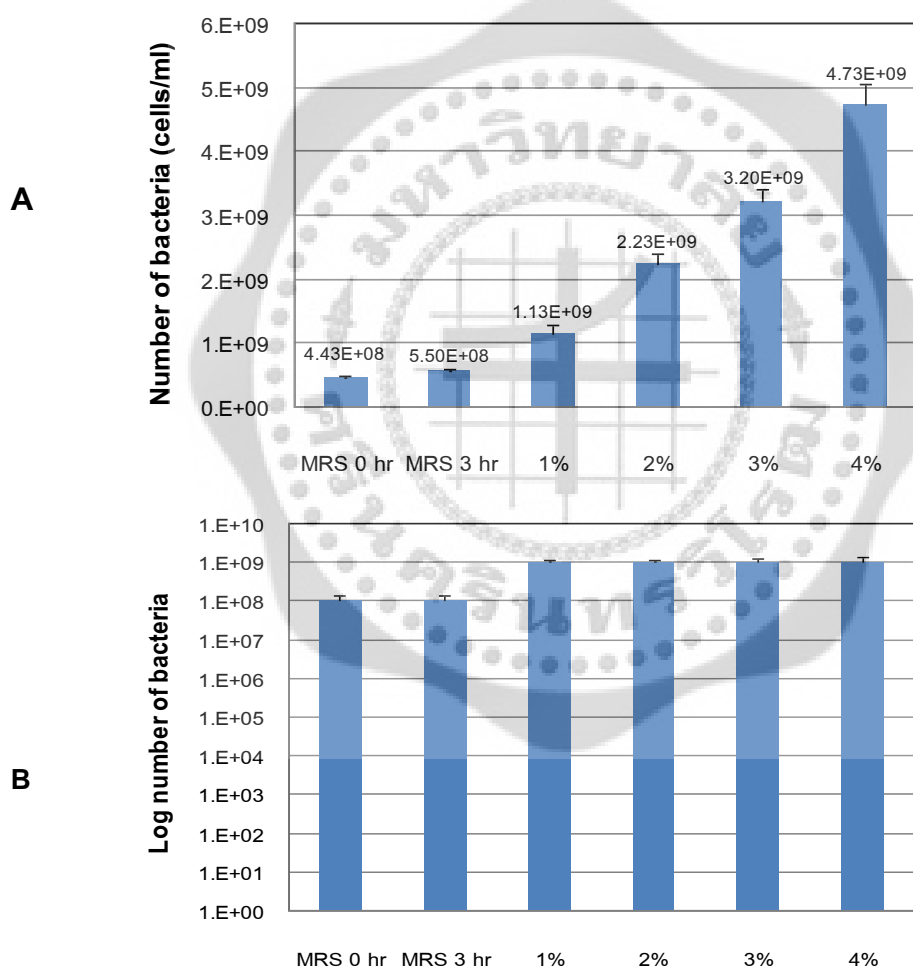


Figure 30 Bile tolerance of H323

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

Table 23 Survival of H415 strain after incubation in various concentration of bile

% Bile	Number of bacteria (cells/ml)	SD
MRS 0 hr	2.1E+08	2.08E+07
MRS 3 hr	2.83E+08	4.04E+07
1%	3.40E+09	3.61E+08
2%	2.27E+09	3.06E+08
3%	5.67E+08	1.53E+08
4%	1.67E+08	5.77E+07

