การคัดเลือกโพรไบโอติกแบคทีเรียเพื่อใช้ในผลิตภัณฑ์นมหมัก

นางสาวนิตยา เมธาวณิชพงศ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2548

ISBN : 974-53-2490-6

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

SELECTION OF PROBIOTIC BACTERIA FOR USE IN FERMENTED MILK PRODUCT

Miss Nittaya Methawanitpong

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotecnology

Faculty of Science

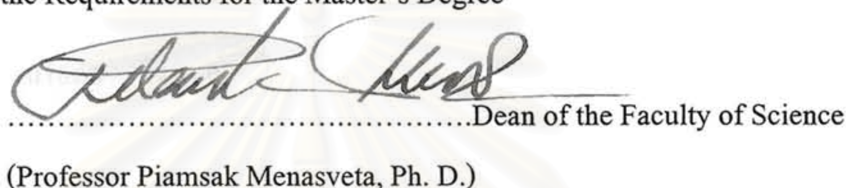
Chulalongkorn University

Academic Year 2005

ISBN : 974-53-2490-6

Thesis Title	Selection of probiotic bacteria for use in fermented milk product
By	Miss Nittaya Methawanitpong
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Romanee Sanguandeekul, Ph. D.
Thesis Co-advisor	Associate Professor Somboon Tanasupawat, Ph. D.
Thesis Co-advisor	Assistant Professor Suttisak Suknaisilp, M. Sc.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree



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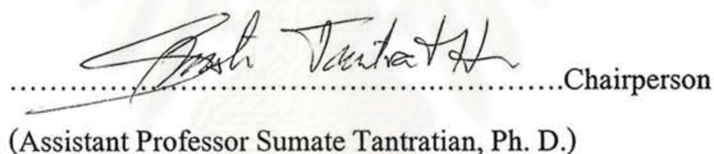

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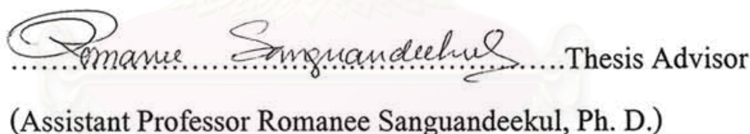

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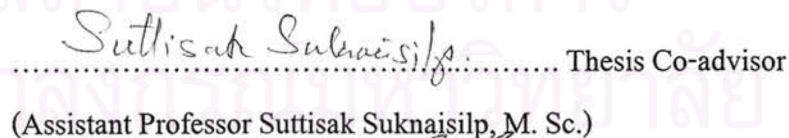
(Assistant Professor Romanee Sanguandeekul, Ph. D.)

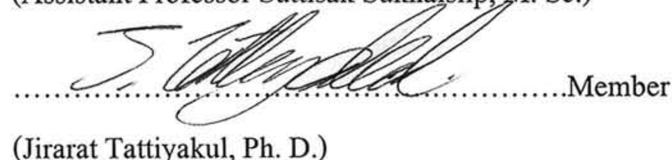
.... Thesis Co-advisor

(Associate Professor Somboon Tanasupawat, Ph. D.)



.... Thesis Co-advisor

(Assistant Professor Suttisak Suknaisilp, M. Sc.)



....Member

(Jirarat Tattiyakul, Ph. D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

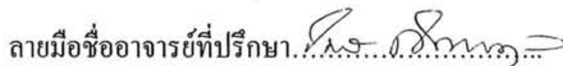
นิตยา เมธาวณิชพงศ์: การคัดเลือกโพรไบโอติกแบคทีเรียเพื่อใช้ในผลิตภัณฑ์นมหมัก
(SELECTION OF PROBIOTIC BACTERIA FOR USE IN FERMENTED MILK PRODUCT)
อ. ที่ปรึกษา: ผศ. ดร. รมณี สงวนดีกุล, อ. ที่ปรึกษาร่วม: รศ. ดร. สมบูรณ์ ธนาศุภวัฒน์,
ผศ. สุทธิศักดิ์ สุขในศิลป์: จำนวน 128 หน้า. ISBN: 974-53-2490-6

การแยกแบคทีเรียกรดแลคติกที่เป็นโพรไบโอติกจากอุจจาระทารก 11 ตัวอย่าง ลำไส้สุกร 13 ตัวอย่าง และลำไส้ไก่ 15 ตัวอย่าง ได้แบคทีเรียจำนวนรวม 379 สายพันธุ์ ผลการตรวจสอบลักษณะทางฟีโนไทป์เบื้องต้น พบว่า เป็น *Lactobacillus* จำนวน 216 สายพันธุ์ *Enterococcus* 122 สายพันธุ์ และแบคทีเรียอื่นๆ ที่ยังไม่จำแนกชนิด จำนวน 41 สายพันธุ์ พบแบคทีเรียที่สามารถทนกรดที่ pH 2.5 และทนน้ำดี 0.3% จำนวน 29 สายพันธุ์ และพบว่า IF2-8, P2-10 และ CK8-11 จากตัวอย่างทารก สุกร และไก่ มีศักยภาพเป็นโพรไบโอติก โดย IF2-8 ทนกรดได้สูงสุดที่ 96.4% และสามารถยับยั้ง *E. faecium, Y. enterocolitica* และ *B. cereus* จากผลลักษณะทางฟีโนไทป์และความคล้ายคลึงของการวิเคราะห์ลำดับเบสในช่วง 16S rDNA พบว่า IF2-8, P2-10 และ CK8-11 มีความคล้ายคลึงกับ *L. gasseri*, *L. johnsonii* และ *L. salivarius* ที่ 99.91, 99.53 และ 99.91% ตามลำดับ และพบว่า P12-3, IF7-5 และ IF8-1 มีความคล้ายคลึงกับ *L. amylovorus*, *E. raffinosus* และ *E. faecalis* ที่ 99.53, 99.75 และ 99.83% ตามลำดับ หลังจากนั้นนำแบคทีเรียที่มีศักยภาพเป็นโพรไบโอติกมาศึกษาการผลิตนมหมัก โดยแบ่งเป็น 3 ชุด ชุดแรกมี 3 ผลิตภัณฑ์ใช้ IF2-8, P2-10 และ CK8-11ในแต่ละผลิตภัณฑ์ ชุดที่สองใช้โพรไบโอติกผสมกับ *S. thermophilus* แบ่งเป็น 3 ผลิตภัณฑ์ คือ IF2-8 ผสม *S. thermophilus*, P2-10 ผสม *S. thermophilus* และ CK8-11 ผสม *S. thermophilus* ชุดสุดท้ายเป็นชุดควบคุม ใช้จุลินทรีย์โยเกิร์ต (*S. thermophilus* และ *L. delbrueckii* subsp. *bulgaricus*) บ่มนมหมักที่ 42 $^{\circ}$C เป็นเวลา 24 ชั่วโมง และเก็บที่ 5 $^{\circ}$C เป็นเวลา 3 สัปดาห์ หลังจากหมักเสร็จ พบว่า IF2-8 อยู่รอดได้สูงสุด (73.0-88.4%) ทั้งในนมหมักชุดที่ใช้เพียงโพรไบโอติกและชุดที่ใช้โพรไบโอติกร่วมกับ *S. thermophilus* นอกจากนี้นมหมักที่ใช้ IF2-8 ร่วมกับ *S. thermophilus* ยังทำให้ IF2-8 มีจำนวนเซลล์สูงขึ้น 2 log cycle เมื่อเทียบกับนมหมักที่ใช้ IF2-8 เพียงชนิดเดียว สำหรับการสร้างกรดของนมหมักชุดควบคุม, ชุดที่ใช้เพียง IF2-8 และชุดที่ใช้ *S. thermophilus* ร่วมด้วย มีค่าเป็น 1.54, 0.62 และ 1.15% ส่วนความหนืดมีค่า 488, 256 และ 601 cPs ตามลำดับ ซึ่งพบว่าชุดที่ใช้เพียง IF2-8 มีการสร้างกรดและความหนืดต่ำกว่าชุดควบคุมมาก ส่วนชุดที่ใช้ *S. thermophilus* ร่วมด้วย มีการสร้างกรดและความหนืดไม่แตกต่างจากชุดควบคุม ดังนั้นแสดงให้เห็นว่าไม่ควรใช้ IF2-8 เพียงชนิดเดียวในการผลิตนมหมัก แต่สามารถใช้ร่วมกับ *S. thermophilus* ในผลิตภัณฑ์นมหมักได้

สาขาวิชา เทคโนโลยีชีวภาพ

ปีการศึกษา 2548

ลายมือชื่อนิสิต.......................................

ลายมือชื่ออาจารย์ที่ปรึกษา.............................


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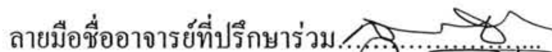

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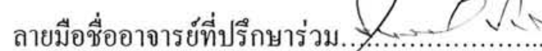

4672302023: MAJOR BIOTECHNOLOGY

KEY WORDS: PROBIOTIC BACTERIA / INFANT / PIG / CHICKEN / FERMENTED MILK

NITTAYA METHAWANITPONG: SELECTION OF PROBIOTIC BACTERIA FOR USE IN FERMENTED MILK PROCUCT. THESIS ADVISOR: ASST. PROF. ROMANEE SANGUANDEEKUL, PH. D., THESIS COADVISOR: ASSOC. PROF. SOMBOON TANASUPAWAT, PH. D. AND ASST. PROF. SUTTISAK SUKNAISILP, M. Sc. 128 pp.

ISBN: 974-53-2490-6

Isolation of probiotic lactic acid bacteria for use in fermented milk product was carried on infant faeces, pig intestine and chickens intestine. Three hundred and seventy-nine isolates were isolated from 11 of infant faeces, 13 of pig intestine, and 15 of chicken intestine. Based on their phenotypic characteristics, 216 isolates were belonged to *Lactobacillus*, 122 isolates were *Enterococcus* and the other 41 isolates were unidentified. Twenty-nine isolates were acid- and bile-resistant at pH 2.5 and 0.3% bile salt, respectively. The potentially probiotic isolates IF2-8, P2-10 and CK8-11 were selected from infant, pig and chicken, respectively. IF2-8 was the best acid-resistant isolate at survival rate 96.4% and could inhibit *E. faecium, Y. enterocolitica* and *B. cereus*. Based on their phenotypic characteristics and 16S rDNA sequence similarity, IF2-8, P2-10, and CK8-11 were closely related to *L.gasseri*, *L. johnsonii*, and *L. salivarius* with 99.91, 99.53 and 99.91%, respectively. Furthermore the other strains P12-3, IF7-5, and IF8-1 were closely related to *L. amylovorus, E. raffinosus*, and *E. faecalis* with 99.53, 99.75 and 99.83%, respectively. Later, the three potential probiotic strains were used for fermented milk trial which divided into 3 sets, the first was inoculated with IF2-8, P2-10, and CK8-11 culture, while the second was inoculated with the mixed culture of each probiotic culture and *S. thermophilus*, and the last was the control that was inoculated with yoghurt starter bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. These products were incubated at $42^{\circ}C$ for 24 h and stored at $5^{\circ}C$ for 3 weeks. Upon the completion of incubation, IF2-8 had greatest survival (73.0-88.4%) in both fermented milk used only probiotic and fermented milk used probiotic and *S. thermophilus*. Furthermore, fermented milk used IF2-8 mixed with *S. thermophilus* resulted increasing in viability of IF2-8 (2 log cycles) compared to fermented milk that used only IF2-8. For acid production, the control fermented milk, only IF2-8 product and IF2-8 mixed with *S. thermophilus* product were 1.54, 0.62 and 1.15%, respectively. Their viscosity were 488,256 and 601 cPs, respectively. Fermented milk used only IF2-8 was found that acid production and viscosity much lower than the control, whereas fermented milk mixed with *S. thermophilus* produced acid not different from the control. Therefore, this results indicated that IF2-8 should be applied along with *S. thermophilus* in fermented milk but it could not be used as starter culture alone in fermented milk.

Field of study Biotechnology

Academic year 2005

Student's signature Nittaya Methawanitpong

Advisor's signature Romanee Sanguandeekul

Co-Advisor's signature Somboon Tanasupawat

Co-Advisor's signature Suttisak Suknaisilp

ACKNOWLEDGEMENTS

I wish to express sincere thanks and gratitude to my thesis advisor, Assistant Professor Dr. Romanee Sanguandeekul and my thesis co-advisor, Associate Professor Dr. Somboon Tanasupawat, and Assistant Professor Suttisak Suknaisilp for their tireless efforts as well as valuable advice and comments throughout the course of this study.

I would also like to thank Assistant Professor Dr. Sumate Tantratian for serving as the thesis committee chairperson and Dr. Jirarat Tattiyakul for serving as thesis committee members and their recommendations for the research.

Special thanks are given to all friends, and all staff members in the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for their help, kindness and friendship during my study.

Finally, the most important, is my sincere and deepest gratitude to my parents and everyone in my family for their great love, constant support, understanding and heartfelt encouragement extended throughout my study.

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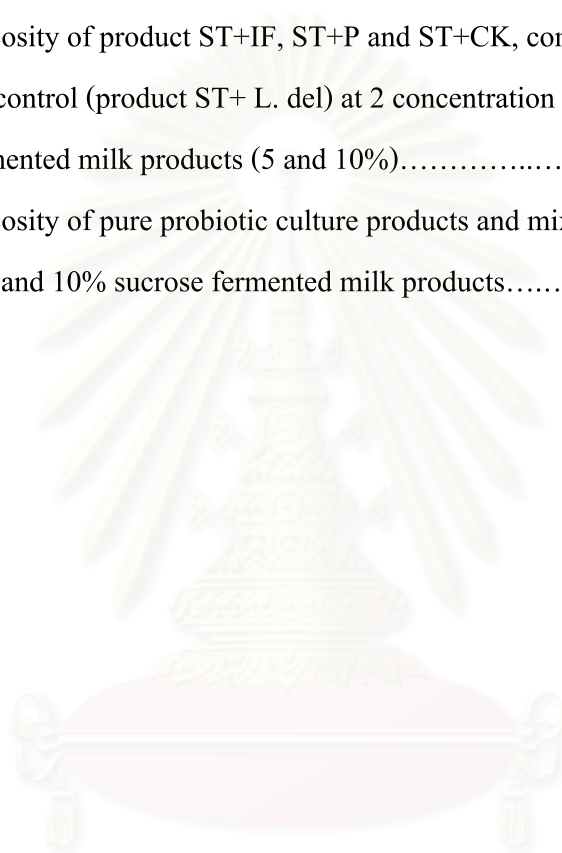

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ABBREVIATIONS

Abbreviations or symbols	Term
%	percent
°C	degree centigrade or Celsius
μg	microgram
N	normality
M	molar
(m, μ) l	(milli, micro) liter
(m, μ) mol	(milli, micro) mole
v/v	volume by volume
v/w	volume by weight
w/v	weight by volume
cps	centipoise
h	hour
cfu	colony forming unit
mm	millimeter
nm	nanometer
rpm	round per minute
sp.	species
min	minute
g/l	gram per liter
PCR	polymerase chain reaction
ATCC	American Type Culture Collection
NRIC	Nadai Research Institute Culture Collection
DSM	Deutsche Sammlung von Mikroorganisms
JCM	Japan Collection of Microorganisms

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Abbreviations or symbols	**Term**
NCIMB	National Collection of Industrial Food and Marine Bacteria
NCDO	National Collection of Dairy Organisms
LMG	Universiteit Gent, Laboratorium voor Mikrobiologic, Gent, Belgium

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CHAPTER I

INTRODUCTION

1.1 Significance of the study

During recent years, an increasing interest has developed in foods that contribute to a positive effect on health beyond their nutritional value. Among these functional foods, much attention has been focused on probiotic products. Probiotic foods contain microorganisms or components of microbial cells that have a beneficial effect on the health and well-being of the consumer host (Salminen *et al*., 1999). The concept of ingesting live microorganisms for the purpose of improving our intestinal health and general well-being can be traced to the beginning of the 20th century (Metchnikoff, 1907; O'Sullivan, 2001). This practice is now refered to as "probiotic" is the subject of intense scientific research directed toward obtaining effective probiotic bacteria and establishing their health benefits. The market for probiotic culture is very significant in Asia, particularly Japan, and has attained significance in Europe during the past decade. The probiotic market is smaller in the United States of America, but it is growing and has tremendous growth potential if the strain is obtained with the required scientific evidence that allows the U.S. Food and Drug Asministration (FDA) to permit specific health claims.

Lactic acid bacteria strains are the major representatives of probiotics, they are considered as GRAS organisms that are safe to consume and have a long history of use in food (Bredholt, Nesbakken and Holck, 2001). During the past two decades, probiotic microorganisms have been increasingly included in various types of food products, especially in fermented milk. Fermented milk products containing viable probiotic bacteria have been used by humans primarily as a prophylactic and their use has been extended for treatment of intestinal infections. Some workers (Alm, 1983; Zychowiez *et al*., 1974) have suggested the use of probiotic to prevent and treat diarrhea induced by *Salmonella* or *Shigella*. Thus, in recent years studies on the lactic acid bacteria have been emphasized and

may play an important role in improving the intestinal flora (Itoh *et al*., 1995) and protecting the host against colonisation of the intestinal tract by non-indigenous microorganisms (Mital and Garg, 1992). In order for a probiotic strain to exert its beneficial effect on the host, it has to be able to survive from the passage through the host's digestive tract. So far, researches have mainly focused on strains sensitivity towards low pH and bile salts (Conway, Gorbach and Goldin, 1987; Charteris *et al*., 1998a; Du Toit *et al*.,1998; Jacobsen *et al*.,1999).

Large populations of lactic acid bacteria inhabit the proximal regions of the digestive tracts of mammalian species and fowl (Arici *et al*.,2004). In this study lactic acid bacteria strains were isolated from infant faeces, pig intestine and chicken intestine and tested for probiotic potential in order to obtain local strains of probiotic that can be used in Thai fermented milk product.

1.2 Research objectives

1. Isolate and identify lactic acid producing bacteria by their phenotypic characteristics and 16S rDNA sequencing.
2. Determine the bile and acid tolerance, and antibacterial activity to select potencially probiotic strain.
3. Produce fermented milk with probiotic bacteria and evaluate the survival of probiotic bacteria in fermented milk during fermentation and refrigerated storage.

1.3 Scope and limitation of the study

This study was aimed to isolate the tolerance of low pH and bile salts of potentially probiotic lactic acid bacteria from infant, pig and chicken. The isolates selected were identified by both phenotypic and molecular method. These isolates were subsequently used in milk fermentation.

1.4 Expected results

Since probiotic bacteria that was used in fermented milk in Thailand was imported, in this study, the isolated probiotic lactic acid bacteria will be obtained from different sources and preserved for future study. In addition, they be applied in the production of fermented milk or various types of dietary supplement for human and animal.

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CHAPTER II

LITERATURE REVIEW

2.1 Definition of probiotics

The word probiotic is derived from the Greek meaning ‘for life’ and the concept of “probiotics”, like many other revolutionary ideas, appeared long before a neologism was proposed to describe it. At the beginning of the 20th century, Nobel Prize-Winning Russian scientist Elie Metchnikoff provided a through description of the concept based on the importance of the intestinal microflora on the general health status of the human body (Metchnikoff, 1907). Lilly and Stillwell (1965) first used the word “probiotic” to describe substances secreted by one protozoan to stimulate the growth of another. Sperti (1971) used it to describe tissue extracts that stimulated microbial growth and Parker (1974) used it to describe animal feed supplement, including organism and substances that had a beneficial effection an animal by contributing to its intestinal flora balance. Parker’s definition included antibiotics-used to promote the growth of farm animals. In 1989, Fuller (1989) defined a probiotic as a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance. This definition stressed the importance of viable cells as a component of an effective probiotic and excluded antibiotics. Huis in’t Veld and Havenaar (1991) definied probiotics as ‘a mono–or mixed culture of live microorganism which when applied to man or animal affects the host beneficially by improving the properties of the indigenous microflora’. This definition developed the concept of probiotics in several ways. It did not restrict probiotic activity to the gut microflora but included the possibility of application to microbial communities at other sites, e.g. respiratory tract, urogenital tract, skin. The probiotic may consist of a mono-culture of a cocktail of cultures and it also introduced the concept of human use. Recently an EC supported group of European scienticts, suggested that probiotics for use in human nutrition are best defined as live microbial food ingredients that are beneficial to health

(Salminen *et al*., 1998). This definition takes into account results from recent research, which demonstrated non-microflora mediated probiotic effects, e.g. probiotic effects on immune parameters.

2.2 Lactic acid bacteria

According to Salminen and Wright (1998), lactic acid bacteria are Gram positive; cocci or rods; anaerobic, microaerophilic, or aero-tolerant; and catalase negative. They produce lactic acid as the major end product during the fermentation of carbohydrate. The genera of lactic acid bacteria are *Aerococcus, Alloiococcus, Bifidobacterium, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagacoccus,* and *Weissella*. The classification of lactic acid bacteria into different genera is largely based on their morphology, growth at different temperatures, configuration of the lactic acid produced, ability to grow at high salt concenrtations, and acid or alkaline tolerance. They are mesophilic. Some can grow below 5 $^{\circ}$C and some as high as 45 $^{\circ}$C, with respect to growth pH, some can grow as low as 3.2, some as high as 9.6, and most grow in the pH range 4.0-4.5 depending on the species, they synthesize either the L(+) or D(-) isomer of lactic acid or both. Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Embden-Meyerhof pathway (Glycolysis) results in almost exclusively lactic acid as end-product under standard conditions, and the metabolism is refered to as homolactic fermentation. The homofermenters are *Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and some species of *Lactobacillus*. In the case of heterolactic fermentatiion, bifidum pathway used by *Bifidobacterium*, and 6-phosphogluconate/ phosphoketolase pathway used by *Leuconostoc, Oenococcus, Weissella*, and some species of *Lactobacillus*, are mainly sugar fermentation pathway that results in significant amounts of other end-products such as ethanol, acetate, and carbon dioxide in addition to lactic acid.