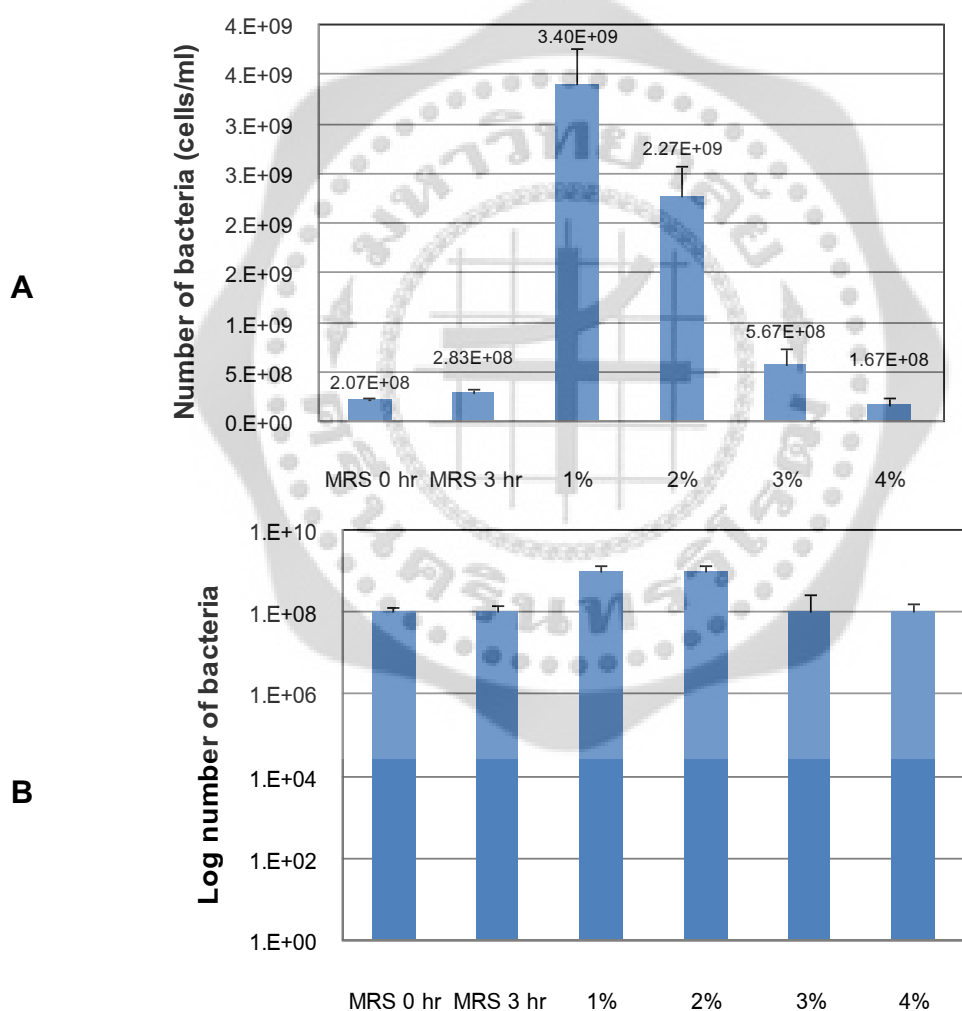


Figure 31 Bile tolerance of H415

(A) Indicated in cell numbers

(B) Indicated in logarithmic numbers

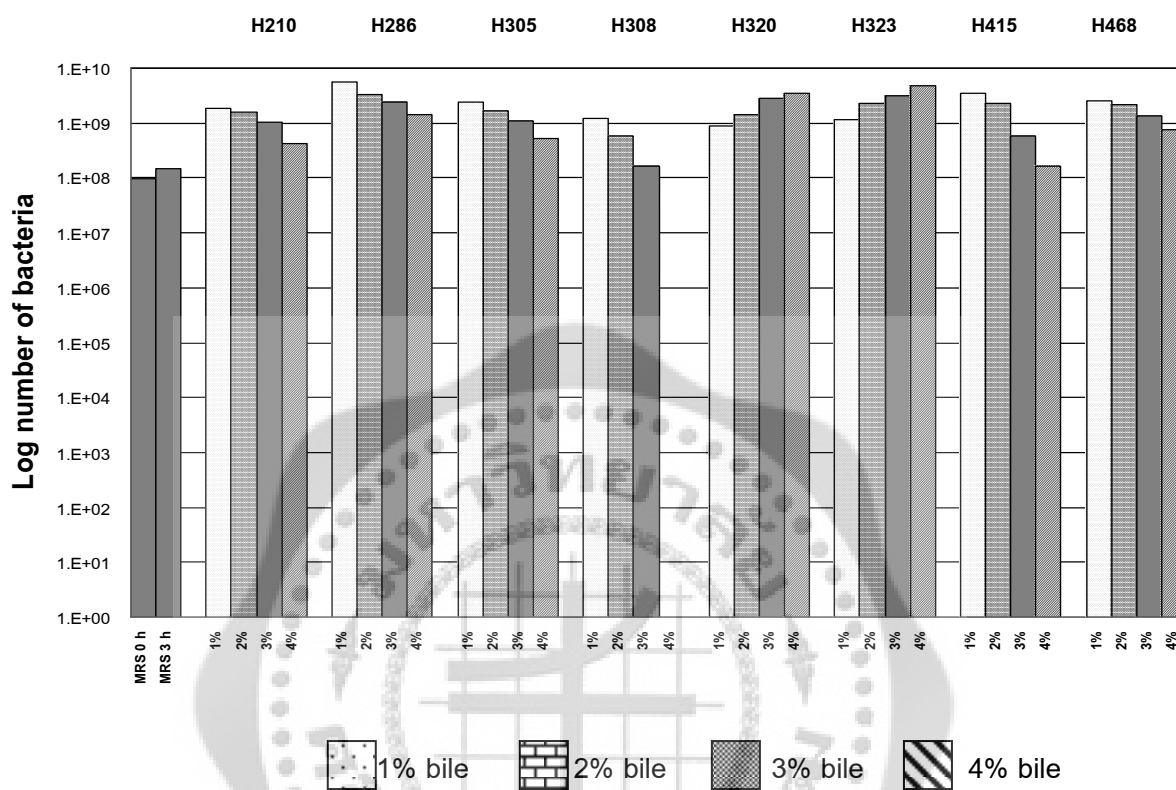


Figure 32 Summary of survival of selected *Lactobacillus* strains after incubation at various bile values

The pH values are numbered on a logarithmic scale

7. Phenotypic characteristics of selected *Lactobacillus* isolates by carbohydrate fermentation profile (API 50 CHL)

Selected *Lactobacillus* strains including one isolate of H210 reuterin-producing strain, which has potent antimicrobial activity and seven isolates have shown strong inhibition on cancer cell proliferation were characterized by API 50 CHL as shown in Figure 33 and Table 24. The carbohydrate fermentation patterns were used to determine the species of selected *Lactobacillus* isolates and analyzed by API 50 CHL V5.1 at <http://apiweb.biomerieux.com/servlet/Identify>. One strain, H210 was able to utilize glycerol, L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, D-tagatose and gluconate as shown in Figure 33A and appendix D table 25. This strain was showed 76.2% identity to *L. plantarum* 1, 23.5% identity to *L. pentosus* and 0.1% identity to *L. brevis* 1.

For the cancer cells proliferation inhibitory strains, 7 strains of H286, H305, H308, H320, H323, H415 and H468 as shown in Tables 25-31. H286, a Caco-2 cell inhibitory strain, as shown in Appendix D table 26, was identified as *L. paracasei* spp. *paracasei* 2, *L. paracasei* spp. *paracasei* 1 and *L. rhamnosus* with 61.5 %, 38.3% and 0.1% identity respectively. H468 and H320, Caco-2 and U937 cell inhibitory strains, as demonstrated in appendix D table 27, 28, was identified as *L. paracasei* spp. *paracasei* 1 and *L. paracasei* spp. *paracasei* 2 with 92.8 % and 6.5% identity respectively. H323, a Caco-2 and U937 cells cell inhibitory strain, as shown carbohydrate utilization patterns by API 50 CHL in Figure 30B, was identified as *L. pentosus* and *L. plantarum* 1 with 99.8% and 0.1% identity respectively (Appendix D table 29). H305, a U937 cells cell inhibitory strain, as shown in appendix D table 30, was identified as *L. paracasei* spp. *paracasei* 1 and *L. paracasei* spp. *paracasei* 3 with 99.9 % and 0.1 % identity respectively. H308, demonstrated in appendix D table 31, was identified as *L. salivarius* and *L. plantarum* 1 with 99.9 % and 0.1 % identity respectively. H415, demonstrated in Table 31 was identified as *L. plantarum* 1, *L. pentosus* and *L. brevis* 1 with 66.6%, 33.2% and 0.1% identity respectively.