Lactic acid bacteria can produce a variety of antimicrobial compounds, which provided these organisms with a competitive advantage over other microorganisms. The

antimicrobial compounds, include lactic acid, acetic acid, hydrogen peroxide, carbon dioxide, diacetyl, as well as bacteriocins (Gould, 1995; Herbin, *et al*., 1997; Mishra and Lambert, 1996; Lee and Paik, 2001). These inhibitory compounds have differences in antimicrobial properties. The pH reduction by lactic acid or acetic acid has effect to cellular metabolism, with retardation of the growth of several contaminated microorganisms (Hill, Driscoll, and Booth, 1995). The antimicrobial effects of hydrogen peroxide resulted from the oxidation of sulfhydryl groups causing denaturing of enzymes that are able to destroy many pathogens. The hydrogen peroxide may also be as a precursor for the production of free radicals, which can damage DNA of other microorganisms (Mishra and Lambert, 1996). Carbondioxide may exert its antimicrobial effect in several ways such as by rendering the environment more anaerobic, inhibiting the enzymatic decarboxylation, and disrupting the cell membrane with the accumulation of the gaseous phase in the lipid bilayer (Eklund, 1984). Diacetyl inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization (Jay,1982; Jay, 1986). Antimicrobial activities of bacteriocins are the insertion and pore formation, the depolarization of the target membrane, and leading to the rapid efflux of low molecular weight compounds of the target cell (Gould, 1995; Mishra and Lambert, 1996).

2.3 The gastrointestinal (GI) tract of human

The gastrointestinal tract of human represents an ecosystem of the highest complexity. The muscosal surface provided a large area for the adherence to and microbial colonisation of the small intestine. When compared to capacity 2 m^2 skin surface of our body , the area of our GI system, calculated to be 150-200 m^2, is huge (Waldeck, 1990). A three-fold increase in the surface area is accomplished by circular fold, 7-10-fold by folding of the epithelium (intestinal villi) and 15-40-fold by the formation of microvilli in the enterocyte resorptive luminal membrane. Thereby the necessary space for interactions during the digestive process and for adhesion to the mucosal wall and concomitant colonisation is provided.

Inspite of rapid research advances in gut microbial ecology, our understanding of this complex ecosystem and the microbial interactions is still limited. The GI tract of the average human adult is colonised by approximately 10^{14} microbial cells (Luckey and Floch, 1972), about 10 times more than all tissue cells of the body taken together. This immense metabolic potential suggests strong regulatory effects on body functions, especially in the colon where the largest concentration of up to 5×10^{11} bacterial cells per gram is found. Representing more than 400 species, these "autochthonous" microorganisms include diverse bacterial genera, of which the Gram-positive, anaerobic genera *Bacteroides*, *Eubacterium* and *Bifidobacterium*, (Table 2.1) predominate in the densely populated large intestine. Other groups such as the *clostridia*, *peptostreptococci*, "*Streptococci*" and *lactobacillus* also seem to play an important role, e.g. in the maintenance of a stable gut mucosa, and in the generation of short chain fatty acid in a beneficial ratio. The numerically predominant genera of bacteria detected in the faeces of different individuals are the same, there is variation in the occurance and population size of bacterial species (Moore, Cato, and Holdeman, 1978)

Table 2.1 Bacterial genera that are commonly detected as components of the intestinal microflora of humans

Strain	Characteristics
Bacteroides	Gram-negative, non-spore-forming bacilli. Obligate anaerobes. Metabolic products include combinations of acetic, succinic, lactic, formic or propionic acids. If N-butyric acid is produced, isobutyric and isovaleric acids are also present.
Bifidobacterium	Gram-positive, non-spore-forming, nonmotile bacilli, sometimes with club-shaped of spatulated extremities. Obligate anaerobe. Acetic and lactic acid are produced primarily, in the molar ratio 3:2. Glucose is degraded exclusively and characteristically by the fructose-6-phosphate 'shunt' metabolic pathway.
Clostridium	Gram-postive bacilli that form endospores. Obligate anaerobes.
Enterococcus	Gram-positive cocci. Facultative anaerobes. Lancefield group D. Can grow in 6.5% NaCl broth and in normal broth at pH 9.6
Eubacterium	Gram-positive bacilli, non-spore-forming. Obligate anaerobes. Produce mixtures of organic acids including butyric, acetic and formic acids.

Table 2.1 Bacterial genera that are commonly detected as components of the intestinal microflora of humans (Continued)

Strain	Characteristics
Fusobacterium	Gram-negative, non-spore-forming bacilli. Obligate anaerobes. N-butyric acid is produced but isobutyric and isovaleric acids are not.
Peptostreptococcus	Gram-positive cocci. Obligate anaerobes. Can metabolize peptone and amino acids.
Ruminococcus	Gram-positive cocci. Obligate anaerobes. Amino acids and peptides are not fermented. Fermentation of carbohydrates produces acetic , succinic and lactic acids, ethanol, carbon dioxide and hydrogen.
Lactobacillus	Gram-positive bacilli, non-spore-forming bacilli. Grow best under anaerobic conditions. Lactic acid is a major product of glucose fermentation.
Escherichia coli	Gram-negative rods, facultatively anaerobic. Citrate not utilized. Carbohydrates fermented to lactic , acetic and formic acids. Part of formic acid is split by a complex hydrogenase system to give equal amounts of carbon dioxide and hydrogen. Lactose is fermented by most strains but fermentation can be delayed of absent. Motile by means of peritrichous flagella or nonmotile.

Source: Tannock (1995).

2.3.1 Intestinal lactobacilli of human

Lactobacilli are ubiquitous gram-positive rod-like bacteria (Kandler and Weiss, 1986) and are important group of lactic acid bacteria in intestine. Since they are acid-resistant, they easily pass the acidic stomach which (with a pH of 1-3) functions as a barrier for many bacteria. As acidophiles, lactobacilli are the first kind of microorganisms that can start growing (even before the enterococci) in the proximal small bowel, i.e., the acidic duodenum, and therefore they are the first microorganisms encountered in the GI tract (Bongaerts and Severijnen, 2001). In contrast to most other intestinal bacteria, such as intestinal streptococci and *Escherichia coli*, most lactobacilli are limited in depolymerising polysaccharides, as starch in food, and /or hydrolysing sugars from glycoproteins, e.g., the human intestinal mucins. In addition they prefer lactose, sucrose, and glucose as energy sources. As facultative anaerobes, these bacteria resist intestinal traces of molecular

oxygen, and gain only a small part of the energy content of the sugars, i.e., the energy released by enzymatic substrate phosphorylation in sugar fermentation. Due to the absence of adequate enzymes and cytochromes in these bacteria, most energy present in lactic acid and ethanol is inaccessible. Thus, lactobacilli ferment suitable sugars to either lactic acid, e.g., the homolactic *L. acidophilus*, or to lactic acid, ethanol and carbondioxide, e.g., the heterolactic *L. fermentum*. Because of the low energy yield they need enormous amounts of sugars for growth, and consequently, lactic adid is abundantly produced. And additional condition for optimal growth is the presence of amino acids in food (Kandler and Weiss, 1986).

2.3.2 The study in lactic acid bacteria from infants

Xanthopoulos, Litopoulou, and Tzanetakis (2000) isolated *lacobacillus* from new born infants in Greece. *L. paracasei* subsp. *paracasei* (six strains), *L. rhamnosus* (six strains), *L. acidophilus* (two strains), *L. gasseri* (three strains) and *L. reuteri* (three strains) isolates were tested for their ability to grow and metabolize in milk and to resist specific conditions of the GI tract. They found that many of the tested strains had desirable properties concerning their ability to withstand adverse conditons of the GI tract. In general, strains of *L. paracasei* subsp. *paracasei* and *L. rhamnosus* were more resistant to low pH of the stomach than all the other strains. Furthermore, *L. paracasei* subsp. *paracasei*, *L. acidophilus* and *L. rhamnosus* strains could be preferably used as starter to produce fermented milk with possibly interesting organoleptic properties, as well as dietary and possible therapertic importance.

Lee, Yu, and Heo (2003) identified and screened for antimicrobial activity against *Clostridium difficile* of *Bifidobacterium* and *Lactobacillus* species isolated from faeces of 32 healthy breast-fed Korean infants (aged 2-15 months), twelve of the 109 lactic acid bacteria showed activity against *C. difficile* and 19 strains were active against *E. coli* 0157:H7, but none against *S. aureus*. Four strains had antimicrobial activity against both *C. difficile* and *E. coli* 0157:H7. Of the 12 strains that had activity against *C. difficile*. Eight of 12 strains were identified as *B. infantis* and *L. salivarius*.

Arici *et al*. (2004) studied some characteristics of *Lactobacillus* isolated from faeces of newborn infants and children under 2 years of age. In this research, 21 strains of lactobacilli were isolated from these samples i.e., *L. rhamnosus* (seven strains), *L. paracasei* subsp. *paracasei* (four strain), *L. fermentum* (four strains), *L. buchneri* (two strains), *L. brevis* (one strain), *L curvatus* (one strain) and *Lactobacillus* sp. (two strains) were analysed for acid production, antibiotic resistances, H_2S productions and antimicrobial activities. Inhibition activity of bacteriocin and/or bacteriocin-like substances of the lactobacilli against some food contaminants and pathogenic bacteria (*E. coli* ATCC 25922, *S. aureus* ATCC 28213, *S. aureus* ATCC 2392, *Y. enterocolitica* and *B. cereus*) were determined by the agar diffustion method.

Ahrne *et al*. (2005) studied lactobacilli in the intestinal of 112 Swedish infants that age ranges from 1-8 weeks and 6-18 months. Lactobacilli reached a peak at 6 months when 45% of the infants were colonised, *L. rhamnosus* and *L. gasseri* were the most common species in this period. *Lactobacillus* isolation reached a nadir of 17% by 12 months but increased to 31% by 18 months of age. Moreover, the results suggest that certain *Lactobacillus* species, especially *L. rhamnosus*, thrive in the intestinal flora of breast-fed infants. After weaning they are replaced by other *Lactobacillus* species of types found in food.

Rinne *et al*. (2005) studied the quantitative and qualitative difference of the gut microbiota in infants. They evaluated gut microbiota at the age of 6 months in 32 infants who were either exclusively breast-fed, formula-fed, nursed by a formula supplemented with prebiotics or breast-fed by mothers who had been given probiotics. Total number of bifidobacteria was lower among the formula-fed group than in other groups (p=0.04). besides, the specific *Bifidobacterium* microbiota composition of the breast-fed infants was achived in infants receiving prebiotic supplemented formula.

Bello and Hertel (2005) isolated lactobacilli from saliva and faecal samples from 3 healthy subjects (male) aged 27-31 years. The results revealed that the species composition of the *lactobacillus* biota of human saliva and faeces was found to be subject-specific and fluctuated to some degree, but the species *L. gasseri*, *L. paracasei*, *L. rhamnosus* and

L. vaginalis were detected in saliva and faecal samples of individual subjects. Their result, together with recent published data (Bello *et al*., 2003; Munson *et al*., 2004) give strong evidence that some lactobacilli found in human faeces are allochthonous to the intestine and originate from the oral cavity.

2.4 The gastrointestinal tract of pig (Kidder and Manners,1978)

If probiotic preparations have to survive and be active in the digestive tract, they have to be suitable for that environment and resist the host's protective mechanisms which are inhibitory to microbes, for example, there are powerful stomach defense such as low pH and proteolytic enzymes (Table 2.2). The retention time as well as the degree of mixing of the ingested materials with the gastric juices and previous digesta also influence the survival of administered strains. In the anterior small intestine, the most important defence is the very fast flow rate of which prevents microbial overgrowth unless the microorganisms can be attached to the epithelium in this site. Among other factors, the presence of bile in this region also negatively influences survival and activity of the microbes. A relatively rapid transit time in the posterior small intestine also protects the host unless invading microbes and adhere to the epithelial mucosa. The caecum and large intestine the passage rate is lower and the microbes can establish, however, they must compete with a stable indigenous microflora in the healthy host. The extent of survival in the stomach, together with the volume of the digest found in the different parts of the digestive tract, influence the numbers of the probiotic organisms required for dosage.

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Table 2.2 pH values in the digestive tract of pigs

Age	Stomach	Small intestine		Caecum	Colon
		Anterior part	Posterior part		
Neonatal	4.0 – 5.9	6.4 – 6.8	6.3 – 6.7	6.7 – 7.7	6.6 – 7.2
Unweaned	3.0 – 4.4	6.0 – 6.9	6.0 – 6.8	6.8 – 7.5	6.5 – 7.4
Weaned	2.6 – 4.9	4.7 – 7.3	6.3 – 7.9	6.1 – 7.7	6.6 – 7.7
Adult	2.3 – 4.5	3.5 – 6.5	6.0 – 6.7	5.8 – 6.4	5.8 – 6.8

Source: Tannock (1992)

2.4.1 Indigenous lactic acid bacteria in pig

The pig is a monogastric animal in which the foregut (stomach and small intestine) is colonized by a relatively rich microflora. The flora is not as rich as in ruminants which have a specialized foregut fermentation system, but stomach contents still contains about 10^7–10^8 bacteria per gram of digesta. As the killing by low pH is not so great bacterial numbers found in the small intestine are generally also high, 10^7– 10^9. See Table 2.3, the microflora of the pig foregut is dominated by lactic acid bacteria, mostly *Lactobacillus* and *Streptococcus* spp. They are found both in the digesta and attached to the epithelia. The non-secreting pars oesophagea area in the stomach is densely colonized with layers of lactic acid bacteria (Fuller *et al.*, 1978). There is a difference in the composition of the microflora in the caecum and colon with Gram-negative organsims dominating the caecum (Robinson, Allison, and Bucklin, 1981) and Gram-positive bacteria dominating the colon (Salanitro, Blake, and Muirhead, 1977)

Several functions within the pig digestive tract enable the lactic acid bacteria microflora to be dominant. These include the fact that lactic fermentation in the stomach is facilitated by the relatively high stomach pH. Furthermore, food entering the stomach is inoculated with the indigenous lactic acid bacteria as the ingesta is mixed with gastric contents and continuous inoculation of lactic acid bacteria is ensured by the sloughing of parsoesophagea cells with attached lactic acid bacteria. The relatively high pH in the greater part of the stomach also means that the killing of lactic acid bacteria in the gastric

content is not so great, hence they will become a major component of the microflora in the small intestine.

The importance of lactic acid bacteria microflora in the foregut relates to physiological, microbiological and digestive functions. It helps the young pig to lower the pH in the stomach by the production of lactic acid and other organic acids formed mainly from lactose in the sow's milk (Cranwell, Noakes, and Hill, 1976). Both the organic acids and the low pH value in the pyloric antrum are important in decreasing the numbers of bacteria passing into the small intestine (Smith, 1965).

Species often found are *Lactobacillus acidophilus, L. delbrueckii, L. fermentum, L. reuteri, L. salivarius* and *Enterococcus bovis, E. durans, E. faecalis, E. faecium, Streptococcus intestinalis, S. porcinus* and *S. salivarius* (Jonsson and Conway, 1992). Bifidobacteria detected from the digesta are *Bifidobacterium adolescentis* and *B. suis*.

Table 2.3 Distribution of members of the normal microfolra in the digestive tract of pigs

Organ	Bacteria	Population level[a]
Crop	Lactobacilli	10^9
	Streptococci	10^6
	E. coli	10^2
	Yeasts	10^4
Small bowel	Lactobacilli	10^7
	Streptococci	10^4
	E. coli	10^4
	Yeasts	10^4
Large bowel	Lactobacilli	10^9
	Streptococci	10^7
	E. coli	10^7
	Yeasts	10^4
	Obligate anaerobes[b]	10^{10}

[a] CFU g^{-1} of organ contents (wet weight).

[b] *Eubacterium, Clostridium, Propionibacterium, Peptostreptococcus, Peptococcus, Megasphaera, and Bacteroides* species.

Source : Tannock (1992)

2.4.2 The study in lactic acid bacteria from pig

Russell (1979) isolated and identified anaerobic bacteria in the large intestine of pigs, using strictly anaerobic culture method. It was found that over 90% of the bacteria isolated were gram-positive and consisted mainly of gram-positive cocci, lactobacilli, eubacteria, and clostridia. Of 192 isolates recovered, only 124 could be assigned to recognized species.

Nemcova *et al.* (1997) isolated 14 strains of lactobacilli from the gut of suckling pigs and determine their susceptibility to antimicrobial feed additives, acid tolerance, adherance to epithelial cells from the porcine intestine and antimicrobial activity. Four strains were identified *as L. casei* subsp. *casei*, two strains as *L. rhamnosus* as well as *L. reuteri* and three strains as *L. salivarius*. The tested lactobacilli were acid resistant at pH 3 and they showed inhibitory activity against indicator bacteria in the presence of glucose.

Gusils, Bujazha, and Gonzalez (2002) isolated, characterized and further selected beneficial *lactobacillus* strains for the elaboration of a pig probiotic feed. One-hundred strains of lactic acid bacteria isolated from the GI tract of pigs only six, four identified as *Enterococcus faecium* and two as *L. acidophilus*, showed inhibition against enteric indicator strains: *Salmonella enteritidis*, *S. cholera* suis, *S. typhimurium* and *Y. enterocolitica*. The selected strains were resistant to pH 3.0 and bile salts. These strains fulfil the conditions of probiotic bacteria and could be seclected for elaborating pig probiotic feed, in order to prevent infectious diseases.

Rodriguez *et al.* (2003) determined the production of reuterin by lactobacilli isolates from pig faeces and evaluated their potential as probiotic bacteria. The results showed that 28 of 165 lactobacilli isolates produced reuterin in the presence of glycerol. Six isolates were identified as *L. reuteri*. They were able to survive at pH 3 and subsequent exposure to cholic acid or oxgall, and presented bile salt hydrolase and bacteriocin-like activities.

Yin and Zheng (2005) isolated and identified the dominant Lactobacilli species in gut and faeces of 9 adult pigs using carbohydrate fermentation and 16s rDNA analysis. The results showed that 52 lactobacilli-like colonies were selected from 387 on the basis of

their shape and Gram staining, and the fermentation of 11 carbohydrates, from which 12 lactobacilli were selected for 16S rDNA analysis. The results showed that *L. ruminis* was the dominant *Lactobacillus* in the stomach, small intestine, large intestine and faeces of pigs.

2.5 The gastrointestinal tract of chicken (Barrow,1992)

The gut microflora of poultry is complex and the interactions between different types of organisms are very complicated. Despite the fact that the flora can be subdivided most conveniently according to the area of the alimentary tract involved it must be remembered that the flora is almost continuous throughout the length of the gut. Microorganisms from the crop which survive the low pH of the gizzard generally multiply in the small intestine (Table 2.4). Organisms from this organ may be taken into the caeca. The microbial content of the cloaca and faeces depends on whether they contain material from the small intestine or from the caeca. Caecal droppings are discharged two to four times every day. The predominant organisms are lactobacilli (Table 2.5) producing mainly lactic and acetic acids such that the crop contents. The pH of the healthy chicken is 4-5 with the result that less aciduric organisms do not normally grow to the same high numbers. A number of metabolic types have been isolated and characterized including *L. salivarius, L. fermentum* and a type resembling *L. acidophilus.*

The caeca are filled with a thick viscous fluid containing no food particles. These organs have the highest viable counts (bacterial counts of 10^{11} g^{-1} of contents) and most complex microflora exist. Smith (1965) attributed this to the slow rate of flow, the kinetics of bacterial growth resembling batch culture. Most of the microorganisms present are obligate anaerobes, more than 200 strains present in the highest dilutions of caecal samples from chickens of more than 4 weeks of age. Gram-positive, anaerobic cocci, including peptosteptococci, comprise up to 30% of the total viable count. Other major components include gram-negative, non-sporing rods (20% of the total) such as the *Bacteriodaceae*. This important group includes *Bacteroides hypermegas*, now reclassified as *Megamonas*, *Bact. microfusus* and many other types distinguished by morphology, biochemical activity

and fermentation products. Few of them can be assigned to known species. gram-positive, non-sporing rods, including several types of *Eubacterium*, comprise up to 16% of the total count. The budding bacterium, *Gemmiger formicalis*, and the budding cocci account for 10% of the total, present at 10^9 to 10^{10} g^{-1}. *Clostridium* sp. and *Bifidobacterium* including *B. gallinarum* are present at similar levels. Facultative anaerobes include *Enterobacteriaceae* such as *E. coli, Citrobacter, Salmonella, Proteus* and *Klebsiella* which are frequently present but in lower numbers. Smaller numbers of other organisms such as the aerobe, *Pseudomonas*, and yeasts may be found throughout the gut from time to time but are never persent in high numbers.