A. H210 strain

B. H323 strain

Figure 33 Carbohydrate utilization patterns of H210 and H323 strains

Reuterin-producing strain and antimicrobial activity and H323,
Cancer cell proliferation inhibitory strain by API 50 CHL

Table 24 Carbohydrate fermentation of selected *Lactobacillus* strain*

Test	Carbohydrate	Strains							
		H210	H286	H305	H308	H320	H323	H415	H468
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	Sorbitol	+	+	+	+	+	+	+	+
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 24 Carbohydrate fermentation of selected *Lactobacillus* strain* (continued)

Test	Carbohydrate	Strains							
		H210	H286	H305	H308	H320	H323	H415	H468
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>

CHAPTER V

DISCUSSION

A well known group of probiotic *Lactobacillus* spp. is commonly shown to have health promoting^(37, 41). Lactobacilli are normally found in the human and animal⁽³⁻⁶⁾ gastrointestinal tracts (GIT) and have been isolated from humans⁽⁴⁻⁵⁾, animals⁽⁷⁻⁸⁾ and fermented products⁽⁹⁻¹¹⁾. In the present study, we proposed to isolate *Lactobacillus* from infant feces and fermented foods to screen for probiotic properties including reuterin production and anti-proliferative activity against the cancer cell lines.

Lactobacilli are known to produce antimicrobial substances such as bacteriocin⁽²⁵⁻²⁶⁾ and reuterin⁽²⁷⁻²⁸⁾. In this study, *Lactobacillus* isolates of infant feces and fermented foods were investigated for reuterin production. Some *Lactobacillus* species produce reuterin, especially *L. reuteri* which resides in the gastrointestinal tract of human and animals^(91, 174) and has been found to be a major component of *Lactobacillus* species found in all hosts⁽¹⁷⁴⁾. Reuterin is a very broad spectrum antimicrobial compound produced during anaerobic fermentation of glycerol by the action of glycerol dehydratase which, converts glycerol to reuterin^(32-33, 73). Consequently, we investigated reuterin production by using spot overlay technique as described by Taweechoatipatr⁽¹¹³⁾. *Lactobacillus* spotted plate was overlaid with soft agar containing glycerol and incubated anaerobically at 37°C for 3 hr for reuterin production and detected by 2, 4-dinitrophenylhydrazine and KOH solution (potassium hydroxide). As shown in Table 1, 6 isolates from 300 tested *Lactobacillus* isolates were obtained from different sample of infants feces and fermented foods. They were Gram-positive rods and catalase-negative. Reuterin was detected in those *Lactobacillus* isolates whereas *L. reuteri* ATCC 25922 was used as positive control of reuterin-producing strain. It had displayed reddish brown coloration around spot (Figure 6).

The 6 reuterin-producing isolates were confirmed of reuterin production by colorimetric assay modified method of Circle and colleagues⁽¹⁶⁹⁾, which is a fast and simple tool for screening a high number of isolates for reuterin production⁽¹⁷⁵⁾. This method measures a dehydration derivative of reuterin in acidic conditions. Figure 34 displays the

schematic pathway of glycerol reduction to reuterin, reuterin dehydration to acrolein at acidic pH ⁽⁷⁴⁾. Reuterin production was determined by using the acid-catalyzed formation of a colored complex between acrolein/ 3-HPA and tryptophan ⁽¹⁶⁹⁾.

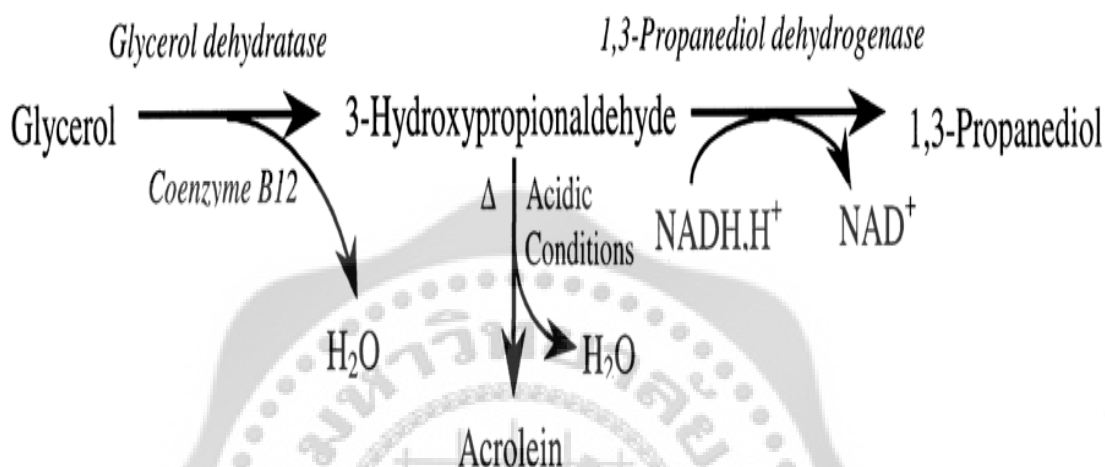


Figure 34 Metabolic pathway from glycerol to 3-HPA (reuterin), acrolein and 1, 3-propanediol (1, 3-PDO) ⁽⁷⁴⁾

In this study, the results showed that only 1 isolate, H210 showed color change from purple to yellow when compared to *L. reuteri* ATCC 55730 positive control, that confirms the presence of reuterin. Five testing *Lactobacillus* isolates could not be detected by this method (Figure 7). This result suggested that in all 5 isolates may be only produce reuterin as a transient metabolite, which is immediately further reduced, spot overlay method used to detect reuterin production may cause it to show false positive results or reuterin is produced in very low amounts and could not be detected by this method. On the other hand, H210 has shown the unique capability to retain and accumulate reuterin. Production of reuterin by H210 isolates is similar to that observed in *L. reuteri*. This featured capability out-competes with other producing strain ^(73, 88).

H210 reuterin-producing strain were tested for antagonistic activities against pathogenic microorganisms including gram-negative bacteria including *E. coli*, *V. cholerae*,

V. parahaemolyticus, *S. Typhimurium*, *S. dysenteriae*, *H. pylori* and gram-positive bacteria including *S. aureus*, MRSA 20651, MRSA 20654 and yeast such as *C. albicans* using the agar well diffusion method⁽¹⁷⁰⁻¹⁷¹⁾. Our data showed that reuterin is effective for most tested pathogenic microorganisms. As shown in Table 2 and Figure 8 and Figure 9, reuterin demonstrated very strong inhibitory (>22 mm) activities against *S. Typhimurium*, *S. aureus* (MRSA) DMST 20651 and *S. aureus* ATCC 6538. Followed by strong inhibitory (16-21 mm) activities against *S. aureus* (MRSA) DMST 20654, *V. parahaemolyticus*, *S. dysenteriae* and *B. subtilis* ATCC 6633. Moderate to weak (<10-15 mm) inhibitory activities well displayed against *E. coli* ATCC 25922, *V. cholerae* DMST 2873, *H. pylori* (H40) and *C. albicans* ATCC 10231. Similar to our findings, Axelsson and colleagues⁽³⁴⁾ demonstrated reuterin is a broad-spectrum antimicrobial which is active against gram-positive, gram-negative bacteria including yeast, fungi, protozoa and viruses. For mechanism action, reuterin is capable of inhibiting DNA synthesis by inhibiting ribonucleotide reductase (NRD), involved in the first step in *de novo* synthesis of deoxyribonucleotide for DNA synthesis. This inhibition of the ribonucleotide reductase activity could describe the broad-spectrum antimicrobial effect⁽⁷⁶⁾. The results indicated antimicrobial activity that reached a variable but potent level against all pathogens. Genetic diversity among strains of pathogens may account for relative differences in susceptibilities of pathogenic isolates to reuterin⁽⁹¹⁾. However, these *Lactobacillus* strains showed that both reuterin production and effective antimicrobial had broad spectrum activity against pathogens. These *Lactobacillus* strain therefore may be used for further investigations characterizing antimicrobial substances.

Probiotic microorganisms are commonly known to have health promoting attributes, including anti-proliferative effects and apoptosis against human cancer cell lines and such properties may be useful for cancer prevention and therapy^(41, 158-159). In this study, a total of three hundred *Lactobacillus* isolates were isolated from human fecal samples and fermented foods were investigated for anti-proliferative activity against Caco-2 colon carcinoma cell line and U937 human monocytic leukemic cell line by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)⁽¹⁶⁸⁾. Our preliminary experiments were to determine the optimal concentration of MRS (bacterial media control). The results

as showed at 15-20% v/v concentration of MRS had significant inhibitory effects on the cell viability in three types of cells as compared control group, while at 5-10% v/v concentration showed no significant inhibitory effects on the cell proliferation (Table 3, Figure 10). Then we tried and tested some of the *Lactobacillus* conditioned media at 5-10% v/v concentration to find the most effective concentration on the cell viability. The results as showed at 5% and 10% v/v no inhibitory effects and had the inhibitory effects on the cell proliferation, respectively (data not shown). Therefore, optimal *Lactobacillus*-conditioned media concentrations at 10% v/v to determined inhibitory effect on cell proliferation.

The inhibitory effect of conditioned medium from healthy infant feces on Caco-2 cell proliferation is demonstrated in Table 4. The result showed that H370 had a moderate inhibitory effect, and three isolates: H286, H323 and H468 showed strong inhibitory effect on Caco-2 cells. Similar results were observed on U937 cell as shown in Table 5. Eight isolates: H61, H114, H115, H121, H267, H268, H450 and H456 showed weak inhibitory effect, eight isolates: H117, H120, H323, H370, H404, H427, H428 and H461 showed moderate inhibitory effect. And four isolates: H305, H308, H320 and H415 showed strong inhibitory effect.

In addition, inhibitory effect of conditioned medium from fermented foods is shown in Table 6. F13 had the moderate inhibitory effect on Caco-2 cells. Twelve isolates: F1-F9 and F17-F19 showed strong inhibitory effect. Interestingly, our experiments have also shown the strong inhibitory effect on U937 cell of twelve isolates: F1-F9, F12-F13 and F17-F19. The *Lactobacillus*-conditioned media which showed inhibitory effect to two cancer cell lines and some had deleterious effect to normal cell line (Vero cells). As the results demonstrate in Figure 11-14 that result showed H323, F1, F2, F3, F4, F5, F7, F9, F13, F17 and F19 have shown exhibited significant inhibitory effects on the growth of Vero cells, when compared to MRS bacterial media control.

Several *Lactobacillus* species have the anti-proliferative effects against cancer cell lines. Ewaschuk and colleagues⁽¹⁷⁶⁾ reported that *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum*, reduced the viability and induced apoptosis of HT-29 and Caco-2 cells. Chin-Feng Liu and colleagues⁽³⁷⁾ showed that the heat-killed cells (HKC) of *L. plantarum* NTU

102, *L. acidophilus* BCRC 14079 were isolated from pickled vegetables and commercial yoghurt respectively, showed the inhibitory effect on Caco-2 cells of more than 50%. Our study, suggests that *Lactobacillus* spp. exerted an *in vitro* inhibitory effect on cancer cell proliferation, which is considered a functional criteria of probiotics ⁽¹⁷⁷⁾. However, inhibitory activities were different in each isolate which it may have produced different biological substances or mechanism different in inhibition of cancer cells. Characterization of cell death in cancer cells is an interesting issue. In previous studies suggested that the soluble polysaccharide from *L. acidophilus* 606 were observed to induce apoptosis in the HT-29 cells by DNA fragmentation and propidium iodine staining ⁽¹⁷⁸⁾. In this study, the mechanism of action was unclear. Therefore, the inhibitory proliferation strains and no inhibitory effects on the cell viability of normal cell line (Vero cells) have been selected as a candidate and studies on the mechanism of inhibition of cancer cells or other properties.

Acid and bile tolerance are selection criteria for the identification of probiotic microorganisms to survive in and colonize the gastrointestinal tract. Probiotic microorganisms should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces ⁽¹⁷⁹⁻¹⁸⁰⁾. The survival of bacteria in the gastric juice depends on their ability to tolerate low pH. The pH of excreted HCl in the stomach is 0.9, but the presence of food raises the pH value to 3.0 ⁽¹⁸¹⁾. In this study, one and seven isolates reuterin-producing strain and the strongest inhibition proliferation strains respectively were tested for acid and bile tolerance, and results showed that H210 strain could survive after incubation for 3 hr at pH 2, 3 and 4 decreased by 4, 1 and 1 log values respectively. H286 strain could survive at pH 2 and 3 but numbers decreased by 4 and 3 log values respectively, but this strain survived well at pH 4 which showed no log difference. H305 strain could survive at pH 2, 3 and 4 decreased by 4, 3 and 1 log values respectively. H308 strain could survive at pH 2, 3 and 4 decreased by 5, 2 and 1 log values respectively. H320 strain could survive at pH 2, 3 and 4 decreased by 6, 4 and 2 log values respectively. H323 strain could survive at pH 2 and 3 decreased by 6 and 3 log values respectively, but this strain survived well at pH 4 which showed no log difference. H415 strain could survive at pH 2, 3 and 4 decreased by 4, 2 and 1 log values respectively. H468 strain could survive at

pH 2, 3 and 4 decreased by 5, 2 and 1 log values respectively. All values were compared with MRS bacteria media control. Wang and colleague⁽¹⁸²⁾ demonstrated the survival test revealed that several *Lactobacillus* strains were resistant to pH 2 even after 3 hr of exposure, while most of the isolates showed reduced viability after being exposed to pH 2. These results are in accordance with those obtained from similar previous studies, where *Lactobacillus* strains isolated from infant feces were viable even after being exposed to pH values of 2.5-4.0, but showed reduce viability at lower pH values^(183, 184).