Table 2.4 pH values in the digestive tract of chickens

Position	pH
Crop	4.00 – 6.30
Proventiculus	3.17 – 4.80
Gizzard	2.50 – 4.74
Duodenum	5.70 – 6.00
Jejunum	5.80 – 5.90
Ileum	6.30 – 6.40
Rectum or colon	6.30 – 6.40
Ceca	5.70 – 8.40
Cloaca	5.40 – 8.40

Source : Sturkie, 1976

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Table 2.5 Distribution of members of the normal microflora in the digestive tract of fowl

Organ	Bacteria	Population level[a]
Crop	Lactobacilli	10^9
	Streptococci	10^4
	E. coli	10^2
Small bowel	Lactobacilli	10^8
	Streptococci	10^4
	E. coli	10^2
Large bowel	Lactobacilli	10^9
	Streptococci	10^7
	E. coli	10^6
	Yeasts	10^2
	Obligate anaerobes[b]	10^{10}

[a] CFU g^{-1} of organ contents (wet weight).

[b] Anaerobic cocci, *Eubacterium, Clostridium, Gemmiger, Fusobacterium and Bacteroides* species.

Source : Tannock (1992)

2.5.1 The study in lactic acid bacteria from chicken

Garriga *et al*. (1998) selected the lactobaciili for chicken probiotic adjuncts. During inhibitory activity screening 296 strains of lactic acid bacteria from the GI tract of chicks, 77 strains showed inhibition against enteric indicator strains (*Salmonella enteritidis* and *E. coli*). Eight strains identified as *L. salivarius* were selected for the following attributes: their ability to inhibit all the indicator strains; a high adhesion efficiency to the epithelial cell of chickens and aslo their resistance to a number of antibiotics, monensin, bile salts and pH 3. It was concluded that *L. salivarius* CTC2183 and *L. salivarius* CTC2197 were capable of becoming predominant over the indigenous flora in the incubated chicken feed mixture.

Gusils, Gonzalez, and Oliver (1999) isolated lactobacilli from chicken intestines and tested for their probiotic properties. *L. fermentum* subsp. *cellobiosus*, *L. fermentum* and *L. animalis* were isolated and indicated that these strains were able to retain their beneficial characteristics in the presence *Salmonella gallinarum* such as presence of lectins, production of antimicrobial compounds, and ability to grow and compete. The selected microorganisms can be considered as potential ingredients for a chicken probiotic feed formulation intended to control salmonellosis and also improve poultry sanitation.

Reque *et al*. (2000) isolated and identified microorganisms for probiotic use in chickens. The strains were isolated from the crop, proventriculus, gizzard, ileum and caeca of chicken. Selection of strains included various criteria such as viability during storage, tolerance to low pH/gastric juice, bile and antimicrobial activity. The identification of the culture was based on characteristics of lactobacilli, carry out morphology, gram-stain, growth at 15 and 45 $^{\circ}$C and fermentation of different carbon sources. Base on these criteria *L. fermentum* LPB was identified and test for probiotic use for chickens.

Miyamota *et al*. (2000) isolated *Lactobacillus* flora in the cloaca and vagina of 40 normal laying hens and investigated their ability to inhibit growth of *S. enteritidis* using a spot-the-lawn technique. In the cloaca, *L. acidophilus* was isolated from 92.5% of hens, and *L. salivarius* was isolated from 85% of hens, whereas *L. fermentum* was isolated from only one hen. In the vagina, *L. acidophilus* and *L. salivarius* were isolated from 42.5% of hens. In the inhibition assay *in vitro*, all strains of *Lactobacillus* from cloaca and vagina inhibited growth of *S. enteritidis*.

Ehrmann *et al*. (2002) studied 112 strains of lactic acid bacteria of duck origin for their use as a probiotic feed supplement in poultry. *In vitro* studied included aggregation, co-aggregation, cell surface hydrophobicity and adhesion activities on poultry crop cells and human Hep 2-cells. Additionally, growth with bile acids and tolerance to acidic pH were tested. Among all the isolates, two strains of *L. animalis* TMW 1.972 and *L. salivarius* TMW 1.992 were selected for a survival test in poultry. The results indicate that two strains of lactobacilli exhibited strong potentials as probiotic adjuncts.

Lu *et al*. (2003) studied on bacterial community succession in the ileal and cecal ecosystems of broiler chickens by fedding a vegetarian corn-soy broiler diet devoid of feed additives and examined by analysis of 1,230 partial 16S rRNA gene sequences. The results revealed that nearly 70% of sequences from the ileum were related to those of *Lactobacillus* i.e., *L. acidophilus, L. crispatus, L. reuteri, L. delbrueckii, L. salivarius* and *L. gasseri*, with the majority of the rest being related to *Clostridiaceae*(11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%). In contrast, *Clostridiaceae*-related sequences (65%) were the most abundant group detected in the cecum, with the other most abundant sequence being related to *Fusobacterium* (14%), *Lactobacillus* (8%) i.e. *L. acidophilus*, *L. Crispatus, L. delbrueckii*, *L. reuteri* and *L. aviarius,* and *Bacteroides* (5%)

2.6 Choice of strains for use as probiotics

Lactic acid producing bacteria are common components of probiotics (Table 2.6). They are popular choices because of the historical belief that these bacteria are desirable member of the intestinal microflora, arising from the fact that lactic acid bacteria have long been used in the manufacture of dairy foods and are thus 'generally regarded as safe: GRAS' and because the consequent large-scale culture and preservation methods for lactic acid bacteria in a viable state have already been developed by the dairy industry. The choice of strains to be included in probiotic products has largely been decided on the basis of whether they are amenable to industrial handing and if they will remain viable for a suitable time in the prepared product. Selecting a preferable probiotic strain criteria, while over 20 criteria have been put forward, there is general agreement regarding the key selection criteria for probiotic bacteria for use in human foods (Huis in't and Shortt, 1996; Charteris *et al*., 1998b; Ouwehand *et al*.,1999; Mattila, Matto, and Saarela, 1999; Salminen, Isolauri, and Salminen, 1996):

1. Human origin.
2. Non-pathogenic
3. Acid and bile tolerant

Table 2.6 Microbial species from which strains find application in probiotic products

Lactobacillus species	*Bifidobacterium* species	Other Lactic acid bacteria	"Non-lactics"[a]
L. acidophilus	*B. adolescentis*	*Ent. faecalis*[b]	*Bacillus cereus* (toyoi ≅)[b]
L. amylovorus (L. casei)	*B. animalis*	*Ent. faecium*	*Escherichia coli Nissle,* 1917 ≅)
L. crispatus	*B. bifidum*	*Sporolactobacillus inulinus*[b]	*Propionibacterium freudenreichii*[b]
L. gallinarum[b]	*B. breve*		*Saccharomyces cerevisiae* (boulardii ≅)
L. gasseri	*B. infantis*		
L. johnsonii	*B. lactis*[c]		
L. paracasei	*B. longum*		
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

[a] Mainly as pharmaceutical preparations.

[b] Mainly applied for animals.

[c] Probably synonymous with *B. animalis*.

Source : Holzapfel *et al*. (1998)

4. Ability to withstand technological processes and remain viable during shelf-life period
5. Evidence of beneficial health effects.

The acid and bile tolerances are two fundamental properties that indicate the ability of a probiotic microorganism to survive the passage through the gastrointestinal tract, resitsting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Prasad *et al*., 1998; Hyronimus *et al*., 2000; Park *et al*., 2002).

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 (Erkkila and Petaja, 2000). After food ingestion, it takes 2-4 h for the stomach to empty. For those bacteria that survive the environmental conditions of the stomach, the further challenge is bile secretion and bile salts in the duodenum. The bile salts are released into the upper small intestine after ingestion of fatty meals and they have a detergent-like function. Since the cell membranes of microorganisms are composed of lipids and fatty acids. The bile salts are critical to them. However, some microorganisms are able to reduce

this detergent effect by their ability to hydrolyse bile salts by bile salt hydrolase enzyme (BSH) and thus to decrease their solubility (Erkkila and Petaja, 2000). BSH activity has been found in many genera including *Lactobacillus* (Gilliland and Speck, 1977b). Bile salts resistance varies a lot between the *Lactobacillus* species and also between strains, and the mechanism is still unknown (Erkkila and Petaja, 2000). Although the bile concentration of the GI tract varies, the mean intestinal bile concentration for the screening of a resistant probiotic strain is believed to be 0.3% w/v (Gilliland, Staley, and Bush, 1984).

2.7 Therapeutic value of probiotics

The claimed beneficial effects from consumption of fermented milks were once a very debatable issue. Research conducted since the turn of the century has however, enhanced the understanding of the resulting therapeutic effects and it is currently widely recognised as wholesome. The consumption of probiotic products is helpful in maintaining good health, restoring body vigour, and in combating intestinal and other disease orders (Mital and Garg, 1992). A list of the main therapeutic benefits attributed to consumption of probioitcs is indicated in Table 2.7. Most scientific papers refer to research using *L. acidophilus* and *Bifidobacterium* species as dietary cultures.

1. Control of intestinal infections

Probiotic bacteria such as bifidobacteria and lactobacilli posses antimicrobial properties (Hughes and Hoover, 1991). Both *L. acidophilus and B. bifidum* have been shown to be inhibitory towards many of the commonly known food borne pathogens (Gilliland and Speck, 1977a; Gilliland, 1979; Sandine, 1979; Rasic and Kurmann, 1983; Lim, Huh, and Back, 1993). Several studies indicated the preventative control of intestinal infections through administering milk cultured with *L. acidophilus* or *B. bifidum* or both (Rasic and Kurmann, 1983; Gorbach, Chang, and Goldin, 1987).

Table 2.7 Claimed beneficial effects and therapertic application of probiotic bacteria in humans

Beneficial effects :
Maintenance of normal intestinal microflora
Enhancement of the immune system
Reduction of lactose-intolerance
Reduction of serum cholesterol levels
Anticarcinogenic activity
Improved nutritional value of foods
Therapeutic applications :
Prevention of urogenital infection
Alleviation of constipation
Protection against traveller's diarrhoea
Prevention of infantile diarrhoea
Reduction of antibiotic-induced diarrhoea
Prevention of hypercholesterolaemia
Protection against colon/bladder cancer
Prevention of osteoporosis

Source : Fuller (1989)

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Mechanisms for the inhibition of pathogens ascribed to lactobacilli and bifidobacteria include :

- the production of inhibitory/antimicrobial substanced such as: organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids;
- their acting as competitive antagonists, i.e. competition for adhesion sites and nutrients;
- stimulation of the immune system.

Production of organic acids by the probiotics lowers the pH and alters the oxidation-reduction potential in the intestine, resulting in antimicrobial action. Combined with the limited oxygen content in the intestine, organic acids inhibit especially pathogenic Gram-negative bacteria types, e.g. coliform bacteria (Sandine, 1979). Bifidobacteria produce both lactic and acetic acids, but higher amounts of acetic acid are produced which exhibits a stronger antagonistic effect against gram negative bacteria than lactic acid (Rasic, 1983). Probiotic microorganisms may prevent harmful bacterial colonisation of a habitat by competing more effectively than an invading strain for essential nutrients or adhesion sites or by making the local environment unfavourable for the growth of the invader by producing antibacterial substanced (Sandine, 1979; Gurr, 1987). Regular consumption of probiotic bacteria may induce an improved immunological response in humans (Rasic, 1983).

2. Reducing lactose intolerance

The inability to digest lactose adequately by certain people is due to the absence of β–D-galactosidase in the human intestine and this leads to various degrees of abdominal discomfort (Kim and Gilliland, 1983). Some lactic acid bacteria used as starter cultures in milk and fermentation, and probiotic bacteria such as *L. acidophilus* and *B. bifidum* produce β–D-galactosidase. This enzyme hydrolyses lactose, which results in increased tolerance for dairy products (Kim and Gilliland, 1983). This utilisation is ascribed to intra-intestinal digestion by β–D-galactosidase. On the other hand, some lactic acid bacteria hydrolyse lactose by means of phospho-β-galactosidase, which may not be as effective in the intestine.

Kim and Gilliland (1983) investigated the effect of *L. acidophilus* as a dietary adjunct in milk to aid lactose digestion in humans. They found that improved digestion of lactose was not caused by hydrolysis of the lactose prior to consumption, indication that the beneficial effect must have occurred in the digestive tract after consumption of milk containing *L. acidophilus*. The continued utilisation of lactose within the GI tract depends on the survival of the lactobacilli in that environment.

3. Reduction in serum cholesterol levels

There are claims that consumption of fermented milk significantly reduces serum cholesterol (Mann and Spoerry, 1974; Gilliland, Nelson, and Maxwell, 1985; Gilliland, 1989). For hypercholesterolemic individuals. Significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks.

The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Claims are strong that certain *L. acidophilus* strains and some bifidobacteria species are able to lower cholesterol levels wihtin the intestine. Cholesterol co-precipitates with deconjugated bile salts as the pH declines as a consequence of lactic acid production by the lactic acid bacteria (Marshall, 1996). The role that bifidobacteria cultures may play in lowering serum cholesterol was lowered by feeding of bifidobacteria in a mechanism that may involve HMG-CoA reductase (Homma, 1988). In this respect Gilliland (1989) reports on various experiments that conclude that a factor is produced in the fermented milk that inhibits cholesterol synthesis in the body.

Another theory is that *L. acidophilus* deconjugates bile acids into free acids, which are excreted more rapidly from the intestinal tract than are conjugated bile acids. As free bile salts are excreted from the body, the synthesis of new bile acids from cholesterol can reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977b). A third hypothesis is that reduction of cholesterol may also be due to a co-precipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the bacteria (Kailasapathy and Rybka, 1997).

According to Marshall (1996) the deconjugation of bile acids can result in the formation of cytotoxic secondary bile salts. The net effect of the probiotic activity towards cholesterol control is therefore questionable.

4. Anticarcinogenic activity

The antitumour action of probiotic is attributed to the inhibition of carcinogens and/or procarcinogens, inhibition of bacteria that convert procarcinogens to carvinogens (Gilliland, 1989; Gorbach *et al.*,1987), activation of the host's immune system (Rasic, 1983) and /or reduction of the intestinal pH to reduce microbial activity.

Kailasapathy and Rybka (1997) reported on several animal studies confirming that the intake of yoghurt and fermented milks containing probiotic bacteria inhibited tumour formation and proliferation.

2.8 Fermented milk

2.8.1 Fermented milk / yoghurt as probiotic carrier food

Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotic microorganisms by which consumers can take in large anounts of probiotic cells for the therapeutic effect. The number of probiotic bacteria required to produce a beneficial effect, has not been established. Kurmann and Rasic (1991) suggested that to achieve optimal potential therapeutic effects, the number of probiotic organisms in a probiotic product should meet a suggested minimum of $>10^6$ cfu ml^{-1}. These numbers required, however, may vary from species to species, and even among strains within a species. Other authors stipulate $>10^7$ and 10^8 cfu ml^{-1} as satisfactory levels (Davis, Ashton, and McCaskill, 1971; Kailasapathy and Rybka, 1997). This criterion is refered to as the 'therapeutic minimum' in literature (Davis *et al.*, 1971; Rybka and Kailasapathy, 1995). One should aim to consume 10^8 live probiotic cells per day. Regular consumption of 400-500 g/week of AB-yoghurt (include *L. acidophilus* and *Bifidobacterium* sp.), containing

10^6 viable cells per ml would provide these numbers (Tamime, Marshall, and Robinson, 1995).

Ishibashi and Shimamura (1993) reported that the Fermented Milks and Lactic acid Bacteria Beverages Association of Japan has developed a standard which requires a minimum of 10^7 viable bifidobacteria cells/ml to be present in fresh dairy products. The criteria developed by the National Yoghurt Association (NYA) of the United States specifies 10^8 cfu g^{-1} of lactic acid bacteria at the time of manufacture, as a prerequisite to use the NYA 'Live and Active Culture' logo on the containers of products (Kailasapathy and Rybka, 1997). The Australian Food Standards Code regulations, requires that the lactic acid cultures used in the yoghurt fermentation must be present in a viable form in the final product, the populations are not specified. At the same time, attainment of pH 4.5 of below is also legally required to prevent the growth of any pathogenic contaminants (Micanel, Haynes, and Playne, 1997).

It has been claimed that only dairy products with viable microorganisms have beneficical health effects. However , in the case of lactose tolerance, treatment of acute gastro-enteritis and treatment of candidated, probiotics used showed the same beneficial effect in viable and non-viable form. Ouwehand and Salminen (1998) gives an overview on this.

Yoghurt has long been recognised as a product with many desirable effects for consumers, and it is also important that most consumers consider yoghurt to be 'healthy'. In recent years, there has been a significant increase in the popularity of yoghurt (Hamann and Marth, 1983) as a food product, accentuating the relevance of incorporation *L. acidophilus* and *B. bifidum* into yoghurt to add extra nutritional-physiological value. The conventional yoghurt starter bacteria, *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, lack the ability to survive passage through the intestinal tract and consequently do not play a role in the human gut (Gilliland, 1979).

2.8.2 Yoghurt production

Yoghurt is a fermented milk product that has been prepared traditionally by allowing milk to sour at 40-45 °C. Modern yoghurt production is a well-controlled process that utilises ingredients of milk, milk powder, sugar, fruit, flavours, colouring, emulsifiers, stabilisers, and specific pure cultures of lactic acid bacteria (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) to conduct the fermentation process.

S. thermophilus and *L. delbrueckii* subsp. *bulgaricus* exhibit a symbiotic relationship during the processing of yoghurt, with the ratio between the species changing constantly (Radke-Mitchell and Sandine, 1984). During fermentation, *S. thermophilus* grows quickly at first, utilizing essential amino acids produced by *L. delbrueckii* subsp. *bulgaricus*. *S. thermophilus*, in return, produces lactic acid, which reduces the pH to an optimal level of growth of *L. delbrueckii* subsp. *bulgaricus*. The lactic acid produced, and lesser amounts of formic acid stimulate the growth of *L. delbrueckii* subsp. *bulgaricus*. The streptococci are inhibited at pH values of 4.2-4.4, whereas lactobaclilli tolerate pH values in the range of 3.5-3.8. After approximately 3 h of fermentation, the numbers of the two organisms should be equal. With longer fermentation, the growth rate of *S. thermophilus* declines while *L. delbrueckii* subsp. *bulgaricus* continues to reduce the pH by producing excessive amounts of lactic acid. The pH of commercial yoghurt is usually in the range of 3.7-4.3 (Hamann and Marth, 1983). Although *S. thermophilus* forms acetaldehyde as a product of metabolism, the pathway is less active at normal fermentation temperatures compared to *L. delbrueckii* subsp. *bulgaricus* that produces acetaldehyde responsible for the characteristic sharp flavour (Davis *et al*., 1971).

2.8.3 Bio-yoghurt

In recent years some yoghurt products have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB-cultures) in addition to the conventional yoghurt organisms, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Therefore, bio-yoghurt is yoghurt that contains live probiotic microorganisms, the presence of which may give rise to claimed beneficial health effects.

2.8.4 Production of bio-yoghurt

For the production of bio-yoghurt, similar processing procedures to traditional yoghurt are applied with the exception of the incorporation of live probiotic starter cultures. Heat treated, homogenised milk with an increased protein content (3.6-3.8%) is inoculated with the conventional starter culture at 45 $^{\circ}$C or 37 $^{\circ}$C and incubated for 3.5 and 9 h, respectively (Anon, 1994). The probiotic culture can be added prior to fermentation simultaneously with the conventional yoghurt cultures of after fermentation to the cooled (4 $^{\circ}$C) product before packaging. The survival of probiotic bacteria in fermented dairy bio-products depends on such varied factors as the strains used, interaction between species present, culture conditions, chemical composition of the fermentation medium (e.g. carbohydrate source), final acidity, milk solids content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen (especially for *Bifidobacterium* sp.), level of inoculation, incubation temperature, fermentation time and storage temperature (Young and Nelson, 1978; Hamman and Marth, 1983; Kneifel, Jaros, and Erhard, 1993).

Shah and Lankaputhra (1997) investigated viability of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt. Five different types of yoghurt were prepared each containing yoghurt bacteria (*L. delbrueckii* subsp. *bulgaricus* 2515, *S. thermophilus* 2010), and probiotic bacteria (*L. acidophilus* 2409 and one species of *Bifidobacterium*; *B. longum* 1941, *B. pseudolongum* 20099, *B. infantis* 1912, *B. bifidum* 1900 or *B. bifidum* 1901). The yoghurt mix was incubated at 42 $^{\circ}$C until the pH reached 4.5 then stored for 6 weeks at 4 $^{\circ}$C. The results were found that all of *Bifidobacterium* spp. decreased during storage but remained above the recommended level of 10^6 cfu g^{-1} for 4 weeks, whereas the population of *L. acidophilus* decreased below this level after storage only two weeks.

Vinderola, Bailo, and Reinheimer (2000) evaluated the survival of lactic acid and intestinal probiotic bacteria in Argentinian commercial yoghurts during refrigerated storage. Yoghurt divided into 2 types, one type was reduce-fat (liquid), while the second type was full-fat (set) yoghurts. Probiotic bacteria (*B. bifidum* BBI and *L. acidophilus* LAI) were added in both 2 types of yoghurts. Samples were storaged at 5 $^{\circ}$C for upto 4 weeks. There

was a great variability in the survival ability of the probiotic cultures in the two yoghurt types. *L. acidophilus* LAI demonstrated, in general, a lower resistance to the yoghurt environment than *B. bifidum* BBI. On the other hand, the full-fat yoghurt was a more inhibitory medium than the reduced-fat one, especially for *B. bifidum* BBI. Ingeneral, pH values of 4.5 or lower jeopardised the cell viability of the probiotic organisms in yoghurt stored at 5 $^{\circ}$C.

Birollo, Reinheimer, and Vinderola (2000) studied viability of lactic acid microflora in different types of yoghurt during refrigerated storage. The lactic acid microflora viability was studied at storage temperatures of 6 $^{\circ}$C and 12 $^{\circ}$C. Cell viability depened on the yoghurt type and the storage temperature on the basis of a minimum value of 10^7 cfu g^{-1}, the shelf life of yoghurts at 6 $^{\circ}$C was longer than 60 days. Both the storage temperature and the yoghurt type should be taken into account when shelf life i.e. specificed on the basis of the lactic acid microflora content.

Oliveira *et al*. (2001) studied on acidification, textural properties, and micro biological stability of fermented milk containing probiotic bacteria. Two strains of probiotic bacteria; *L. acidophilus* (LA5) and *L. rhamnosus* (LC35), were used in pure culture, and in mixed culture with *S. thermophilus* (ST7). Acidifying activity was enhanced with mixed cultures, compared to pure cultures resulting in a shorter time to reach pH 4.5. The stability of probiotic bacteria in pure cultures were more stable than mixed cultures. The texture of the fermented products was not dependent on culture composition, but strong by dependent on milk supplementation. It was observed that all products containing probiotic counts over 2.2 x 10^7 cfu ml^{-1}.

Martin *et al*. (2003) develop a goat's milk fermented product (set-type style) of a satisfactory quality, in terms of sensory characteristics and survival of bacteria. Milk was fermented employing acommercial probiotic starter culture, which contained *S. thermophilus, L. acidophilus,* and *Bifidobacterium*. After 21 days of storage at 4 $^{\circ}$C, the results were found that the population counts being maintained at 10^8 cfu g^{-1} in all samples. All counts of *L. acidophilus* dropped under 10^6 cfu g^{-1}.

Krasaekoopt, Bhandari, and Deeth (2004) investigate the survivality of probiotics, *L. acidophilus* 547, *B. bifidum* ATCC1994, and *L. casei* 01, in two types of yoghurts : one prepared from conventionary treated milk and other prepared from UHT-treated milk with high total solids. After 3.5 h of fermentation, the products were kept at $4^{\circ}C$ for 4 weeks. The number of probiotic bacteria was maintained above the recommended therapeutic minimum (10^{7} cfu g^{-1}) throughout the storage except for *B. bifidum* which decreased below this level after 2 weeks. The viability of probiotic bacteria in yoghurts from both UHT and conventionally treated milks were not significantly ($P>0.05$) different.

Gueimonde *et al*. (2004) assessed the viability of *Lactobacillus* and *Bifidobacterium* strains included as probiotics in a variety of fermented milks commercialized in Spain. The viability of probiotic microorganisms was evaluated throughout the refrigerated storage of the product at $4^{\circ}C$ for 30 days. Counts of *Lactobacillus* spp. always remained higher than 10^{5} cfu ml^{-1}, whereas the population of *Bifidobacterium* spp. decreased below this level in two products.

Awaisheh, Haddadin, and Robinson (2005) evaluate the sensory qualities of the yoghurt and the viability of the probiotic species during storage at $4^{\circ}C$ The cultures employed to make the yoghurts were single probiotic strains of *L. gasseri* or *B. infantis* and, to achieve a short production time, a two-stage fermentation procedure was used with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* providing the rapid acidification. Yoghurt containing counts of $> 1.0 \times 10^{8}$ cfu ml^{-1} of the individual probiotics. Storage trials at $5^{\circ}C$ showed that the viability of the probiotic cultures was retained over 15 days.

Maragkoudakis *et al*.(2005) examine probiotic *Lactobacillus* strains (*L. plantarum* ACA-DC146 and *L. paracasei* subsp. *tolerans* ACA-DC4037) for their potential application in Greek yoghurt production as starters or starter adjuncts. The yoghurt produced was evaluated with respect to its microbiological, physicochemical and sensory properties. Both strains displayed low milk acidification activity, while no inhibition was observed towards or from the yoghurt starters used. Yoghurt produced with *L. paracasei* subsp. *tolerans* ACA-DC 4037 exhibited the best sensory properties, and the strain was selected for further trials. After 2 weeks of refrigerated storage, microbial loads (>7.0 log cfu g^{-1}) reached the level that accordance with international recommendation and guidelines for probiotic and starter cultures in milk products.

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CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Reagents (Analytical grade)

- Acetone: Merck, Germany
- Bromocresol purple: May & Baker, England
- Calcium carbonate: May & Baker, England
- Calcium chloride: Carlo Erba, Italy
- Chloroform: Mallinckrodt, Gernany
- Copper (II) sulfate pentahydrate: Sigma, USA
- D(-) lactate dehydrogenase from *L. leichmannii* : Boehringer Mannheim, Germany
- Di-Potassium hydrogen orthophosphate: Merck, Germany
- Di-Sodium hydrogen phosphate anhydrous: May & Baker, England
- Ethylene diamine tetraacetic acid (EDTA): Merck, Germany
- Ethanol: Carlo Erba, Italy
- Ferric chloride: Carlo Erba, Italy
- Ferric sulphate: Carlo Erba, Italy
- Glucose: Merck
- Glycine: Carlo Erba, Italy
- Hydrogenperoxide: Carlo Erba, Italy
- L-arginine monohydrochloride: Fluka, Switzerland
- L-cystein-hydrochloride monohydrate: Wako, Japan
- L-glutamic acid sodium salt: BDH, England
- L(+) Lactate dehydrogenase from rabbit muscle, Boehringer Mannheim, Germany
- Manganese sulfate tetrahydrate: Carlo Erba, Italy
- Magnesium chloride hexahydrate: Sigma, USA

- Magnesium sulfate: May & Baker, England
- Methanol: Merck, Germany
- Neutral red: May & Baker, England
- Phenol: Carlo Erba, Italy
- Phenol red: May & Baker, England
- Phenolpthalein: Merck, Germany
- Sodium chloride: Merck, Germany
- Sodium citrate: Merck, Germany
- Sodium hydroxide: Merck, Germany
- Tween 80: Carlo Erba, Italy
- Zinc sulphate heptahydrate: Carlo Erba, Italy

3.2 Instruments

- Analytical balance: Satorius, model BA 610, Germany
- Analytical balance: Satorius, model 518, Germany
- Autoclave: Hirayama, model HA-3D, Japan.
- Cellulose TLC plastic sheet Art.5577: Merck, Germany
- Deep freezer –20 °C : Kelvinator, model CFM209 P6W0, USA
- Hot air oven: Haraeus, model T5090E, Germany
- Incubator: Precision, model Thelco 6, USA
- Lyophilizer: FTSsymtem, model dura-dry μp, science engineer, Japan
- Microscope: Olympu,s model CHS, Japan
- Minicentrifuge : Shelton scientific, model VSMC-13, USA
- pH meter: Eutech, model Cyberscan500, Singapore
- Refrigerated centrifuges: Hitachi, model SCR20B, Japan
- Refrigerated incubator shaker: Innova, model 4230, USA
- Spectrophotometer: Shimadzu, model UV-160A, Japan
- Viscometer: Brookfield, model DV-I+ , Massachusetts, USA
- Vortex mixer: Scientific, model K-550-GE, USA
- Water bath: Thelco, model p/s, USA

3.3 Isolation and screening of lactic acid producing bacteria

3.3.1 Sample collection

Faecal samples were obtained from 11 healthy infants under 1 years of age; 5 samples were collected from nursery of Chulalongkorn University, 4 samples were collected from nursery of Kasetsart University, and 2 samples were collected from Soi Raewadee, Nonthaburi. Intestines of 13 adult pigs were collected from Nonthaburi market. Intestines of 15 adult chickens were collected from slaughterhouse, Phra-nung-kloa market, and Raewadee market in Nonthaburi province. Each sample was placed in a sterile container and was tightly close. The samples were stored at 4 $^{\circ}$C until processed or immediately examine.

3.3.2 Isolation and screening

A sample was divided into two parts. One part was isolated directly by diluting 1 g of sample in 9 ml Reduced physiological salt solution (RPS) (Hartemink and Rombouts, 1999) and then serially diluting to obtain a sensible dilution for plating. Three different dilutions were plated on MRS (De Man *et al*, 1960) agar (add 0.3% $CaCO_3$). The plates were incubated aerobically at 37 $^{\circ}$C for 72 h. The second part was taken to enrich with Trypticase phytone yeast extract (TPY) (Biavati, Sgorbati, and Scardovi, 1992) broth which was overlayed with 10 ml of 3% agar and incubated at 37 $^{\circ}$C for 24-48 h. The sample was streaked on TPY agar (add 0.3% $CaCO_3$) , incubated under anaerobic condition in anaerobic jar at 37 $^{\circ}$C for 72 h using the BBL GasPak anerobic system (Becton Dickinson Microbiology Systems, Spards, MD). The isolated colonies with clear zone on both MRS and TPY agar were selected and purified on the same media. All pure isolates were initially tested for morphology, isolates of gram-positive, catalase-negative, rods or cocci shape were maintained in 10% skim milk and stored at –20 $^{\circ}$C for further analyses.

3.4 Screening of potentially probiotic strain

3.4.1 Acid tolerance (Hyronimus *et al.*, 2000)

Primary screening for acid tolerance of the isolates was performed by cultivation the culture in 3 ml of MRS broth adjusted to pH 4 with 6 N HCl and incubated at 37 °C for 72 h. Turbid tubes that showed cell growth were selected for further testings.

Samples of overnight cultures were made to the concentration of 10^8 cfu ml^{-1} by comparing turbidity with Mcfarland No.0.5. Then 20 μl of these cultures were inoculated into 10 ml of MRS broth adjusted to pH values of 2.5 with 6 N HCl. The initial bacterial concentration was 10^6 cfu ml^{-1}. Samples were incubated for 3 h at 37 °C. Cells were serially diluted 10-fold in phosphate buffer (0.1 M, pH 6.2) in order to neutralize the medium acidity. The residual viable count was determined by dilution and plate counting on MRS agar after 72 h of incubation. The survival rate was calculated as the percentage of colonies grew on MRS agar compared to the initial bacterial concentration as this equation :

$$\text{Survival rate (\%)} = \frac{\log N}{\log N_0} \times 100$$

when N = residual viable count

N_0 = initial count

3.4.2 Bile tolerance (Gilliland *et al.*, 1984)

Bile tolerance was determined by inoculation 20 μl of cell (about 1×10^6 cfu ml^{-1}) in 5 ml of MRS broth containing 0.3%(w/v) bile salt. All samples were incubated at 37 °C for 24 h. Growths of control (no bile) and test cultures (0.3% bile salt) were monitored after 0 and 24 h of incubation by measuring the absorbance at 600 nm using a spectrophotometer (Shimadzu model UV-160A, Japan)

3.4.3 Antibacterial activities

3.4.3.1 Preparation of inocula

The sample cultures grown in 10 ml MRS broth with 0.2% glucose at 37 $^{\circ}C$ for 24 h was obtained by centrifugation of culture supernatant at 6,000 rpm at 4 $^{\circ}C$ for 15 min. One of the cell free supernatant was unadjusted for pH in order to study general inhibitory effect and the second was neutralized with 1 N NaOH to pH 6.5 in order to study bacteriocin and bacteriocin-like metabolites. Then both samples were treated with catalase (5 mg/ml) to neutralize hydrogen peroxide neutralized by incubated at 25 $^{\circ}C$ for 30 min.

3.4.3.2 Preparation of indicator strains

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 29737, *Bacillus cereus* ATCC 11778, *Yersinia enterocolitica* ATCC 27799, *Enterococcus faecium* NRIC 1145^{T} and *Lactobacillus plantarum* NRIC 1067^{T} were used as indicator strains. They were cultivated on appropriate media and incubated at 37 $^{\circ}C$ for 18 h. The test plates were prepared by inoculation with 0.2 ml an overnight culture into 20 ml of TSA soft agar (0.75% agar) to the final concentration 10^{6} cfu ml^{-1} and poured in sterile petri dish. The plates were dried at room temperature for 30 min. Wells were punched in the plates by using a sterilized 8 mm diameter cork borrer.

3.4.3.3 Determination of antibacterial activity

The agar well diffusion assay was performed as described by Fleming, Etchells, and Costilow (1985) with some modifications. A 100 µl sample of the unneutralized and neutralized supernatants was filled in 8 mm diameter sealed wells which were cut in the test plates. Inhibition zones were observed and recorded after incubation at 37 $^{\circ}C$ for 48 h.

3.5 Identification of the isolates

3.5.1 Phenotypic characteristics (Tanasupawat, *et al*., 2000)

Gram reaction and colonial appearance was performed on MRS agar for 24 h. Cell morphology characteristics was examined microscopically. Growth at different starting pHs (3.5, 4.0, 8.5 and 9.6) ; the ability to grow at different temperatures 15, 45 and 50 $^{\circ}$C ; tolerance to NaCl 4, 6, 8 and 10% NaCl and production of gas from glucose determined in MRS broth were observed after incubation for 3 days. Arginine hydrolysis, casein hydrolysis, reactions in litmus milk, and acid production from carbohydrates were determined in each medium according to the appendix A.

3.5.2 Isomers of lactic acid (Okada, Toyoda, and Kozaki, 1978)

Isolates tested were cultured in glucose yeast extract peptone beef extract (GYPB) broth for 3 to 5 days and then centrifuged at 3,000 rpm for 10 min to obtain supernatant. The supernatant was adjusted to be neutral with 1 N NaOH and determined enzymatically using D-lactate dehydrogenase, L-lactate dehydrogenase, nicotinamide adenine dinucleotide (NAD; 10 mg/ml), phenazine methosulfate (0.8 mg/ml) and nitro blue tetrazolium chloride (4 mg/ml). The dark blue color appeared within 20 min showed the positive result of the enzyme reaction.

3.5.3 Peptidoglycan type of the cell wall (Komagato and Suzuki, 1987)

Diaminopimelic acid in the cell wall was detected by hydrolysis of 3 mg dried cells grown in GYPB broth. The cells were hydrolyzed with 1 ml 6 N HCl at 100 $^{\circ}$C for 18 h, and the hydrolyzate was applied to a cellulose TLC plate (Merck no.5577). The TLC plate was developed with the system of methanol-water-6 N HCl-pyridine (80 : 26 : 4 : 10, v/v), then sprayed with 0.2% ninhydrin solution and the yellow bands were visualized.

3.5.4 Sequencing of 16S rDNA gene and phylogenetic analysis

DNAs were isolated and purified according to Saito and Miura (1963) and Yamada and Komagata (1970). The 16S rDNA of the isolates was sequenced at DNA technology laboratory, Kasetsart University, Kamphaengsaean campus, Nakornpathom. The sequence was multiply aligned with selected sequences obtained from the GenBank/ EMBL/ DDBJ database by using the CLUSTAL W version 1.83; the alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining method (Saito and Nei, 1987) in the MEGA programme version 2.1 (Komur *et al*., 2001). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

3.5.5 DNA-DNA hybridization

DNAs were isolated and purified following the method of Saito and Miura (1963) and Yamada and Komagata (1970). Photobiotin labelling DNA-DNA hybridization was carried out in 2xSSC (Saline trisodium citrate) and 50 % formamide solution at 38°C for 15 h (Ezaki, Hashimoto, and Yabuuchi, 1989). DNA relatedness was determined by using the colorimetric method, as described by Verlander (1992) and Tanasupawat *et al.* (2000).

3.6 Milk fermentation

One strain of probiotic bacteria from each sample source was selected to produce fermented milk as described follows:

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Starter cultures preparation

The starter cultures were commercial yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* ; Chr Hansen Arpajon, France) and selected probiotic bacteria. Overnight cultures (10^{8} cfu ml^{-1}) were inoculated in 5 ml of fresh milk, and subsequent incubation at 37 $^{\circ}$C until milk coagulation occurred (6-24 h).

Milk fermentation modified Shah and Lankaputhra, 1997

Fermented milk preparations were divided into three sets. The first set was pure probiotic culture from each sample source as starter culture. The second was the mixture of each probiotic culture and *S. thermophilus*. The third was the control that contained only yoghurt bacteria.

UHT milk with 5 and 10% sucrose were heated at 95 °C in boiling water bath for 30 min, cooled and stored for 24 h at room temperature before use in order to check the sterility. The sterilized milk was heated to 45 °C, and added with 5% (v/v) of the starter cultures. Inoculated milk was incubated at 42 °C for 24 h. Fermentation was stopped by rapidly cooling the fermented milk in refrigerator 5 °C and samples were taken during fermentation at 0, 6 and 24 h for measurement of pH, titratable acidity, viscosity and enumeration of yoghurt and probiotic bacteria. The fermented milk were then stored for 3 weeks at 5 °C and pH, titratable acidity, viscosity and enumeration of yoghurt and probiotic bacteria were done at weekly intervals. All fermented milk trials were repeated twice. Samples from each fermented milk were determined as follows :

3.6.1 Enumeration of yoghurt and probiotic bacteria

Fermented milk samples (1 ml) were added to 9 ml of sterile RPS. Appropriate dilutions were made and subsequently pour-plated onto selective media. *S. thermophilus* was enumerated on Lee's (Lee, Vedamuthu, and Washam, 1974) agar, *L. delbrueckii* subsp. *bulgaricus* was enumerated on Tomato juice agar (Difco, USA) and the probiotic strain was enumerated on MRS agar. Bacteria were incubated at 37 °C under aerobic conditions for 72 h. Under these growth conditions, it was possible to differentiate and thus enumerate both yoghurt starter cultures and probiotics based on the different colony morphology. Furthermore, cell viability was calculated in survival rate as mentioned in 3.4.1.

3.6.2 Acid production

The samples were taken for pH measurment using Cyberscan 500 pH meter (Eutech, Singapore). In addition, acidity was determined by titration of the sample with 0.1 N NaOH solution and expressed in percentage (%) according to AOAC method (AOAC, 1990).

3.6.3 Viscosity

The samples were taken for measuring the viscosity using a Brookfield DV-I+ viscometer (Brookfield, Massachusetts, USA), equipped with a T-spindle NO.S63 head. At 60 rpm and at 30-35 $^{\circ}C$, and expressed in centipoise (cps.)

3.7 Statistics

The results were statistically compared using the Duncan's new multiple range test and Student's t-test.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation and screening of lactic acid producing bacteria

Three hundred and seventy-nine lactic acid producing bacteria were isolated from 11 of infant (aged 3-11 months) feces, 13 intestine of pigs, and 15 intestine of chickens. The lactic acid producing bacteria isolated from these samples were 77, 121, and 181 isolates, respectively, based on the appearance of clear zone around the colonies on MRS and TPY agar plates with the addition of $CaCO_3$ (3 g/L). This was also reconfirmed with gram-staining, catalase test and cell morphology as described in 3.3.2. These screened isolates were divided into 3 groups based on their cell shape in MRS and TPY medium.

In the isolation of lactic acid producing bacteria from infants, cocci and pleomorphic form bacteria were found more than rods shape bacteria (Table 4.1). The pleomorphic form bacteria were specific characteristics in bifidobacteria which was anaerobic bacteria. Therefore, some of these pleomorphic form bacteria may identify as *Bifidobacterium*. Benno and Mitsuoka (1986) reported that bfidobacteria appeared after birth and within a week after, the dominant bacterial group in healthy infants were *B. infantis, B. longum* and *B. breve* (Matsuki *et al*.,1999). Regarding to rod shape bacteria Ahrne *et al*. (2005) reported that lactobacilli reached a peak at 6 months when 45% of the infants were colonised and *L. rhamnosus* and *L. gasseri* were the most common species in this period.

Table 4.1 Sample number, sex, age, place and isolate number of lactic acid producing bacteria from infant faeces

Sample number	Infant sex	Age (month)	Place	Isolate no.		
				Rods	Cocci	Pleomorphic form
IF1	Male	7	CU	IF1-5	IF1-2, IF1-3, IF1-4, IF1-7, IF1-9, IF1-10, IF1-11	IF1-1, IF1-6, IF1-8, IF1-12
IF2	Female	7.5	CU	IF2-2, IF2-8, IF2-10	IF2-1, IF2-3, IF2-4, IF2-11	IF2-5, IF2-6, IF2-7, IF2-9
IF3	Female	8	CU	IF3-2, IF3-3 IF3-5, IF3-6	-	IF3-1, IF3-4
IF4	Female	6	RN	-	IF4-1, IF4-2, IF4-3	-
IF5	Female	11	CU	-	IF5-1, IF5-2, IF5-4, IF5-5	IF5-3, IF5-6
IF6	Male	8	KU	-	IF6-1, IF6-3, IF6-5, IF6-6	IF6-2, IF6-4
IF7	Female	3	KU	-	IF7-1	IF7-2, IF7-3, IF7-4, IF7-5
IF8	Male	10	KU	-	IF8-2, IF8-3, IF8-6	IF8-1, IF8-4, IF8-5
IF9	Male	4	CU	-	IF9-2, IF9-4, IF9-6	IF9-1, IF9-3, IF9-5
IF10	Male	6	RN	IF10-3, IF10-4, IF10-6	IF10-1, IF10-2,IF10-5, IF10-7, IF10-8	-
IF11	Male	3	KU	IF11-1	IF11-2, IF11-3,IF11-4, IF11-5, IF11-6,IF11-7, IF11-8	-
Total				12 isolates	41 isolates	24 isolates

CU, Nursery of Chulalongkorn University

KU, Nursery of Kasetsart University

RN, Soi raewadee, Nonthaburi

In pig intestinal, few cocci shape bacteria were isolated (9 isolates), whereas the rod shape bacteria were higher in number (103 isolates) as shown in Table 4.2. They consisted of both short and long rods, and they are arrange in single, in pair, or short/long chains. The results could be attributed to the parts of pig's intestine which were taken for bacterial isolation which may have these rod shape bacteria as dominant bacteria, thus the other group of bacteria has a lower chance to be found. It has been reported that *L. fermentum* and *L. acidophilus* are the dominant lactobacilli in the gut of pigs (Smith *et al*, 1999). However, the recent study from Yin and Zheng (2005) reported that the dominant *Lactobacillus* in the pig gut was *L. ruminis*.