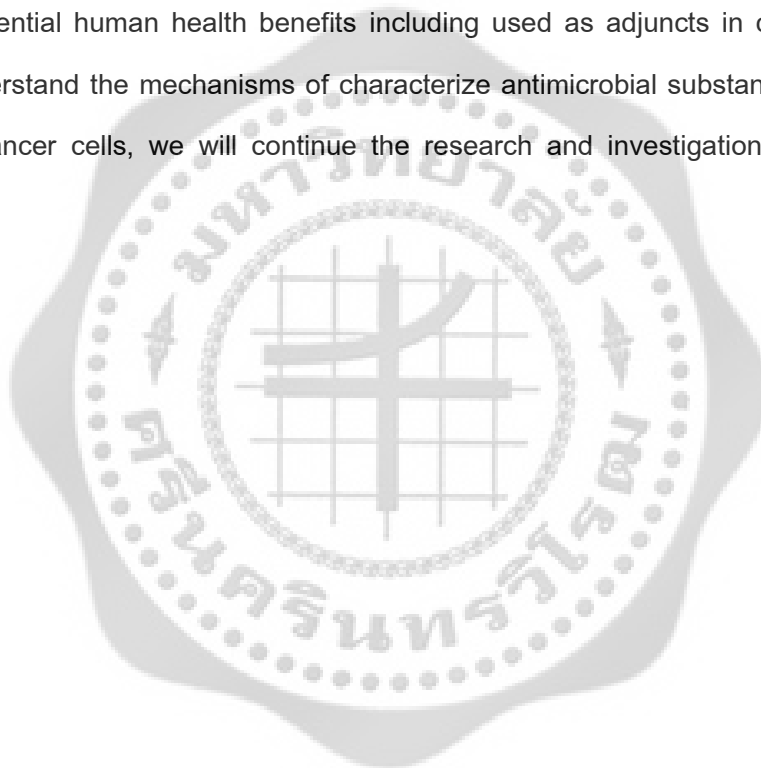
The bile in human intestine is also an important which affects the viability of *Lactobacillus*⁽¹⁸⁵⁾. In the human gastrointestinal tract, the mean bile concentration is 0.3% w/v⁽²¹³⁾. In this study, H210, H305 and H468 strain the viable count were increased by 1 log differences in 1%, 2%, 3% bile, whereas in 4% bile showed no log difference. H286 strain showed no log difference in 1%, 2%, 3%, 4% bile. H308 strain the viable count were increased by 1 log differences in 1% bile, whereas in 2%, 3% bile showed no log difference, and no growth occurred after incubating in 4% bile. H320 strain showed no log difference in 1% bile, whereas in 2%, 3%, 4% bile increased by 1 log differences. H323 strain the viable count were increased by 1 log differences in 1%, 2%, 3%, 4% bile. H415 strain the viable count were increased by 1 log differences in 1%, 2% bile, whereas in 3%, 4% bile showed no log difference. All values were compared with MRS bacteria media control. Wang and colleague⁽¹⁸²⁾ reported that *Lactobacillus* strain isolated from infant feces and pickled cabbages were able to grow in the presence of 1% bile.

In this study, selected *Lactobacillus* strains including one isolate of H210 reuterin-producing strain, which has potent antimicrobial activity and seven isolates have showed strongest inhibition on cancer cell proliferation. All *Lactobacillus* strains tested were characterized by API 50 CHL. In our study, one isolate of H210 reuterin-producing strain, were identified as *L. plantarum*.

For the cancer cells proliferation inhibitory strains, 4 strains, H286, H305, H320 and H468 was identified as *L. paracasei* spp. *paracasei*. And 3 strains, H308, H323 and H415 were identified as *L. salivarius*, *L. pentosus* and *L. plantarum* respectively. Ewaschuk and colleague⁽¹⁸⁶⁾ reported that *L. plantarum* reduced the viability and induced apoptosis of HT-

29 and Caco-2 cells. Liu and demonstrated that *L. paracasei* spp. *paracasei* NTU 101 and *L. plantarum* NTU 102 isolated from infant feces and pickled vegetables respectively give a strong anti-proliferative activities against colon cancer cell lines *in vitro*.

In conclusion, *Lactobacillus* candidates were isolated from infant feces and fermented foods that show satisfactory properties for probiotic application. These candidates may be applied to treating, preventing diarrhea (such as *E. coli*, *S. aureus*, *B. subtilis*, *S. aureus* MRSA 20651, *S. aureus* MRSA 20654, *V. cholerae*, *V. parahaemolyticus*, *S. Typhimurium*, *S. dysenteriae*, *H. pylori* and *C. albicans*) or useful in the production of dairy foods for potential human health benefits including used as adjuncts in cancer therapy. In order to understand the mechanisms of characterize antimicrobial substances and inhibitory effects on cancer cells, we will continue the research and investigation in the molecular perspective.





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APPENDIX A

REAGENT

1. 1X Phosphate buffered saline (PBS buffer)

For preparation of solution, all compounds in the following order are dissolved in distilled water.

NaCl	8	g
KCL	0.2	g
Na ₂ HPO ₄	1.44	g
KH ₂ PO ₄	0.24	g
H ₂ O (distilled water)	800	ml
H ₂ O (distilled water) adjust volume to	1000	ml

Adjust the pH to 7.4 with HCl

The solution was sterilized by autoclave 15 minutes, 121° C. Stored at 4° C.

2. 50 mM Sodium phosphate buffer

1M Na ₂ HPO ₄	77.4	ml
1M NaH ₂ PO ₄	22.6	ml
pH with each buffer to pH 7.4		
H ₂ O (distilled water) adjust volume to	1000	ml

Dilute 500 ml into 500 ml H₂O (distilled water) to give 1L 50 mM solution

The solution was sterilized by autoclave 15 minutes, 121° C. Stored at 4° C.

3. 250 mM glycerol solution

Glycerol	9.14	ml
H ₂ O (distilled water)	500	ml

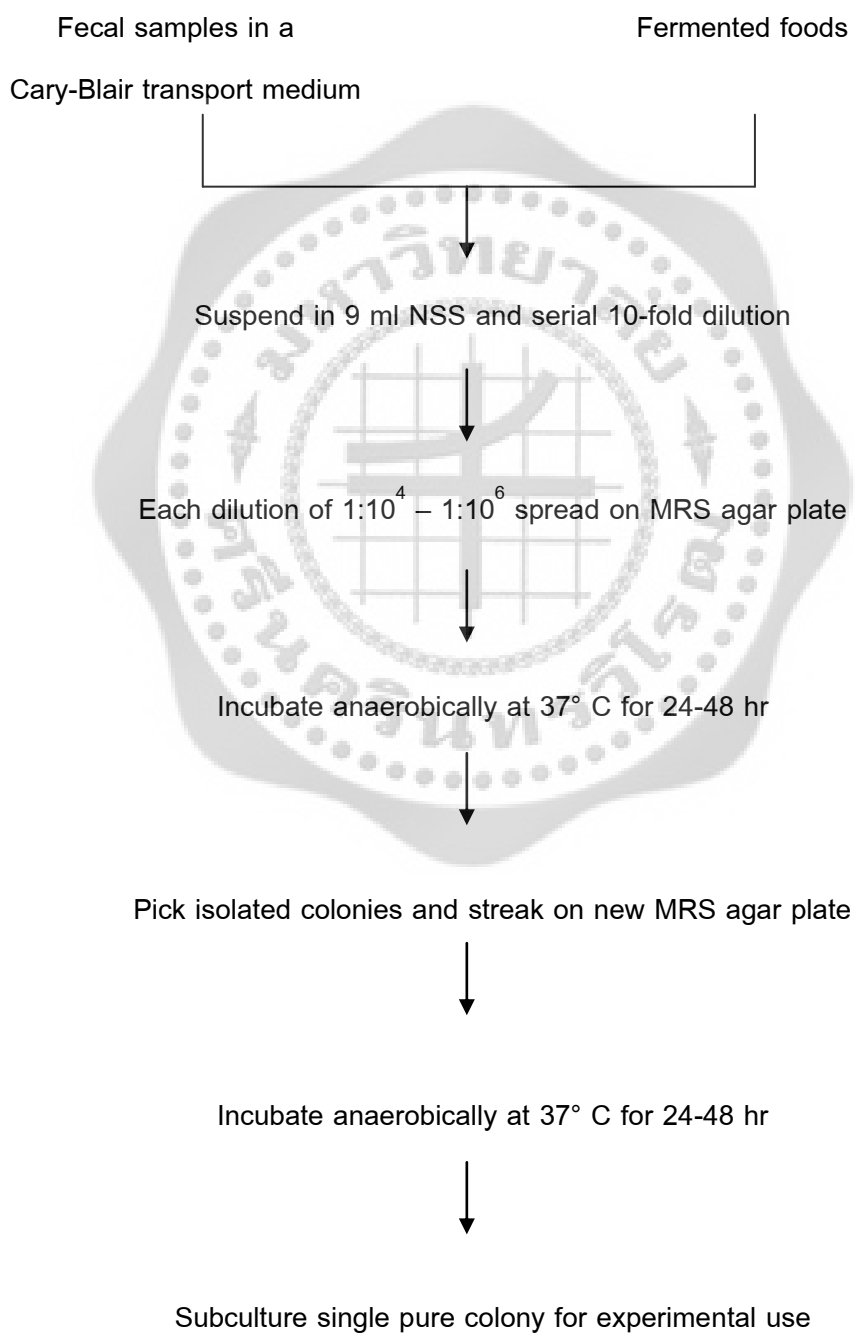
The solution was sterilized by autoclave 15 minutes, 121° C. Stored at 4° C.



APPENDIX B

FLOW CHART OF PROTOCOL

1. Isolation of Lactobacilli from Human Feces and Fermented foods



2. Spot Overlay Technique for the Detection of Reuterin

Inoculate each *Lactobacillus* strain in 200 μ l MRS broth
in each well of 96-well plate, incubate anaerobically at 37°C for 24-48 hr



Using Frogger, transfer of each culture into 175 μ l MRS broth
in each well of 96-well plate, incubate anaerobically at 37°C for 24 hr



Using Frogger, spot each culture onto BHI 20 mM glucose in plate
Incubate anaerobically at 37°C for 24 hr



Add of the soft agar (0.9% agar) with 2% glycerol over the plate
anaerobically at 37°C for 3 hr



Remove the plates and flood the surface with 2, 4-dinitrophenylhydrazine



Wait 5 minutes and discard the 2, 4-dinitrophenylhydrazine



Add the KOH solution and watch for reddish brown color
to develop around the reuterin producing strain

3. Confirmation of the Reuterin Production by Colorimetric Assay

3.1 Reuterin Production

Inoculate 1×10^8 cells/ml *Lactobacillus* isolate in MRS broth
incubate anaerobically at 37°C for 24 or 48 hr



Collect *Lactobacillus* cell pellets
by centrifugation at 4,000xg for 10 min at 4°C