Table 4.2 Sample number and isolate number of lactic acid producing bacteria from pig intestines*

Sample number	Isolate no.		
	Rods	Cocci	Pleomorphic form
P1	P1-1, P1-2, P1-3, P1-4, P1-5, P1-6, P1-7, P1-8, P1-9, P1-10, P1-11, P1-13 P1-15	-	P1-12, P1-14
P2	P2-1, P2-2, P2-3, P2-4, P2-5, P2-6, P2-7, P2-8, P2-9, P2-10, P2-11	-	-
P3	P3-1, P3-2, P3-4	-	P3-3
P4	P4-1, P4-2, P4-3, P4-4	-	-
P5	P5-1, P5-2, P5-3, P5-4	-	-
P6	P6-1, P6-2, P6-3, P6-4, P6-5, P6-6, P6-7, P6-8, P6-9, P6-10, P6-11	-	-
P7	P7-1, P7-2, P7-3, P7-4, P7-5, P7-6, P7-7, P7-8, P7-9, P7-10, P7-11	-	-
P8	P8-2, P8-3, P8-4, P8-6, P8-7, P8-8, P8-9, P8-10, P8-11	-	P8-1, P8-5
P9	P9-2, P9-3, P9-4, P9-5, P9-6, P9-7, P9-8	-	P9-1
P10	P10-1, P10-2 P10-5, P10-6, P10-7, P10-8, P10-9	P10-3, P10-4	-

* Samples were collected from Nonthaburi market.

Table 4.2 Sample number and isolate number of lactic acid producing bacteria from pig intestines* (Continued)

Sample number	Isolate no.		
	Rods	Cocci	Pleomorphic form
P11	P11-4 P11-6, P11-7, P11-8	P11-1, P11-2, P11-3	P11-5
P12	P12-3, P12-4, P12-5, P12-6, P12-7, P12-8, P12-9, P12-10, P12-11, P12-12	P12-1, P12-2	P12-13
P13	P13-2, P13-3, P13-4, P13-5, P13-6, P13-8, P13-9, P13-10, P13-11	P13-1, P13-7	P13-12
Total	103 isolates	9 isolates	9 isolates

* Samples were collected from Nonthaburi market.

The chicken intestine (ileum) was used to isolate in this study. The rod shape bacteria were found more than coccal shape bacteria (Table 4.3). This result was consistent with the research of Lu *et al*. (2003), who indicated that *lactobacillus* species were most abundant group in the ileum of chicken, at 68.5% of total bacteria. Moreover, Tannock (1997) reported that lactobacilli were the predominant organisms in the alimentary tract of the chicken (Tannock, 1997).

Table 4.3 Sample number, place, and isolate number of lactic acid producing bacteria from chicken intestines

Sample number	Place	Isolate no.		
		Rods	Cocci	Pleomorphic form
CK1	SN	CK1-10, CK1-11, CK1-12	CK1-1, CK1-2, CK1-3, CK1-4, CK1-5, CK1-6, CK1-7, CK1-8, CK1-9	-
CK2	SN	CK2-10	CK2-1, CK2-2, CK2-3, CK2-4, CK2-5, CK2-6, CK2-7,CK2-8, CK2-9, CK2-11, CK2-12, CK2-13, CK2-14, CK2-15, CK2-16, CK2-17, CK2-18, CK2-19, CK2-20, CK2-21, CK2-22, CK2-23	-

SN, Slaughterhouse Nonthaburi

PN, Phra-nung-kloa market Nonthaburi

RN, Raewadee market Nonthaburi

Table 4.3 Sample number, place, and isolate number of lactic acid producing bacteria from chicken intestines (Continued)

Sample number	Place	Isolate no.		
		Rods	Cocci	Pleomorphic form
CK3	PN	CK3-3, CK3-6, CK3-7, CK3-8, CK3-9	CK3-1, CK3-2, CK3-4, CK3-5, CK3-10	-
CK4	PN	CK4-1, CK4-2, CK4-3, CK4-4, CK4-5, CK4-6	CK4-7, CK4-8, CK4-9	-
CK5	RN	CK5-1, CK5-2, CK5-3, CK5-4, CK5-5, CK5-6, CK5-7, CK5-8, CK5-9, CK5-10,CK5-11,CK5-12, CK5-13, CK5-14, CK5-15	-	-
CK6	RN	CK6-1, CK6-2, CK6-3, CK6-4 CK6-6, CK6-7, CK6-8, CK6-9, CK6-10,CK6-11,CK6-12,CK6-13	CK6-5	-
CK7	RN	CK7-3, CK7-4, CK7-5, CK7-6, CK7-7, CK7-8, CK7-9,CK7-10, CK7-12, CK7-13, CK7-14, CK7-15, CK7-16	CK7-11	CK7-1, CK7-2
CK8	RN	CK8-11, CK8-12	CK8-1, CK8-2, CK8-3, CK8-4, CK8-5, CK8-6, CK8-7,CK8-8, CK8-9, CK8-10, CK8-13, CK8-14	-
CK9	RN	CK9-8, CK9-9, CK9-11	CK9-1, CK9-2, CK9-3, CK9-4, CK9-5, CK9-6, CK9-7,CK9-10	CK9-12
CK10	RN	CK10-6, CK10-7, CK10-8, CK10-9 CK10-11	CK10-1, CK10-2, CK10-3, CK10-4, CK10-5 CK10-10	-
CK11	RN	CK11-1, CK11-3, CK11-4, CK11-8	CK11-2 CK11-5 CK11-7	CK11-6
CK12	RN	CK12-1, CK12-3, CK12-4, CK12-5, CK12-6, CK12-7, CK12-8, CK12-9, CK12-12	CK12-2	CK12-10, CK12-11, CK12-13

SN, Slaughterhouse Nonthaburi

PN, Phra-nung-kloa market Nonthaburi

RN, Raewadee market Nonthaburi

Table 4.3 Sample number, place, and isolate number of lactic acid producing bacteria from chicken intestines (Continued)

Sample number	Place	Isolate no.		
		Rods	Cocci	Pleomorphic form
CK13	RN	CK13-1, CK13-2, CK13-3, CK13-4, CK13-5	-	-
CK14	RN	CK14-1, CK14-2, CK14-3, CK14-4, CK14-5, CK14-6	-	-
CK15	RN	CK15-1, CK15-2, CK15-3,CK15-4, CK15-5, CK15-6, CK15-7,CK15-9, CK15-10, CK15-11, CK15-12, CK15-13, CK15-14, CK15-15	-	CK15-8
Total		101 isolates	72 isolates	8 isolates

SN, Slaughterhouse Nonthaburi

PN, Phra-nung-kloa market Nonthaburi

RN, Raewadee market Nonthaburi

4.2 Screening of potentially probiotic strain

4.2.1 Acid tolerance

The isolated lactic acid producing bacteria were tested for their ability to grow at low pH (4). In this study the isolation of 77, 121 and 181 isolates of lactic acid producing bacteria from infants, pigs and chickens, respectively, were screened. Primary screening at low pH resulted in 20, 32 and 38 isolates from infants, pigs, and chickens, respectively. Secondary screening were tested in MRS broth adjusted to pH 2.5 after 3 h of incubation at 37 $^{\circ}$C. A total of 61 isolates showed a survival rate < 50% (data not shown) and 29 isolates showed survival rate ≥ 50% as shown in Table 4.4. It can be observed that isolates from infant displayed the highest survival at pH 2.5. Furthermore, the isolates from pig and chicken showed survival rate > 50% in the less number compared to isolates from infant. In infants, 15 isolates have percentage of survival ranged from 57.3-96.4% which were the best percentage of survival. While in pigs, there were 6 isolates have percentage of survival ranged from 67.2-88.5%, and in chicken, there were 8 isolates have percentage of survival ranged from 52.8-73.1%.

Table 4.4 Survival rate of selected isolates when exposure in MRS pH 2.5 for 3 h at 37 °C

Isolates	Incubation time (h)		Survival rate (%)
	0	3	
1. IF2-2	2.2×10^6	1.1×10^6	95.3
2. IF2-8	4.3×10^5	2.7×10^5	96.4
3. IF3-2	6.0×10^5	2.8×10^4	77.0
4. IF3-5	3.5×10^4	6.4×10^3	83.8
5. IF3-6	7.8×10^5	4.7×10^4	79.3
6. IF4-3	1.6×10^6	8.5×10^3	63.3
7. IF6-2	5.6×10^6	7.4×10^3	57.3
8. IF6-4	8.4×10^6	1.7×10^4	61.1
9. IF8-2	4.5×10^6	7.4×10^4	73.2
10. IF8-3	6.5×10^6	9.6×10^4	73.1
11. IF8-6	9.8×10^5	4.4×10^4	77.5
12. IF9-2	2.1×10^6	5.6×10^4	75.1
13. IF10-3	6.7×10^5	8.6×10^3	67.5
14. IF10-8	4.4×10^6	3.6×10^3	63.0
15. IF11-4	7.3×10^6	8.8×10^3	57.5
16. P2-2	8.4×10^6	6.3×10^5	83.8
17. P2-8	1.3×10^5	3.1×10^4	87.8
18. P2-10	6.0×10^5	1.3×10^5	88.5
19. P7-7	5.2×10^6	2.6×10^5	80.6
20. P9-6	2.3×10^6	3.8×10^4	72.0
21. P13-8	8.6×10^6	4.6×10^4	67.2
22. CK3-3	1.5×10^6	6.4×10^3	61.6
23. CK6-7	7.1×10^6	5.6×10^3	54.7
24. CK8-10	2.5×10^6	2.4×10^3	52.8
25. CK8-11	1.5×10^7	5.6×10^4	66.2
26. CK8-13	1.3×10^7	1.6×10^5	73.1
27. CK10-10	8.0×10^5	1.7×10^4	71.7
28. CK13-3	1.4×10^7	6.7×10^3	53.5
29. CK14-2	1.8×10^6	4.5×10^3	58.4

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Total 90 isolates of bacteria grown at pH 4 for 24 h were analysed for acid resistant at pH 2.5 for 3 h. The results demonstrated that 15 isolates from infant showed a survival rate > 50% which was higher than the survival number of 6 isolates from pigs and 8 isolates from chikens. Moreover, the highest acid resistant isolate from infant was IF2-8 with the highest resistant at 96.4%. This result did not differ significantly ($p>0.05$) from the acid-resistant isolates from pig (88.5%).

The acid-resistant isolate from chicken (73.1%) was lower than the isolate from infant and pig. These results associated with the fact that the acidic condition in the stomach of human and pig are approximately at pH 1 to 3 (Bongaerts and Severijnen, 2001), and the gizzard of chicken has pH value as 2.5 to 4.74 (Sturkie, 1976). However, when compare with human and pig, GI tract of chicken was shorter, thus food can pass through the whole gut in only 2 h and 30 min. Jin *et al*. (1998) reported that the resistance against acid of bacteria from chicken may not be important as in the other animals which the food may remain in the GI tract for a long time.

Several *in vitro* assays have been described to select acid resistant strains, i.e., exposure to pH-adjusted PBS (Conway *et al*., 1987; Park *et al*., 2002), incubation in gastric contents (Conway *et al*., 1987; Fernandez, Boris, and Barbes, 2003) and use of a dynamic model of the stomach (Marteau *et al*., 1997). Conway *et al*. (1987) found survival of lactobacilli to be slightly lower when PBS was used rather than gastric juice, because components in the gastric juice may confer some protective effect on the bacterial cell. Moreover, the probiotic strains could be buffered by food or other carrier matrix molecules following consumption and are thus not likely to be exposed to the pH of the stomach (Prasad *et al*., 1998).

The pH value (2.5) used in this study for the selection of potentially probiotic strains is very selective. Even though it is not the most common pH value of the human stomach it assured the isolation of the very acid-tolerant strains (Pennacchia *et al*., 2004).

4.2.2 Bile tolerance

The total of 379 isolates were preliminary selected based on colony morphology, cell shape and cell arrangement. The different characteristics isolates from each sample were selected, 151 isolates were obtained to be representative isolates of the total isolates. This preliminary selected isolates were determined in MRS broth without bile salts (control) and MRS broth containing 0.3% bile salts and the growth capacity was monitored by measuring the absorbance at 600 nm at 0 and 24 h of incubation. The respective control of each isolate showed good growth during the first 24 h of incubation, reaching OD_{600} value >1.0. Total of 112 isolates were able to grow in MRS broth supplemented with 0.3% bile salts (Table 4.5).

Table 4.5 Isolates that grew in the presence of 0.3% bile salts

Origin	Isolate no.	Number of isolate
Infant	IF1-1, IF1-5, IF1-8, IF2-2, IF2-8, IF2-10, IF2-11, IF3-1, IF3-3, IF3-5, IF4-1, IF5-2, IF5-3, IF6-1, IF6-4, IF7-2, IF7-5, IF8-1, IF8-5, IF9-1, IF9-3, IF9-5, IF10-3, IF10-4, IF10-6, IF11-2	26
Pig	P1-4, P1-7, P2-2, P2-5, P2-8, P2-10, P3-1, P3-4, P4-1, P4-4, P5-1, P6-6, P6-11, P7-2, P7-7, P7-10, P8-3, P8-6, P8-8, P8-11, P9-3, P9-6, P10-1, P10-5, P11-2, P12-1, P12-3, P12-6, P13-1, P13-3, P13-6, P13-8, P13-12	33
Chicken	CK1-1, CK1-11, CK2-5, CK2-10, CK2-13, CK3-2, CK3-3, CK3-6, CK3-9, CK4-2, CK4-6, CK4-8, CK5-1, CK5-5, CK5-12, CK5-15, CK 6-3, CK6-7, CK6-10, CK7-1, CK7-3, CK7-5, CK7-10, CK8-3, CK8-11, CK8-12, CK8-13, CK9-1, CK9-5, CK9-7, CK9-9, CK9-12, CK10-1, CK 10-3, CK10-4, CK10-6, CK11-1, CK11-4, CK11-6, CK12-1, CK12-2, CK12-5, CK12-6, CK 12-10, CK12-13, CK13-1, CK13-2, CK13-5, CK14-2, CK14-3, CK15-1, CK15-8, CK15-13	53
	Total	112

Bile plays a fundamental role in specific (Marteau *et al.*, 1997) and non-specific (Kalambaheti, Cooper, and Jackson, 1994) defense mechanism of the gut. The magnitude of its inhibitory effect is determined primarily by the bile salts concentration (Charteris *et al.*,

2000). In the human GI tract, the mean bile concentration is believed to be 0.3% w/v and it is considered as critical and high enough to screen for resistant strains (Gilliland *et al*., 1984). In this study most of the isolates from pig and chicken were able to grow in the medium containing 0.3% bile salts. However, only half of isolates from infant were able to grow in that medium. It may be due to the fact that most isolates from infant are microaerophilic and can not grow well under aerobic condition. Chateau, Deschamps, and Hadj Sassi (1994) reported that there was extreme variability of resistance to bile salts in the *lactobacillus* isolates and all the strains test showed a delayed growth when compared to reference culture without bile salts. This delay was found in other studies on several *L. acidophilus* strains, when the isolates were inoculated in a medium containing 0.3% bile salts (Gilliland *et al*., 1984; Gupta, Mital, and Garg, 1996; Mustapha, Jiang, and Savaiano, 1997).

4.2.3 Antibacterial activities

Of the total 90 lactic acid producing bacteria isolated, which are able to growth at pH 4 within 24 h, forty-nine isolates showed antimicrobial activity against some indicator bacteria with an inhibition ranged from 8.5 to 14.7 mm in diameter (Table 4.6). *E. faecium* NRIC 1145^T were sensitive against most of the lactic acid producing bacteria, whereas *E. coli* ATCC 25922 and *S. aureus* ATCC29737 were resistant to most of these lactic acid producing bacteria isolates. The 46 out of the 49 isolates of lactic acid producing bacteria were found to produce inhibition zones against *E. faecium*, while 12 isolates produced inhibition zone against *L. plantarum*, 6 isolates produced inhibition zone against *Y. enterocolitica*, 9 isolates against *B. cereus*, and only 1 isolate against *S. aureus*. Furthermore, 8 isolates, IF2-2, IF2-8, P2-8, P6-11, P7-2, P8-8, CK6-3, and CK10-6 produced inhibition zones against several indicator organisms. Nevertheless, when the supernatants pH was adjusted to 6.5 and catalase was added, there was no inhibition zone against any indicator bacteria.

Table 4.6 Inhibition zone (mm) against test organisms of the isolates

Isolate no.	pH	Inhibition zone (mm)					
		E. faecium NRIC 1145^T	*L. plantarum* NRIC 1067^T	*Y. enterocolitica* ATCC 27799	*B. cereus* ATCC 11778	*S. aureus* ATCC 29737	*E. coli* ATCC 25922
IF 1-9	4.07	11.5	-	-	-	-	-
IF 2-2	3.84	10.2	-	9.0	9.1	-	-
IF 2-8	3.88	9.7	-	9.3	9.5	-	-
IF 2-10	3.97	11.2	-	-	-	-	-
IF 2-11	4.73	9.8	-	-	-	-	-
IF 3-1	4.04	10.5	-	-	-	-	-
IF 3-3	3.83	-	-	-	-	9.1	-
IF 3-5	3.82	10.0	-	-	-	-	-
IF 5-4	4.85	10.8	-	-	-	-	-
IF 6-1	4.17	11.3	-	-	-	-	-
IF 7-1	4.01	10.5	-	-	-	-	-
IF 8-2	4.18	10.5	-	-	-	-	-
IF 8-6	4.17	10.5	-	-	-	-	-
IF 9-2	4.12	10.3	-	-	-	-	-
IF 10-1	4.64	9.8	-	-	-	-	-
P 2-2	4.45	11.5	-	-	-	-	-
P 2-5	4.16	11.3	-	-	-	-	-
P 2-8	4.31	11.3	-	9.2	9.7	-	-
P 2-10	4.72	12.0	-	-	-	-	-
P 3-1	4.21	10.5	11.5	-	-	-	-
P 4-1	4.31	13.0	11.1	-	-	-	-
P 5-1	4.45	10.5	-	-	-	-	-
P 5-3	4.36	11.5	-	-	-	-	-
P 6-6	4.52	11.0	-	-	-	-	-
P 6-8	4.56	10.8	-	-	-	-	-
P 6-11	4.52	14.0	10.5	11.0	-	-	-
P 7-1	4.23	13.5	14.7	-	-	-	-
P 7-2	4.41	11.3	11.5	-	9.8	-	-
P 8-3	4.06	10.5	-	-	-	-	-
P 8-8	4.19	12.2	14.5	-	9.2	-	-
P 9-6	4.58	11.0	-	-	10.0	-	-
P 10-4	4.44	11.2	-	-	-	-	-
P 10-6	4.39	10.9	8.5	-	-	-	-
CK 4-6	4.25	9.8	-	-	-	-	-
CK 5-5	4.36	10.2	-	-	-	-	-
CK 5-7	4.11	11.1	-	-	-	-	-
CK 5-12	3.97	11.6	-	-	-	-	-
CK 6-3	4.09	14	-	11.3	9.0	-	-

- ; No inhibition zone

ϕ cork borrer = 8 mm

Table 4.6 Inhibition zone (mm) against test organisms of the isolates (Continued)

Isolates	pH	Inhibition zone (mm)					
		E. faecium NRIC 1145[T]	*L. plantarum* NRIC 1067[T]	*Y. enterocolitica* ATCC 27799	*B. cereus* ATCC 11778	*S. aureus* ATCC 29737	*E. coli* ATCC 25922
CK 6-5	4.02	-	11.8	-	-	-	-
CK 6-7	3.98	-	11.5	-	-	-	-
CK 7-3	4.28	13.1	-	-	9.5	-	-
CK 8-2	4.76	12.4	-	-	-	-	-
CK 8-11	4.03	11.2	-	-	-	-	-
CK 9-10	4.18	10.3	-	-	-	-	-
CK 10-6	4.07	13.2	11.5	11.5	-	-	-
CK 10-9	4.03	12.7	10.6	-	-	-	-
CK 10-10	3.96	11.5	-	-	12.0	-	-
CK 13-2	3.82	12.8	10.4	-	-	-	-
CK 14-2	4.57	11.0	-	-	-	-	-

- ; No inhibition zone

ϕ cork borrer = 8 mm

Forty-nine lactic acid producing bacterial produced inhibition zone against some indicator bacteria. However, when the pH value of the suppernatants was adjusted to 6.5 and catalase was added, there was no inhibition zone occurred. This indicated that the antibacterial activities of the isolates were affected by hydrogen peroxide (H_2O_2) or acid production along with the low pH. This results were similar to the results of Maragkoudakis *et al*. (2005), the study used *E. coli, S. thyphimurium* and *H. pyroli*, in the well diffusion assay similar to this study. This results showed that the growth of all pathogens was inhibited at pH 4.5. No inhibition was observed when the pathogens were grown in the presence of near-neutral supernatants (pH 6.5).