Wash twice with 50 mM sodium phosphate buffer



Centrifuged and resuspended in 250 mM glycerol solution



Incubate anaerobically at 37°C for 3 hr



Collect *Lactobacillus* supernatant containing reuterin



Filter-sterilized by using a 0.22 μ m pore size filter and stored at 4°C

3.2 Colorimetric Assay

Add 40 μ l of each sample in 96-well plate



Add 30 μ l of 10 mM L-tryptophan solution



Add 120 μ l of 37% HCl

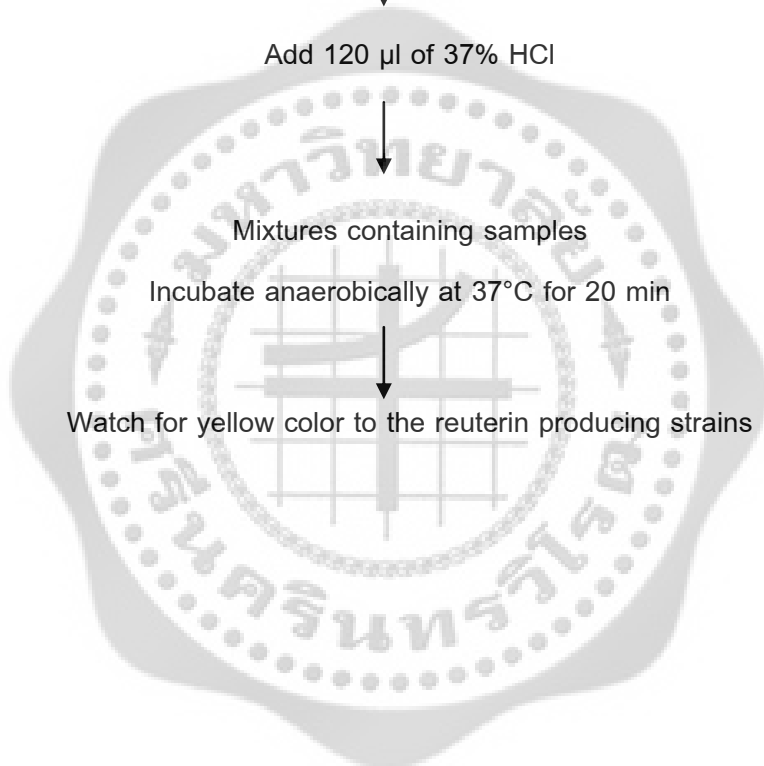


Mixtures containing samples

Incubate anaerobically at 37°C for 20 min



Watch for yellow color to the reuterin producing strains



4. Assessment of Antagonistic Activity by Agar Well Diffusion Method

Using sterile cork borer cut in the agar circular wells of 4 mm diameter



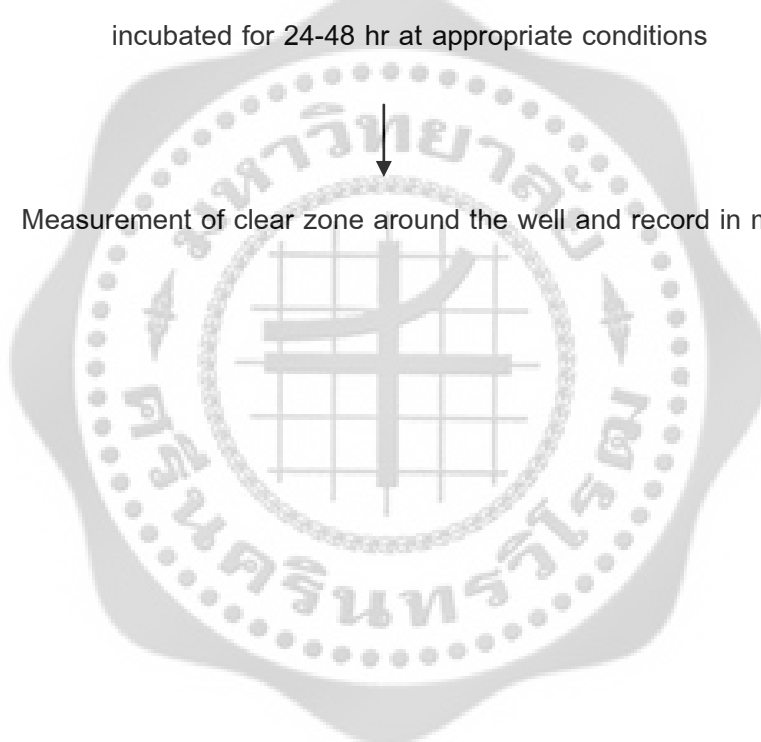
Swabbed of pathogen onto each plate



Well were filled reuterin-containing supernatants in water glycerol solution
incubated for 24-48 hr at appropriate conditions



Measurement of clear zone around the well and record in mm



5. 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 4,000xg for 10 min



Filter-sterilized by using a 0.22 μ m pore size filter



Drying by speed-vacuum

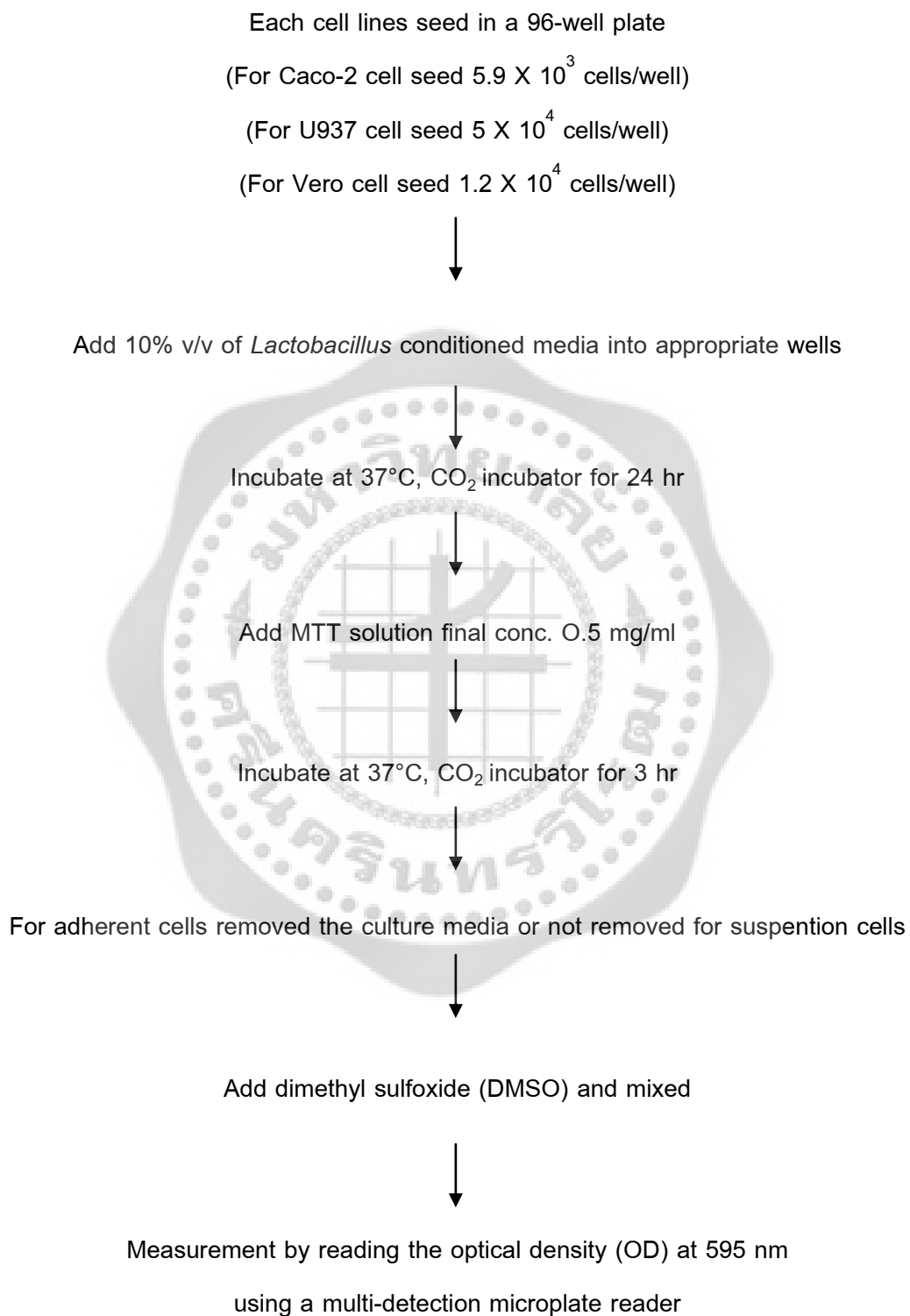


Re-suspend pellet in equal volume of cell culture media



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

6. MTT cell proliferation assay





APPENDIX C

CULTURE MEDIUM

1. MRS Broth (MRS)

'Lab Lemco' Powder	8.0	gm
Glucose	2.0	gm
Yeast extract	4.0	gm
Peptone	10	gm
Sodium acetate	5.0	gm
Triammoniumcitrate	2.0	gm
Tween 80	1.0	ml
Magnesium sulfate	0.2	gm
Manganese sulfate	0.05	gm
Dipotassium hydrogen phosphate	2.0	gm
Distilled water to	1,000	ml
Adjust pH to 6.5 ± 0.2		
Autoclave at 121°C for 15 minutes		

2. MRS Agar (MRS)

Proteose peptone	10.00	gm
Beef extract	10.00	gm
Yeast extract	5.00	gm
Dextrose	20.00	gm
Polysorbate 80	1.00	gm
Ammonium citrate	2.00	gm
Sodium acetate	5.00	gm
Magnesium sulfate	0.10	gm

Manganese sulfate	0.05	gm
Dipotassium phosphate	2.00	gm
Agar	12.00	gm
Distilled water to	1,000	ml
Adjust pH to 6.5 ± 0.2		
Autoclave at 121°C for 15 minutes		





APPENDIX D

Table 25 Carbohydrate fermentation of H210 *Lactobacillus* strain*

Strain			Strain		
Test	Carbohydrate	H210	Test	Carbohydrate	H210
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	Sorbitol	+	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 76.2% identity to *L. plantarum* 1, 23.5% identity to *L. pentosus* and 0.1% identity to *L. brevis* 1

Table 26 Carbohydrate fermentation of H286 *Lactobacillus* strain*

Test	Carbohydrate	Strain H286	Test	Carbohydrate	Strain H286
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 61.5 % identity to *L. paracasei* spp paracasei 2, 38.3% identity to *L.*

paracasei spp paracasei 1 and 0.1% identity to *L. rhamnosus*

Table 27 Carbohydrate fermentation of H468 *Lactobacillus* strain*

Test	Carbohydrate	Strain H468	Test	Carbohydrate	Strain H468
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 92.8 % identity to *L. paracasei* spp. *paracasei* 1 and 6.5% identity to

L. paracasei spp. *paracasei* 2

Table 28 Carbohydrate fermentation of H320 *Lactobacillus* strain*

Test	Carbohydrate	Strain H320	Test	Carbohydrate	Strain H320
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 92.8 % identity to *L. paracasei* spp. *paracasei* 1 and 6.5% identity to

L. paracasei spp. *paracasei* 2

Table 29 Carbohydrate fermentation of H323 *Lactobacillus* strain*

Test	Carbohydrate	Strain H323	Test	Carbohydrate	Strain H323
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 99.8% identity to *L. pentosus* and 0.1% identity to *L. plantarum* 1

Table 30 Carbohydrate fermentation of H305 *Lactobacillus* strain*

Test	Carbohydrate	Strain H305	Test	Carbohydrate	Strain H305
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 99.9 % identity to *L. paracasei* spp. *paracasei* 1 and 0.1 % identity

to *L. paracasei* spp. *paracasei*

Table 31 Carbohydrate fermentation of H308 *Lactobacillus* strain*

Test	Carbohydrate	Strain H308	Test	Carbohydrate	Strain H308
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 99.9 % identity to *L. salivarius* and 0.1 % identity to *L. plantarum* 1

Table 32 Carbohydrate fermentation of H415 *Lactobacillus* strain*

Test	Carbohydrate	Strain H415	Test	Carbohydrate	Strain H415
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 66.6% identity to *L. plantarum* 1, 33.2% identity to *L. pentosus*

and 0.1% identity to *L. brevis* 1



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List of Presentation

Poster presentation

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. 2012 January 17-18; Faculty of Medicine Srinakharinwirot University, Nakhonnayok, Thailand; 2012. p. 93.

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. 2012 February 24-26; Polat Renaissance, Istanbul, Turkey. p. 84.

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. 2012 January 17-18; Faculty of Medicine Srinakharinwirot University, Nakhonnayok, Thailand; 2012. p. 92.

Ladda B, **Chimchang J**, Triviroj T, 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. 2012 February 24-26; Polat Renaissance, Istanbul, Turkey. p. 83.

Oral presentation

Chimchang J, Wongsatayanon B, Taweechotipatr M. Isolation and detection of reuterin production and antimicrobial activity of lactic acid bacteria. The 6th National Conference "Srinakharinwirot Vichakarn". 2012 May 29-30; Srinakharinwirot University, Bangkok, Thailand. p. 26.