Furthermore, Mishra and Lambert (1996) found that lactic acid bacteria produced many inhibitory compounds i.e. lactic acid and other volatile acids, H_2O_2, CO_2 and diacetyl. They also reported about inhibition mechanisms that the inhibitory effect of lactic acid produced by lactic acid bacteria. Low pH affected every aspect of cellular metabolism and retarded the growth of unwanted microbes in culture media. Undissociated lactic acid acetic acid penetrated the cell membrane and disturbed the transmembrane potential, resulting in inhibition of substrate transport and membrane bound activity (Maloney, 1990). The

minimum inhibitory concentration of undissociated lactic acid shows strain specificity. H_2O_2 produced by lactic acid bacteria was inhibitory to both Gram-negative and Gram-positive. Because lactic acid bacteria do not possess catalase (Kandler and Weiss, 1986), H_2O_2 accumulates in the surrounding medium, resulting in anaerobic conditions. The lethal effect of H_2O_2 might be due to the inactivation of essential biomolecules by the superoxide anion chain reaction (Hollang, Knapp, and Shoesmith, 1987). It might also function via the lactoperoxidase-thiocyanate system. The H_2O_2 oxidised the thiocyanate to release toxic oxidation products that were detrimental to foodborne pathogens (Fernandez and Shahni, 1987). The H_2O_2 was more effective as a sporicide than as a bactericide (Bardry, 1983). CO_2 might exert its antimicrobial effect in several ways such as by rendering the environment more anaerobic, by inhibiting enzymatic decarboxylation and by disrupting the cell membrane with the accumulation of the gaseous phase in the lipid bilayer (Eklund, 1984). Diacetyl (2,3 butanedione) was synthesised from pyruvate by certain species of lactic acid bacteria. It inhibited the growth of Gram-negative bacteria and Gram-positive bacteria other than lactic acid bacteria and yeasts (Jay, 1982). Diacetyl interfered with arginine utilisation by reacting with arginine-binding proteins of Gram-negative organism (Jay, 1986).

4.3 Identification of the isolates

4.3.1 Phenotypic characteristics

The isolates were Gram-positive rods and cocci. The colonies showed white or cream white in color, circular shape, convex, smooth to rough edges, creamy consistency and diameter between 0.5–3 mm. The results of growth, physiological and biochemical characteristics of the lactic acid producing bacteria isolates were shown in Table 4.7 – 4.9. Most isolates could grow at 45 $^{\circ}$C, in 4% NaCl, and pH 8.5, but rarely at 15 and 50 $^{\circ}$C, and in 6 and 8% NaCl. In addition only 4, 25, and 30 isolates from infants, pigs, and chickens, respectively, produced gas in MRS broth. The results of acid production from carbohydrates are shown in Table 4.10-4.12. Most of rod shape bacteria produced acid from

glucose, galactose, lactose, fructose, maltose, mannose, raffinose, and sucrose but not from gluconate, glycerol, inulin, ∝-methyl-D-glucoside, and xylose. Most of cocci shape bacteria produced acid from many types of carbohydrates except inulin, ∝-methyl-D-glucoside, and xylose. Ninety isolates that could grow in MRS pH 4 within 24 h were selected, i.e., 20 isolates from infants, 32 isolates from pigs, and 38 isolates from chickens.

Moreover, on the basis of cell shape, cell arrangement and phenotypic characteristics, 216 isolates were belonged to *Lactobacillus*, 122 isolates were *Enterococcus,* and the other 41 isolates were not suspected to be either strains mentioned above.

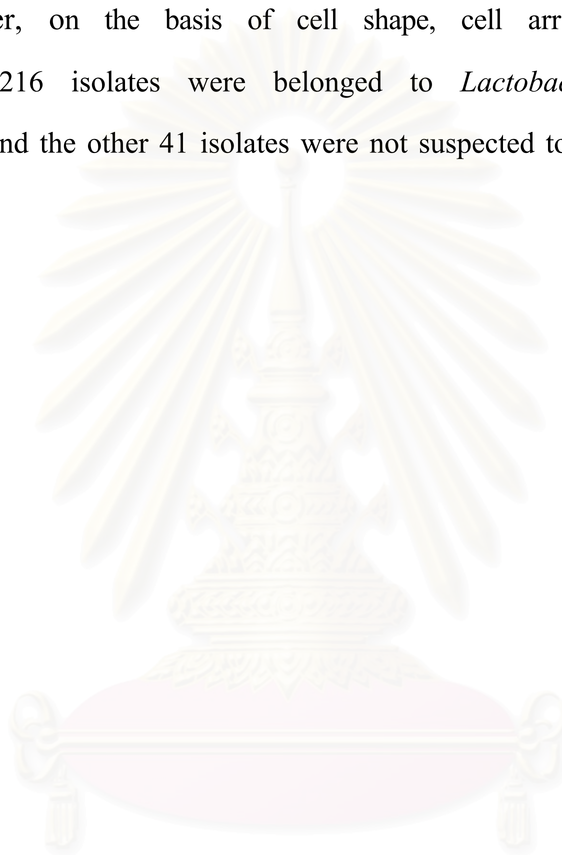

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Table 4.7 General characteristics of the isolates from infant

Characteristics	Isolates																
	IF2-2	IF2-8	IF3-2	IF3-3	IF3-5	IF3-6	IF10-4	IF10-5	IF4-1	IF4-3	IF6-1	IF6-5	IF6-6	IF9-2	IF10-1	IF10-3	IF10-7
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gas from glucose	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+
Nitrate reduction	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Reaction in litmus milk																	
Acidification	+	+	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-
Coagulation	+	+	-	+	+	+	-	-	-	-	+	+	+	+	-	-	-
Reduction	+	+	-	-	+	-	+	+	-	-	+	+	+	+	+	+	+
Growth at pH 3.5	-	-	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-
pH 4.0	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
pH 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	-	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+
8% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Growth at 15 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-

+, positive; -, negative reaction

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Table 4.7 General characteristics of the isolates from infant (Continued)

Characteristics	Isolates			
	IF10-8	IF11-2	IF11-4	IF11-6
Cell form	Cocci	Cocci	Cocci	Cocci
Gas from glucose	-	-	-	-
Arginine hydrolysis	+	+	+	+
Nitrate reduction	-	-	-	-
Reaction in litmus milk				
Acidification	-	+	+	+
Coagulation	-	+	+	+
Reduction	+	+	+	+
Growth at pH 3.5	-	-	-	-
pH 4.0	+	+	-	-
pH 8.5	+	+	+	+
pH 9.6	+	+	+	+
Growth in 4% NaCl	+	+	+	+
6% NaCl	+	+	+	+
8% NaCl	-	+	-	-
Growth at $15^{\circ}C$	-	-	-	-
$45^{\circ}C$	+	+	+	+
$50^{\circ}C$	-	-	-	-

+, positive; -, negative reaction

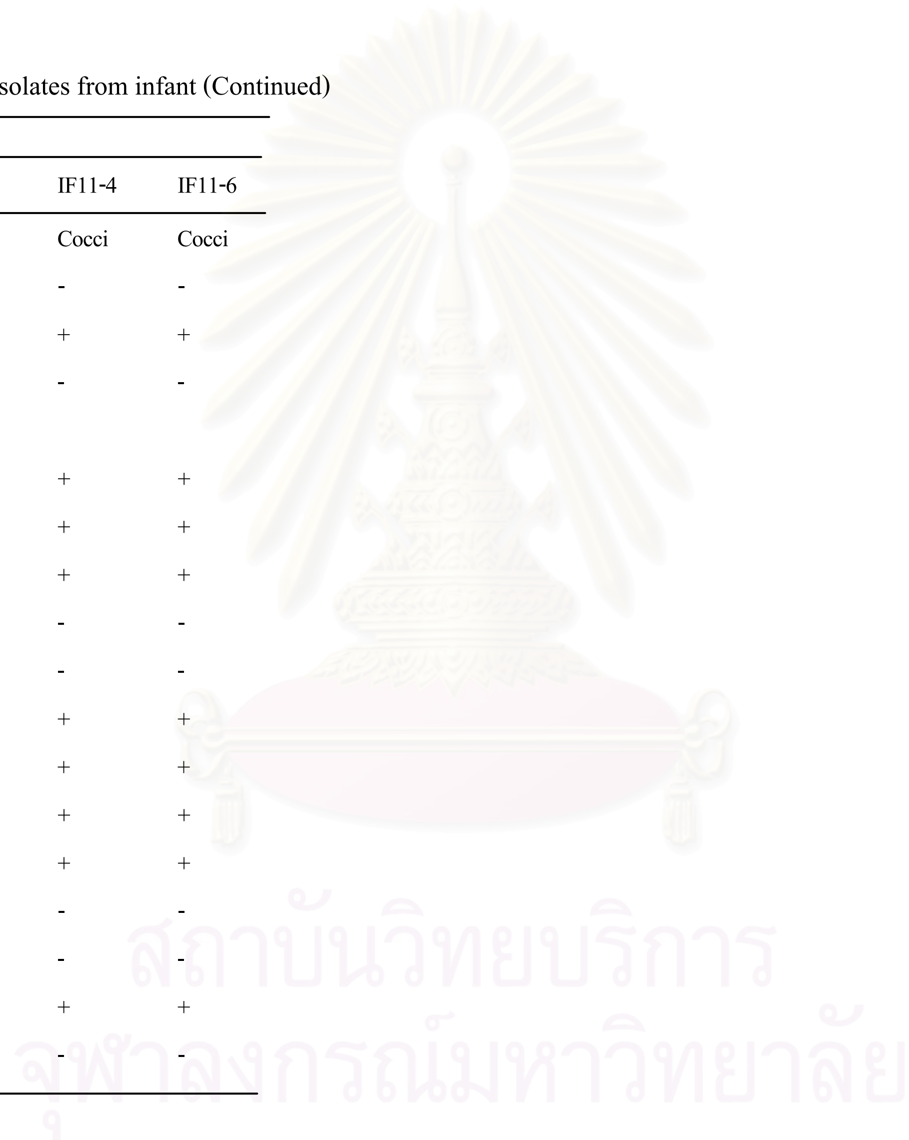
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Table 4.8 General characteristics of the isolates from pig

Characteristics	Isolates																	
	P1-9	P2-1	P2-2	P2-3	P2-4	P2-5	P2-8	P2-10	P3-1	P3-3	P4-2	P4-4	P5-1	P5-2	P5-3	P6-6	P6-8	P6-11
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reaction in litmus milk																		
Acidification	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulation	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Reduction	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at pH 3.5	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-
pH 4.0	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+
pH 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	+	+	+
8% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Growth at 15 °C	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

+, positive; -, negative reaction

Table 4.8 General characteristics of the isolates from pig (Continued)

Characteristics	Isolates																	
	P7-1	P7-2	P7-3	P7-7	P7-10	P8-3	P8-6	P8-8	P8-10	P9-3	P9-4	P9-6	P10-5	P12-3	P12-6	P13-8	P10-4	P13-1
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Cocci	Cocci
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reaction in litmus milk																		
Acidification	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Coagulation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Growth at pH 3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
pH 4.0	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
pH 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	-	+	-	-	+	-	+	-	-	-	+	-	-	-	+	-	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	-	+	-	+	+	+	-	-	-	-	-	+	+	-	+	+	+	+
8% NaCl	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	-
Growth at 15 °C	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

+, positive; -, negative reaction

Table 4.9 General characteristics of the isolates from chicken

Characteristics	Isolates													
	CK2-10	CK3-3	CK3-9	CK4-4	CK4-6	CK5-5	CK5-12	CK6-3	CK6-7	CK7-3	CK8-11	CK8-13	CK13-3	CK14-2
Cell form	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Arginine hydrolysis	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Reaction in litmus milk														
Acidification	-	+	+	-	+	+	-	-	+	+	+	+	+	+
Coagulation	-	+	+	-	+	+	-	-	+	+	+	+	+	+
Reduction	-	+	+	-	+	+	-	-	+	+	+	+	+	+
Growth at pH 3.5	-	+	+	-	-	-	-	-	+	-	-	+	+	+
pH 4.0	+	+	+	+	-	-	+	+	+	+	+	+	+	+
pH 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH 9.6	-	-	-	-	-	+	-	+	-	+	-	-	-	-
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	-	+	+	-	+	-	-	+	+	-	+	-	+	+
8% NaCl	-	-	-	-	-	-	-	-	-	-	+	-	+	-
Growth at 15 °C	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	-	-	-	+	-	-	-	-	-

+, positive; -, negative reaction

Table 4.9 General characteristics of the isolates from chicken (Continued)

Characteristics	Isolates									
	CK1-1	CK1-9	CK2-1	CK3-1	CK8-2	CK8-10	CK8-14	CK10-2	CK10-4	CK10-10
Cell form	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gas from glucose	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	-	-	+	-	+	+	+	+
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Reaction in litmus milk										
Acidification	-	-	+	-	+	+	+	+	+	+
Coagulation	-	-	+	-	+	+	+	+	+	+
Reduction	-	-	+	+	+	+	+	+	+	+
Growth at pH 3.5	-	-	-	-	-	-	-	-	-	-
pH 4.0	-	-	-	-	-	-	-	-	-	-
pH 8.5	+	+	+	+	+	+	+	+	+	+
pH 9.6	+	+	+	+	+	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	-	+	+	+	-	+	+	+
8% NaCl	-	-	-	-	-	-	-	-	-	+
Growth at 15 °C	-	-	-	-	-	-	-	-	-	-
45 °C	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	-	-	-	-	-

+, positive; -, negative reaction

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Table 4.10 Acid production from carbohydrates of the isolates from infant

Characteristics	Isolates																
	IF2-2	IF2-8	IF3-2	IF3-3	IF3-5	IF3-6	IF4-1	IF4-3	IF6-1	IF6-5	IF6-6	IF9-2	IF10-1	IF10-3	IF10-4	IF10-5	
Growth in carbohydrate																	
D-Amygdalin		-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	+
L-Arabinose	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	
D-Cellobiose	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Esculin	W	W	-	W	-	-	+	+	+	+	+	+	+	-	-	+	
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D-Gluconate	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	
Glycerol	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	
Lactose	+	+	-	+	+	+	+	+	W	-	-	+	+	+	+	+	
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
D-Mannitol	-	-	-	-	-	-	+	+	+	+	+	+	+	W	-	+	
D-Mannose	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	
D-Melezitose	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	
D-Melibiose	-	-	-	-	-	-	W	-	+	+	+	+	+	+	+	W	
α-Methyl-D-glucoside	-	+	+	-	+	-	W	W	+	+	+	+	+	-	-	-	
Raffinose	+	-	-	-	-	-	W	+	W	-	-	-	+	+	+	-	
L-Rhamnose	-	-	-	-	-	-	W	-	W	-	-	-	+	-	W	-	
D-Ribose	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	
Salicin	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	
D-Sorbitol	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	
Sucrose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	

+, positive; w, weakly positive; -, negative reaction; ND, not determined

Table 4.10 Acid production from carbohydrates of the isolates from infant (Continued)

Characteristics	Isolates					
	IF10-7	IF10-8	IF11-2	IF11-4	IF11-6	
Growth in carbohydrate						
D-Amygdalin		+	+	W	+	+
L-Arabinose	-	-	+	-	-	
D-Cellobiose	+	+	+	+	+	
Esculin	+	+	+	+	+	
Fructose	+	+	+	+	+	
Galactose	+	+	+	+	+	
Glucose	+	+	+	+	+	
D-Gluconate	+	+	+	+	+	
Glycerol	+	+	+	+	+	
Inulin	-	-	W	-	-	
Lactose	+	+	W	W	-	
Maltose	+	+	+	+	+	
D-Mannitol	+	+	W	-	-	
D-Mannose	+	+	+	+	+	
D-Melezitose	+	+	+	+	+	
D-Melibiose	-	-	-	-	-	
∝-Methyl-D- glucoside	-	-	-	-	-	
Raffinose	-	-	-	-	-	
L-Rhamnose	-	-	-	-	-	
D-Ribose	+	+	+	+	+	
Salicin	+	+	+	+	+	
D-Sorbitol	+	+	+	+	+	
Sucrose	+	+	+	+	+	

+, positive; w, weakly positive; -, negative reaction; ND, not determined

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Table 4.11 Acid production from carbohydrates of the isolates from pig

Characteristics	Isolates																	
	P1-9	P2-1	P2-2	P2-3	P2-4	P2-5	P2-8	P2-10	P3-1	P3-3	P4-2	P4-4	P5-1	P5-2	P5-3	P6-6	P6-8	P6-11
Growth in carbohydrate																		
D-Amygdalin		W	-	-	-	-	-	-	-	-	-	-	-	W	-	-	-	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
D-Cellobiose	+	+	-	+	+	-	-	+	-	-	-	-	+	-	-	-	+	-
Esculin	+	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	+	-
Fructose	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
Galactose	+	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	W	-	-	-	-	-	-	W	-	-	-	-	-	-	-	-	W	-
Glycerol	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	W	-
Inulin	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-
Maltose	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	W	+
D-Mannitol	-	-	-	-	-	-	+	-	-	-	W	+	-	-	-	-	+	-
D-Mannose	+	+	W	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-
D-Melezitose	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-
D-Melibiose	-	+	-	+	+	+	+	+	+	-	+	+	-	+	-	+	-	-
α-Methyl-D- glucoside	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Raffinose	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+
L-Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Ribose	+	+	-	+	+	-	+	-	-	-	-	-	-	-	-	+	+	W
Salicin	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-
D-Sorbitol	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	W	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-Trehalose	W	-	W	-	-	+	+	+	-	-	-	-	-	-	-	+	+	-

+, positive; w, weakly positive; -, negative reaction; ND, not determined

Table 4.11 Acid production from carbohydrates of the isolates from pig (Continued)

Characteristics	Isolates																	
	P7-1	P7-2	P7-3	P7-7	P7-10	P8-3	P8-6	P8-8	P8-10	P9-3	P9-4	P9-6	P10-4	P10-5	P12-3	P12-6	P13-1	P13-8
Growth in carbohydrate																		
D-Amygdalin		-	-	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	W	-
D-Cellobiose	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
Esculin	-	-	-	-	W	-	+	-	+	-	-	-	+	-	+	+	+	W
Fructose	+	+	+	+	+	+	+	+	+	+	W	-	+	-	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Glucose	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
D-Gluconate	-	-	-	-	-	-	-	-	-	+	-	-	W	-	-	-	+	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	W	-	-	-	W	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W	-
Lactose	-	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+
Maltose	W	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
D-Mannitol	W	W	W	-	W	-	-	+	-	-	-	-	W	-	-	-	-	-
D-Mannose	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+
D-Melezitose	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-
D-Melibiose	-	+	+	-	+	+	+	+	+	-	-	+	-	+	+	+	+	+
α-Methyl-D- glucoside	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Raffinose	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
L-Rhamnose	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	W	-
D-Ribose	-	-	-	-	+	+	+	-	+	-	-	W	+	+	-	+	+	W
Salicin	-	W	-	W	+	+	+	-	+	+	-	+	+	-	+	+	+	+
D-Sorbitol	-	W	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	W
Sucrose	+	+	+	+	+	+	-	-	+	-	+	+	W	+	-	+	+	+

+, positive; w, weakly positive; -, negative reaction; ND, not determined

Table 4.12 Acid production from carbohydrates of the isolates from chicken

Characteristics	Isolates															
	CK1-1	CK1-9	CK2-1	CK2-10	CK3-1	CK3-3	CK3-9	CK4-4	CK4-6	CK5-5	CK5-12	CK6-3	CK6-7	CK7-3	CK8-2	CK8-10
Growth in carbohydrate																
D-Amygdalin		-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
L-Arabinose	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+
D-Cellobiose	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Esculin	+	+	+	-	+	-	-	-	-	W	-	-	-	-	+	+
Fructose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Lactose	+	+	-	+	+	+	+	+	+	+	-	-	+	+	W	+
Maltose	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	-	-	-	-	-	+	+	-	+	+	W	+	+	+	+	-
D-Mannose	+	+	+	-	+	+	+	+	+	+	+	+	+	+	W	+
D-Melezitose	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	+
D-Melibiose	-	-	-	-	-	+	+	ND	+	+	+	+	+	+	-	+
∝-Methyl-D- glucoside	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Raffinose	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	+
L-Rhamnose	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-
D-Ribose	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Salicin	+	-	+	-	+	-	-	+	-	+	-	-	+	-	-	+
D-Sorbitol	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+
Sucrose	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose			+					+	+	+	+	+	+	+	-	+

+, positive; w, weakly positive; -, negative reaction; ND, not determined

Table 4.12 Acid production from carbohydrates of the isolates from chicken (Continued)

Characteristics	Isolates								
	CK8-11	CK8-13	CK8-14	CK10-2	CK10-4	CK10-10	CK13-3	CK14-2	
Growth in carbohydrate									
D-Amygdalin		-	-	W	+	+	W	-	-
L-Arabinose	-	-	-	-	-	-	-	-	
D-Cellobiose	-	-	+	+	+	+	-	-	
Esculin	-	-	+	+	+	+	-	-	
Fructose	+	+	+	+	+	+	+	+	
Galactose	+	+	W	+	+	+	+	+	
Glucose	+	+	+	+	+	W	+	+	
D-Gluconate	-	-	W	+	+	-	-	-	
Glycerol	-	-	-	-	-	-	-	-	
Inulin	-	-	-	-	-	-	-	-	
Lactose	+	+	-	-	-	+	+	+	
Maltose	+	+	W	+	+	+	+	+	
D-Mannitol	+	-	-	W	W	-	-	-	
D-Mannose	+	-	+	+	+	+	-	-	
D-Melezitose	-	-	W	W	W	-	-	-	
D-Melibiose	+	+	-	-	-	+	+	+	
∝-Methyl-D- glucoside	-	-	-	-	-	-	-	-	
Raffinose	+	+	-	-	-	-	+	+	
L-Rhamnose	-	-	-	-	-	-	-	-	
D-Ribose	W	+	+	+	+	+	+	+	
Salicin	-	-	+	+	+	+	-	-	
D-Sorbitol	+	-	-	W	W	-	-	-	
Sucrose	+	+	-	+	+	+	+	+	
D-Trehalose	+	-	-	W	W	-	-	-	

+, positive; w, weakly positive; -, negative reaction; ND, not determined

4.3.2 Isomer of lactic acid

Most of tested isolates produced L-lactic acid (Table 4.13). Only 8 isolates, i.e., IF2-2, IF2-8, IF3-5, IF10-3, P2-8, P7-7, CK2-10, and CK14-2, produced both D- and L-lactic acid.

Table 4.13 Isomer of lactic acid of various isolates

Isolates	Isomer of lactic acid	Isolates	Isomer of lactic acid
IF2-2	DL	P8-8	L
IF2-8	DL	P12-6	L
IF3-5	DL	P13-8	L
IF6-1	L	CK2-10	DL
IF9-2	L	CK3-3	L
IF10-3	DL	CK3-9	L
P2-2	L	CK4-6	L
P2-8	DL	CK5-5	L
P2-10	L	CK6-3	L
P5-1	L	CK6-7	L
P6-6	L	CK8-11	L
P6-11	L	CK8-13	L
P7-1	L	CK9-12	L
P7-2	L	CK10-6	L
P7-7	DL	CK13-3	L
P8-6	L	CK14-2	DL

Lactic acid bacteria produced L(+) lactic acid (Dextro rotatory), D(-) lactic acid (Levo rotatory) and DL-lactic acid (Racemic). DL-lactic acid gets converted in to any of the other 2 isomers inside the human body. In human, both isomers were absorbed from the intestinal tract. Whereas L(+) lactic acid was completely and rapidly metabolized in glycogen synthesis, D(-) lactic acid was metabolized at a lesser rate, and the unmetabolized acid was excreated in the urine (Sabinsa Corporation, 2000). Therefore the L- or DL-lactic acid bacteria was selected for use in food products. In this study, the isolates tested were L- and

DL-lactic acid bacteria, but D-lactic acid bacteria was not detected from all of the isolates. It indicated that the L- or DL-lactic acid bacteria in this study were able to be used in food products.

4.3.3 Peptidoglycan type of cell wall

Only 3 isolates, i.e., IF2-8, P2-10 and CK8-11, were selected for use in milk and anylysis of their peptidoglycan type of cell wall. The results showed that all of them did not have *meso*-DAP in the cell wall. Peptidoglycan was an essential component of the cell wall of virtually all bacteria that preserved cell integrity by withstanding the internal osmotic pressure. It was also responsible for the maintainance of cell shape and was intimately involved in cell division. Peptidoglycan was especially abundant in Gram-positive bacteria, in which it accounted for approximately half of the cell wall mass. (Chowdhury and Boons, 2005). Diaminopimelic acid (DAP) was a composition of peptidoglycan. It was a molecule with 2 asymmetric carbon atoms which allowed the formation of different stereoisomer: the L-, the D-, and the *meso*-configurations (Borruat *et al*., 2001). Lactic acid bacteria was separated into 2 groups based on presence or absence of *meso*-DAP in the cell wall. Therefore the cell wall anylysis was a method for idenfying lactic acid bacteria.

4.3.4 Sequencing of 16S rDNA and phylogenetic analysis

Three isolates that were selected for milk fermentation and 3 isolates from representative of different groups were analysed for 16S rDNA sequencing (1,400-1,500 bases) and phylogenetic analysis. The results are shown in Fig. 4.1–4.2 and Table 4.14 – 4.15. The selected isolate from infant faeces, IF2-8 showed identical 16S rDNA nucleotide sequences and was most closely related to identical 16S rDNA nucleotide sequences of *L. gasseri* with 99.91% similarity. P2-10 from intestine of pig showed 16S rDNA similarity value of 99.53% similar to *L. johnsonii*. CK8-11 from intestine of chicken shared the similarity percentage of 99.91% with *L. salivarius*. Regarding the other isolates, P12-3 was closely related to *L. amylovorus* and which subported by a similarity value of 99.53%.

Furthermore, *Enterococcus* isolates, IF7-5 and IF8-1 from infant faeces, were most closely related to *E. raffinosus* and *E. faecalis* with similarity values of 99.83 and 99.75, respectively. IF2-8, P2-10, CK8-11 and P12-3 were identified as *L. gasseri*, *L. johnsonii*, *L. salivarius* and *L. amylovorus*, respectively. Futhermore, IF7-5 and IF8-1 were identified as *E. raffinosus* and *E. faecalis*, respectively (Stackebrandt *et al*., 2002).

This result obtained was consistent with the studies of Xanthopoulos *et al*. (2000); Ahrne *et al*. (2005), and Bello and Hertel (2005) that they reported the isolation of *L. gasseri* from infant faeces. *L. johnsonii* was also found in human (Holzapfel and Schillinger, 2002; Tannock, 1997; Ostlie, Helland, and Narvhus, 2003; Reuter, Klein, and Goldberg, 2002). This result was consistent with that from Garriga *et al*. (1998), who found *L. salivarius* from GI of chicken. Miyamota *et al*. (2000) also reported this species in cloaca of chicken while Enrmann *et al*. (2002) found it in intestine of duck which was closely related with that from chicken intestine. Furthermore, Lu *et al*. (2003) studied on bacterial community succession in the ileal and cecal ecosystems of broiler chicken. He found that nearly 70% of bacteria from ileum were *Lactobacillus* including *L. salivarius*.

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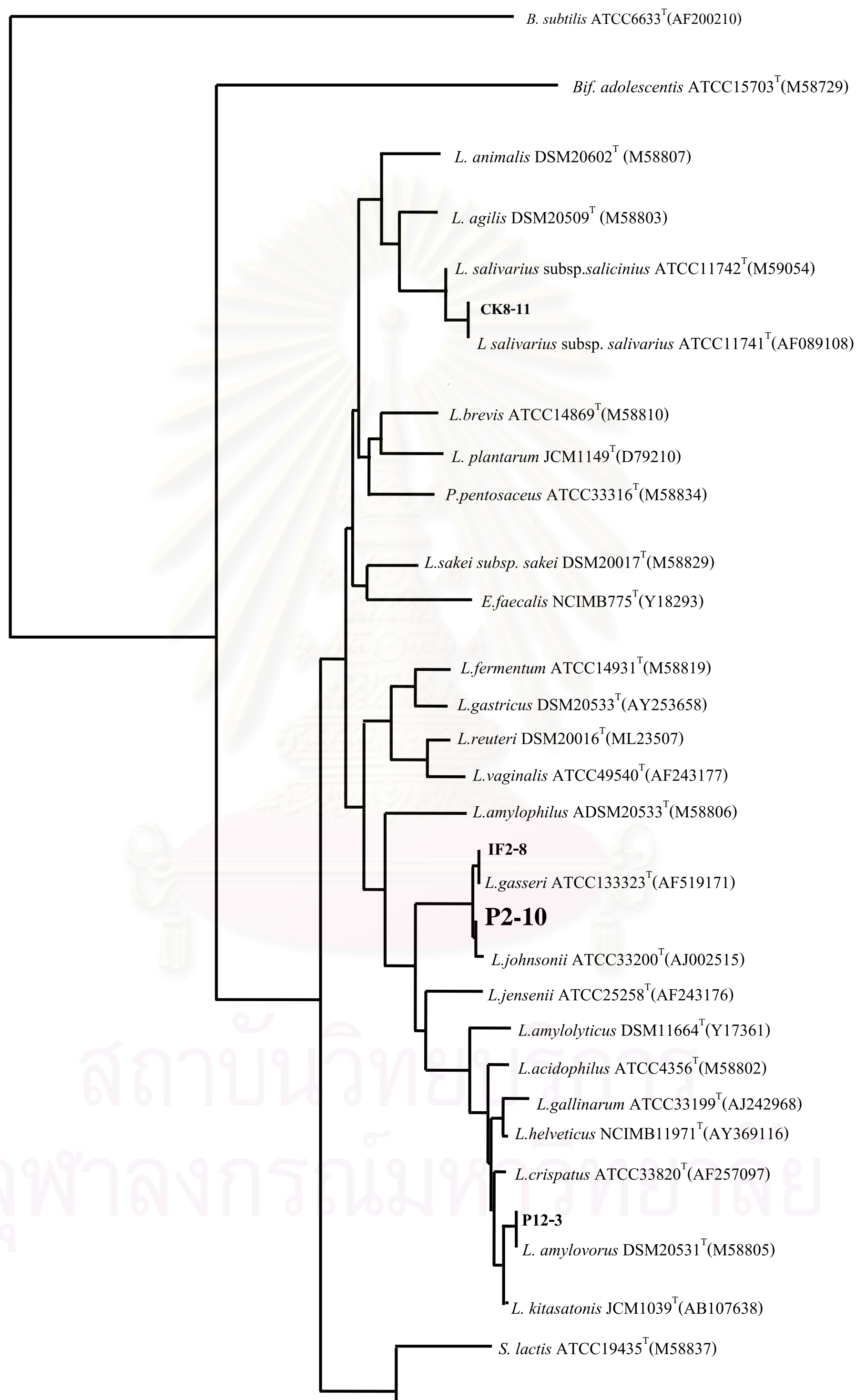

Fig. 4.1 Phylogenetic relationships of *Lactobacillus* strains isolates from infants, pigs and chickens.

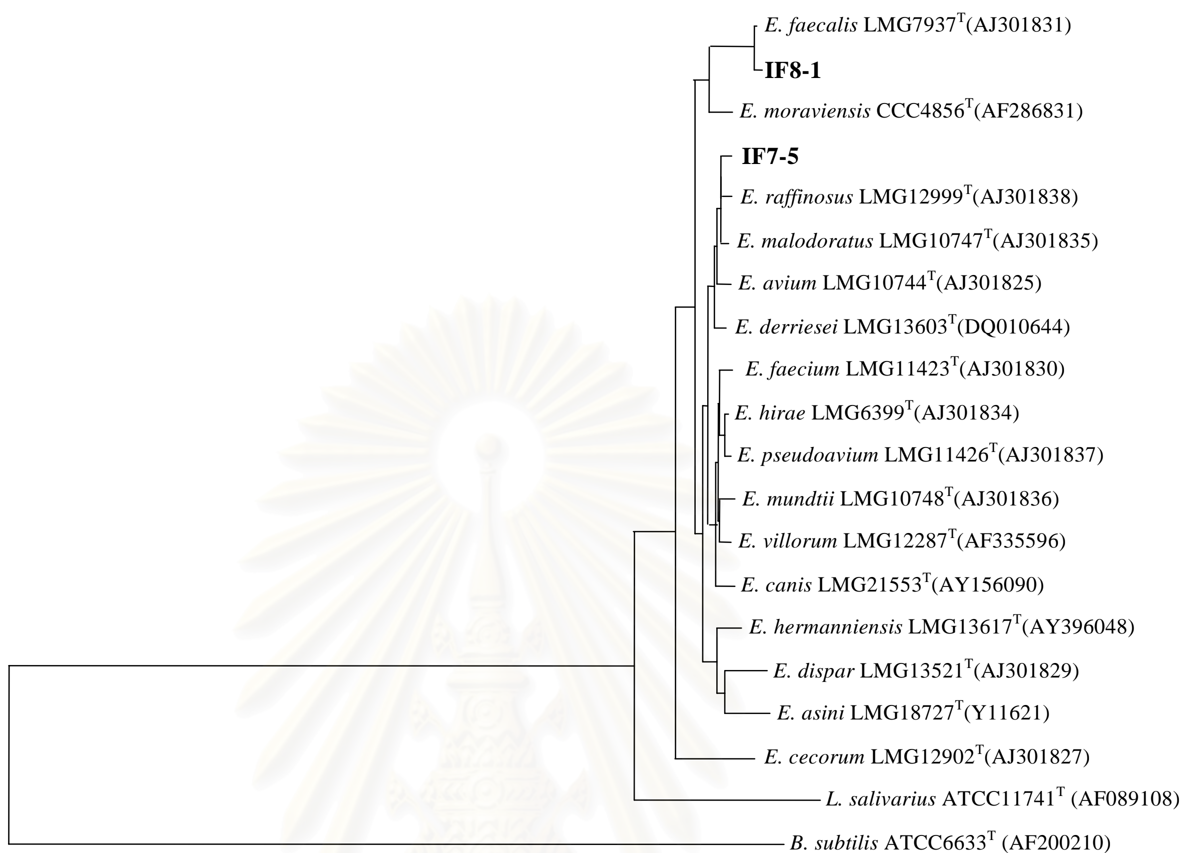

Fig. 4.2 Phylogenetic relationships of *Enterococcus* strains isolates from infants.

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Table 4.14 Percentage similarities among strains of *Lactobacillus* based on 16S rDNA sequences

Accession No.	% Similarity																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. AF257097	100																						
2. AY369116	98.68	100																					
3. AJ242968	97.91	98.68	100																				
4. M58802	98.49	98.49	97.53	100																			
5. M58805	98.87	98.2	97.42	98.11	100																		
6. **P12-3**	98.87	98.3	97.53	98.4	99.53	100																	
7. Y17361	96.85	96.46	95.66	96.85	97.34	97.63	100																
8. AF243176	94.43	94.64	93.81	94.23	94.32	94.23	94.25	100															
9. AJ002515	93.50	93.82	92.78	93.41	93.29	93.31	93.21	94.55	100														
10. **P2-10**	93.71	94.34	93.30	93.92	93.61	93.23	93.72	94.85	99.53	100													
11. AF519171	94.03	94.65	93.61	93.82	93.72	93.94	93.62	94.75	99.25	99.72	100												
12. **IF2-8**	93.92	94.54	93.51	93.71	93.61	93.83	93.52	94.74	99.16	99.63	99.91	100											
13. M58806	91.74	91.32	90.45	91.11	91.54	91.36	91.68	93.31	92.90	93.22	93.01	92.91	100										
14. M58810	89.90	89.46	88.33	89.79	89.45	89.26	89.36	91.32	91.41	91.63	91.31	91.20	92.60	100									
15. AF243177	90.66	91.53	90.44	90.56	90.44	90.57	90.99	90.36	91.00	91.53	91.53	91.42	91.88	91.89	100								
16. ML23507	91.21	91.21	90.12	91.11	91.20	91.12	91.75	90.89	91.73	92.05	91.95	91.84	92.62	92.73	97.73	100							
17. AY253658	90.35	90.25	89.14	90.56	90.03	90.05	90.36	90.91	91.67	91.99	92.09	91.98	92.82	92.95	95.38	95.79	100						
18. M58819	90.45	90.24	89.13	90.56	90.23	90.15	90.46	91.12	91.87	92.19	92.29	92.18	92.83	92.74	94.18	95.29	97.34	100					
19. M59054	88.35	89.05	87.92	89.12	88.45	88.26	88.15	90.61	89.83	90.16	90.06	89.95	89.83	92.52	90.13	91.27	91.08	91.47	100				
20. **CK8-11**	88.46	89.15	88.02	89.23	88.56	93.83	88.26	90.71	89.94	90.27	90.16	90.05	89.94	92.62	90.02	91.17	91.19	91.57	99.91	100			
21. AF089108	88.57	89.26	88.13	89.12	88.45	88.26	88.15	90.81	90.04	90.37	90.27	90.16	90.04	92.72	90.13	91.07	91.29	91.68	99.81	99.91	100		
22. M58803	88.94	89.06	87.93	89.60	88.61	88.63	89.17	90.21	90.07	90.61	90.71	90.61	90.20	93.15	90.66	91.72	92.15	92.04	96.56	96.66	96.56	100	
23. M58807	89.03	88.71	87.57	89.26	88.47	88.49	88.96	89.79	89.54	89.75	89.63	89.52	90.30	93.45	90.72	91.57	92.01	91.49	94.28	94.38	94.28	96.18	100

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Table 4.15 Percentage similarities among strains of *Enterococcus* based on 16s rDNA sequences

Accession No.	% Similarity																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. IF8-1	100																	
2. AJ301831	99.75	100																
3. AF286831	97.68	97.76	100															
4. AJ301835	96.61	96.79	97.68	100														
5. AJ301838	96.52	96.70	97.59	99.58	100													
6. IF7-5	96.70	96.88	97.77	99.75	99.83	100												
7. AJ301827	95.36	95.54	95.45	96.34	96.25	96.44	100											
8. AJ301825	96.61	96.79	97.41	99.23	99.23	99.41	96.61	100										
9. DQ010644	96.70	96.88	97.50	99.23	99.23	99.41	96.34	99.15	100									
10. Y11621	95.81	95.90	96.54	96.98	97.07	97.24	94.90	96.89	97.16	100								
11. AJ301829	95.71	95.80	96.43	97.32	97.32	97.50	95.45	97.05	97.23	97.33	100							
12. AY396048	95.89	96.07	96.88	98.12	98.20	98.38	95.44	98.20	98.46	97.51	97.77	100						
13. AF335596	96.80	97.06	97.68	98.81	98.89	99.06	95.71	98.63	99.15	97.07	97.14	98.11	100					
14. AJ301836	96.70	96.79	97.68	98.72	98.80	98.98	95.53	98.63	98.63	96.71	96.70	97.85	99.15	100				
15. AJ301837	96.80	97.06	97.95	98.72	98.81	98.98	95.54	98.55	98.72	96.81	96.88	98.03	99.15	99.15	100			
16. AJ301834	96.88	97.15	98.03	98.81	98.89	99.06	95.62	98.63	98.81	96.89	96.79	98.11	99.24	99.23	99.75	100		
17. AJ301830	96.98	97.06	97.69	98.46	98.55	98.72	95.36	98.38	98.64	97.51	97.06	97.77	99.15	98.98	99.32	99.24	100	
18. AY156090	97.24	97.33	98.46	98.46	98.55	98.72	95.99	98.29	98.46	97.16	97.32	97.41	98.81	98.81	98.98	99.06	98.98	100

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4.3.5 DNA-DNA hybridization

Table 4.16 DNA similarity of isolates and *Lactobacillus* species

Isolates	DNA-DNA similarity with labeled strains (%)
	L. gasseri IF2-8
IF2-2	17.2
IF2-8	100.0
IF3-5	70.9
	L. johnsonii P2-10
P2-10	100.0
P9-6	45.2
P12-6	29.0
	L. salivarius NRIC 1072^T
CK3-3	72.8
CK5-5	43.2
CK6-3	67.0
CK8-11	76.7
CK10-6	15.1
NRIC 1072	100.0

DNA hybridization is acknowledged as the superior method for the elucidation of relationships between closely related taxa, such as strains and species, in which a DNA-DNA similarity over 70% plays a dominant role (Wayne *et al*., 1987). In this study, IF3-5 showed DNA-DNA similarity values of 70.9% similar to IF2-8. CK3-3, CK6-3 and CK8-11 showed DNA-DNA similarity within the range 67.0 to 76.7 % similar to *L. salivarius* NRIC 1072^T. These results indicated that they should be identified as *L. gasseri* and *L. salivarius*, respectively, whereas IF2-2, P9-6, P12-6, CK5-5, and CK10-6, had DNA-DNA similarity value (< 45.2%) that less than the 70%. So, they could not be identified as any known species of *Lactobacillus* in this study.

4.4 Milk fermentation

4.4.1 Microbial viability

In this study, viability of probiotic and yoghurt bacteria in three sets of fermented milk were investigated. The first used pure probiotic culture as starter culture, the second used mixed culture of each probiotic culture and *S. thermophilus*, and the third was the control that used only yoghurt bacteria. All sets of fermented milk were studied between 5 and 10% sucrose concentration.

4.4.1.1 The viability of probiotic bacteria was compared among 3 fermented milk products within set 1 (Table 4.17-4.18). The results showed that during fermentation (24 h), the cell counts of IF2-8 and CK8-11 were nearly the same whereas the cell counts of P2-10 was lower than those products. After cold storage for 3 weeks, (Table 4.19-4.20), cell viability of IF2-8 and P2-10 were not differ significantly ($p>0.05$) and CK8-11 showed a much lower population than IF2-8 and P2-10.

4.4.1.2 The viability of probiotic bacteria was compred among 3 fermented milk products within set 2. During fermentation, IF2-8 had higher cell counts than P2-10 and CK8-11. After storage for 3 weeks, the cell counts of IF2-8, P2-10 and CK8-11 differed significantly ($p>0.05$). Furthermore, viability of *S. thermophilus* in all of products during fermentation and storage did not differ significantly ($P>0.05$).

4.4.1.3 The viability of probiotic bacteria was compared between products within set 1 and set 2 (Fig. 4.3-4.5). Afters storage for 3 weeks, viable cells of IF2-8 in set 1 was slightly lower than in set 2. On the other hand, viable cells of P2-10 in set 1 higher than in set 2, and viable cell of CK8-11 in set 1 and 2 had same level.

4.4.1.4 The viability of probiotic bacteria was compared between 5 and 10% sucrose fermented milk products (Fig. 4.3-4.5). number of probiotic cells in 5% sucrose fermented milk did not differ from 10% sucrose fermented milk significantly ($P>0.05$).

Table 4.17 Viability (log cfu g^{-1}, mean[a] ± SD) of probiotic bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the 5% sucrose fermented milk during the fermentation process at 42 °C for 24 h

Set no.	Fermented milk	Viability (log cfu g^{-1}, mean[a] ± SD)								
		Probiotic bacteria			*S. thermophilus*			*L. delbrueckii* subsp. *bulgaricus*		
		0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h
1	IF	7.88 ± 0.17	8.86 ± 0.02	12.79 ± 0.95						
	P	7.41 ± 0.53	7.64 ± 0.40	10.09 ± 0.75						
	CK	7.69 ± 0.03	8.26 ± 0.38	12.78 ± 0.16						
2	ST + IF	6.99 ± 0.18	10.57 ± 0.11	13.42 ± 0.08	6.99 ± 0.13	10.81 ± 0.22	11.38 ± 1.39			
	ST + P	6.65 ± 0.33	10.09 ± 0.55	11.74 ± 0.62	7.03 ± 0.07	11.24 ± 0.47	11.74 ± 0.19			
	ST + CK	7.35 ± 0.49	9.95 ± 0.27	11.38 ± 0.68	7.33 ± 0.35	10.70 ± 0.05	12.03 ± 1.06			
3 (control)	ST + L.del				7.17 ± 0.27	10.60 ± 0.15	11.19 ± 1.08	6.02 ± 0.34	8.33 ± 0.01	11.96 ± 0.26

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

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Table 4.18 Viability (log cfu g^{-1}, mean[a] ± SD) of probiotic bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the 10% sucrose fermented milk during the fermentation process at 42 °C for 24 h

Set no.	Fermented milk	Probiotic bacteria			*S. thermophilus*			*L. delbrueckii* subsp. *bulgaricus*		
		0 h	6 h	24 h	0 h	6 h	24 h	0 h	6 h	24 h
1	IF	7.95 ± 0.43	8.92 ± 0.17	12.53 ± 0.59						
	P	7.33 ± 0.64	7.71 ± 0.05	10.18 ± 0.88						
	CK	7.44 ± 0.30	8.43 ± 0.28	12.04 ± 0.06						
2	ST + IF	7.16 ± 0.06	10.67 ± 0.11	13.65 ± 0.36	7.25 ± 0.44	10.94 ± 0.08	12.60 ± 0.12			
	ST + P	6.22 ± 0.06	9.47 ± 0.06	11.09 ± 0.42	7.06 ± 0.03	10.35 ± 0.17	11.09 ± 0.75			
	ST + CK	6.97 ± 0.15	9.39 ± 0.35	11.15 ± 0.47	7.19 ± 0.02	10.83 ± 0.52	11.61 ± 0.17			
3 (control)	ST + L.del				7.22 ± 0.15	10.99 ± 0.58	9.00 ± 0.00	6.44 ± 0.37	9.30 ± 0.99	11.29 ± 0.67

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

Table 4.19 Viability (log cfu g^{-1}, mean[a] ± SD) of probiotic bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the 5% sucrose fermented milk during storage at 5 °C for 3 weeks

Set no.	Fermented Milk	Viability (log cfu g^{-1}, mean[a] ± SD)											
		Probiotic bacteria				*S. thermophilus*				*L. delbrueckii* subsp. *bulgaricus*			
		Week 1	Week 2	Week 3	Survival rate (%)	Week 1	Week 2	Week 3	Survival rate (%)	Week 1	Week 2	Week 3	Survival rate (%)
1	IF	12.43 ± 1.59	10.49 ± 1.35	9.37 ± 1.65	73.0								
	P	9.27 ± 0.85	8.32 ± 0.54	7.45 ± 0.84	73.7								
	CK	10.68 ± 2.82	5.50 ± 0.71	2.00 ± 0.00	15.6								
2	ST + IF	13.96 ± 1.06	13.09 ± 1.65	11.87 ± 2.53	88.4	10.40 ± 0.57	7.89 ± 0.16	5.83 ± 1.17	50.9				
	ST + P	10.18 ± 0.47	7.87 ± 0.29	6.63 ± 0.24	56.5	10.09 ± 0.57	7.79 ± 1.12	6.15 ± 0.94	52.3				
	ST + CK	9.35 ± 1.91	5.00 ± 0.00	2.00 ± 0.00	17.6	11.32 ± 0.51	8.46 ± 0.45	7.08 ± 0.60	59.3				
3 (control)	ST + L.del					7.00 ± 0.00	4.15 ± 0.21	2.00 ± 0.00	17.9	11.61 ± 1.04	9.22 ± 0.54	8.24 ± 0.56	69.0

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

Table 4.20 Viability (log cfu g^{-1}, mean[a] ± SD) of probiotic bacteria, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the 10% sucrose fermented milk during storage at 5 °C for 3 weeks

Set no.	Fermented Milk	Viability (log cfu g^{-1}, mean[a] ± SD)											
		Probiotic bacteria				*S. thermophilus*				*L. delbrueckii* subsp. *bulgaricus*			
		Week 1	Week 2	Week 3	Survival rate(%)	Week 1	Week 2	Week 3	Survival rate (%)	Week 1	Week 2	Week 3	Survival rate (%)
1	IF	12.09 ± 0.78	10.62 ± 1.72	8.69 ± 1.00	69.2								
	P	9.51 ± 0.32	8.46 ± 0.12	7.53 ± 0.38	74.0								
	CK	9.00 ± 0.00	5.50 ± 0.71	2.00 ± 0.00	16.6								
2	ST + IF	12.43 ± 2.02	11.78 ± 1.42	9.95 ± 1.49	72.7	9.89 ± 0.16	6.98 ± 1.39	6.57 ± 0.13	52.1				
	ST + P	9.70 ± 0.29	7.39 ± 0.86	6.80 ± 0.40	61.4	10.70 ± 0.05	8.32 ± 0.96	6.48 ± 0.00	58.5				
	ST + CK	8.92 ± 0.11	5.98 ± 1.38	2.00 ± 0.00	17.9	11.54 ± 0.90	7.80 ± 0.71	5.94 ± 0.65	51.1				
3 (control)	ST + L.del					7.00 ± 0.00	3.50 ± 0.71	2.00 ± 0.00	22.2	11.52 ± 0.68	8.96 ± 0.52	8.46 ± 0.82	74.9

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

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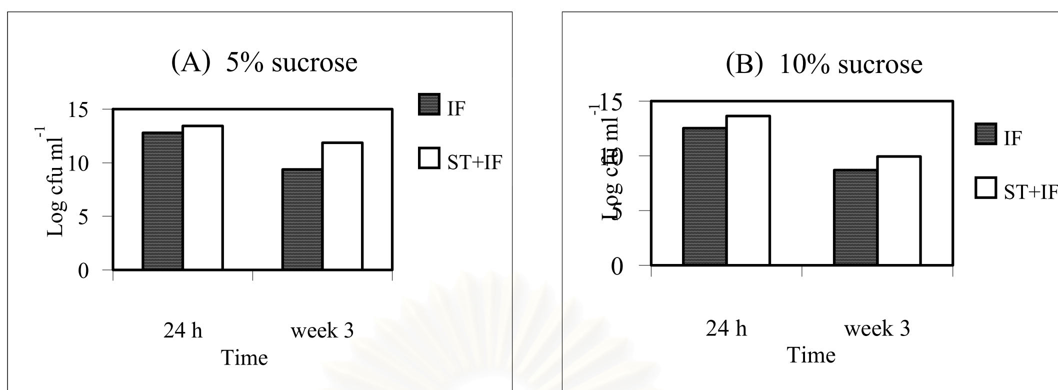

Fig. 4.3 Viable counts of IF2-8 in pure probiotic culture and mixed culture products at (A) 5 and (B) 10% sucrose added, after fermentation (24 h) and storage (3 weeks).

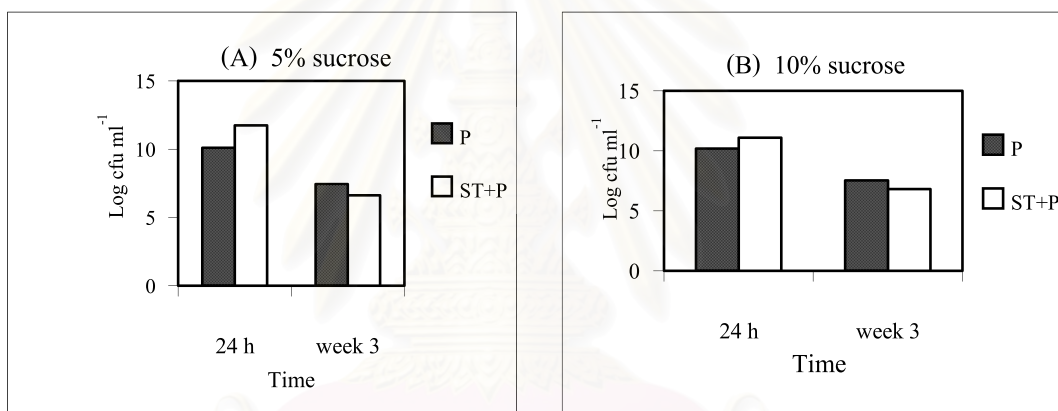

Fig. 4.4 Viable counts of P2-10 in pure probiotic culture and mixed culture products at (A) 5 and (B) 10% sucrose added, after fermentation (24 h) and storage (3 weeks).

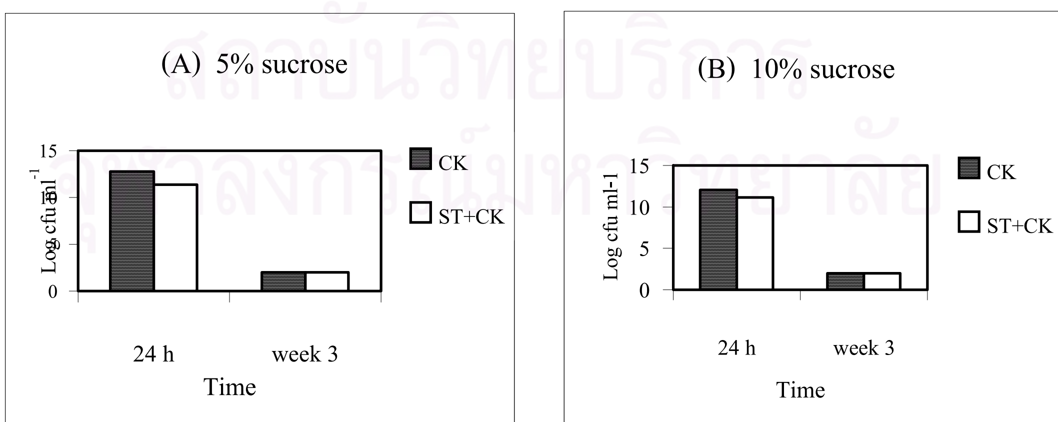

Fig. 4.5 Viable counts of CK8-11 in pure probiotic culture and mixed culture products at (A) 5 and (B) 10% sucrose added, after fermentation (24 h) and storage (3 weeks).

4.4.2 Survival rate of probiotic cell

After storage the fermented milks for 3 weeks, the cell viability was assessed in % survival rate. The results are shown in Table 4.19-4.20.

4.4.2.1 In set 1, the survival rate of IF2-8 and P2-10 ranged from 73.0-73.7%, while the CK8-11 cells survived slightly (15.6%).

4.4.2.2 In set 2, IF2-8 had the highest survival rate (88.4%), and higher than the control (*L. delbrueckii* subsp. *bulgaricus* survived 69%), while P2-10 and CK8-11 survived less than IF2-8 at 56.5 and 17.6%, respectively.

4.4.2.3 In set 1 and 2, the survival rate of IF2-8 and CK8-11 in set 1was less than IF2-8 and CK8-11 in set 2, whereas P2-10 in set 1 had higher survival rate than P2-10 in set 2.

4.4.2.4 The survival rate of cell cultures in 5 and 10% sucrose fermented milk were similar.

After cold storage at 5 $^{\circ}$C for 3 weeks, the fermented milk in set 1, IF2-8 (*L. gasseri*) showed highest survival rate (Table 4.19), which coincide the results of acid-resistant assay (IF2-8 was the highest acid-resistant) as shown in Table 4.4.

P2-10 (*L. johnsonii*) survived slightly lower than IF2-8, while CK8-11 (*L. salivarius* subsp. *salivarius*) showed very low survival rate (Table 4.19), which was not consistented with the acid-resistant results in the CK8-11 survived well (66.2%) in MRS medium at low pH (Table 4.4). Besides product acidity, the survival of probiotic bacteria in fermented dairy products depends on several factors such as the strains used, interaction between species present, culture condition, chemical composition of the fermentation medium (e.g. carbohydrate source), milk solids content, availability of nutrients, growth promoters and inhibitors, concentration of sugars (osmotic pressure), dissolved oxygen, level of inoculation, incubation temperature, fermentation time and storage temperature (Young and Nelson, 1978; Hamman and Marth, 1983; Kneifel *et al*., 1993)

When comparing between pure probiotic culture products and mixed culture with *S. thermophilus* products. The results indicated that *S. thermophilus* affected the survival of

IF2-8 by increasing the survival rate slightly, while the rate of P2-10 was lower and the rate of CK8-11 was very low.

The increasing survival of IF2-8 suggested that , during fermentation, *S. thermophilus* grew quickly first and it might improve the growth conditions of probiotic by producing substances favourable to the growth of probiotic or by reducing the oxygen pressure. Thus probiotic were easily grown and led to the increase in population and also their survival rate (Saarela, 2000).

Nonetheless, during the 3 weeks of cold storage the population of IF2-8 and P2-10 remained above 10^6 cfu ml^{-1}, the minimum level suggested by some authors (Kurmann and Rasic, 1991; Samona and Robinson, 1994; Rybka and Kailasapathy, 1995) for probiotic microorganisms in fermented milks in order to produce therapeutic benefits.

4.4.3 Acid production

Acid production and pH values of products are shown in Table 4.21-4.22. pH values were related to titratable acidity values, thus only the results of titratable acidity will be discussed.

4.4.3.1 The comparison among fermented milk products in set 1, IF2-8 and CK8-11 producted acid nearly at the same level but lower than the control, while P2-10 produced the lowest acid (Fig. 4.6).

4.4.3.2 The comparison among fermented milk products in set 2, all of probiotic bacteria produced comparable amount of acid, but lower than the control (Fig. 4.7).

4.4.3.3 The comparison between fermented milk products in set 1 and set 2, products in set 2 produced titratable acid twice as much as in set 1 (Fig. 4.8-4.10).

4.4.3.4 The acid production in 5 and 10% sucrose fermented milk product was not significantly different ($p>0.05$) (Fig. 4.8-4.10).

Table 4.21 pH (mean[a] ± SD) of fermented milk products at 5 and 10% sucrose added, during the fermentation process at 42 °C for 24 h and storage at 5 °C for 3 weeks

Set no.	Fermented Milk	pH (mean[a] ± SD)											
		0 h		6 h		24 h		Week 1		Week 2		Week 3	
		5%	10%	5%	10%	5%	10%	5%	10%	5%	10%	5%	10%
	IF	6.31 ± 0.11	6.36 ± 0.02	5.85 ± 0.05	5.84 ± 0.02	4.65 ± 0.18	4.75 ± 0.14	4.66 ± 0.19	4.77 ± 0.16	4.66 ± 0.18	4.75 ± 0.16	4.65 ± 0.19	4.76 ± 0.14
1	P	6.36 ± 0.06	6.32 ± 0.01	6.00 ± 0.00	5.91 ± 0.01	5.04 ± 0.28	5.05 ± 0.15	5.04 ± 0.25	5.04 ± 0.13	5.03 ± 0.25	5.03 ± 0.13	4.73 ± 0.71	5.04 ± 0.12
	CK	6.33 ± 0.11	6.41 ± 0.04	6.00 ± 0.01	5.99 ± 0.01	4.66 ± 0.04	4.81 ± 0.11	4.66 ± 0.02	4.81 ± 0.08	4.64 ± 0.03	4.80 ± 0.08	4.66 ± 0.02	4.79 ± 0.08
	ST + IF	6.27 ± 0.04	6.33 ± 0.00	4.54 ± 0.06	4.51 ± 0.06	3.91 ± 0.12	4.00 ± 0.02	3.91 ± 0.13	3.97 ± 0.01	3.92 ± 0.13	4.00 ± 0.01	3.97 ± 0.11	4.02 ± 0.04
2	ST + P	6.28 ± 0.02	6.33 ± 0.02	4.66 ± 0.27	4.45 ± 0.17	3.99 ± 0.01	4.02 ± 0.03	4.00 ± 0.01	4.04 ± 0.01	4.00 ± 0.01	4.05 ± 0.02	4.05 ± 0.03	4.04 ± 0.03
	ST + CK	6.21 ± 0.06	6.34 ± 0.04	4.61 ± 0.22	4.45 ± 0.12	4.10 ± 0.01	4.12 ± 0.08	4.13 ± 0.01	4.11 ± 0.04	4.13 ± 0.01	4.14 ± 0.06	4.17 ± 0.01	4.13 ± 0.05
3 (control)													
	ST + L.del	6.22 ± 0.08	6.29 ± 0.06	4.62 ± 0.07	4.31 ± 0.04	3.70 ± 0.21	3.66 ± 0.01	3.70 ± 0.18	3.64 ± 0.01	3.70 ± 0.16	3.63 ± 0.02	3.72 ± 0.13	3.64 ± 0.03

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

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Table 4.22 Titratable acidity (%, mean[a] ± SD) of fermented milk products at 5 and 10% sucrose added, during the fermentation process at 42 °C for 24 h and storage at 5 °C for 3 weeks

Set no.	Fermented milk	Titratable acidity (%, mean[a] ± SD)											
		0 h		6 h		24 h		Week 1		Week 2		Week 3	
		5%	10%	5%	10%	5%	10%	5%	10%	5%	10%	5%	10%
1	IF	0.16 ± 0.02	0.16 ± 0.03	0.22 ± 0.01	0.22 ± 0.01	0.59 ± 0.08	0.55 ± 0.08	0.62 ± 0.05	0.55 ± 0.11	0.65 ± 0.02	0.60 ± 0.08	0.62 ± 0.06	0.56 ± 0.12
	P	0.16 ± 0.01	0.15 ± 0.01	0.20 ± 0.02	0.18 ± 0.00	0.41 ± 0.10	0.39 ± 0.03	0.42 ± 0.10	0.41 ± 0.01	0.43 ± 0.10	0.39 ± 0.00	0.42 ± 0.10	0.40 ± 0.01
	CK	0.16 ± 0.03	0.15 ± 0.03	0.20 ± 0.03	0.18 ± 0.01	0.56 ± 0.03	0.54 ± 0.04	0.59 ± 0.06	0.56 ±0.08	0.60 ± 0.02	0.57 ± 0.08	0.60 ± 0.03	0.59 ± 0.06
2	ST + IF	0.17 ± 0.04	0.15 ± 0.01	0.57 ± 0.01	0.53 ± 0.02	1.15 ± 0.35	1.08 ± 0.06	1.14 ± 0.23	0.90 ± 0.11	1.09 ± 0.27	0.95 ± 0.06	1.15 ± 0.22	0.97 ± 0.09
	ST + P	0.15 ± 0.01	0.15 ± 0.01	0.52 ± 0.04	0.69 ± 0.03	0.92 ± 0.12	0.72 ± 0.05	1.00 ± 0.01	1.03 ± 0.05	0.94 ± 0.05	0.94 ± 0.05	0.90 ± 0.00	0.84 ± 0.01
	ST + CK	0.18 ± 0.01	0.14 ± 0.01	0.55 ± 0.08	0.62 ± 0.23	0.79 ± 0.04	0.81 ± 0.13	0.88 ± 0.03	0.83 ± 0.01	0.87 ± 0.04	0.83 ± 0.04	0.92 ± 0.10	0.89 ± 0.07
3 (control)													
	ST + L.del	0.18 ± 0.01	0.16 ± 0.02	0.54 ± 0.06	0.73 ± 0.18	1.49 ± 0.45	1.30 ± 0.24	1.46 ± 0.36	1.35 ± 0.01	1.49 ± 0.32	1.47 ± 0.15	1.54 ± 0.37	1.41 ± 0.08

rom two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

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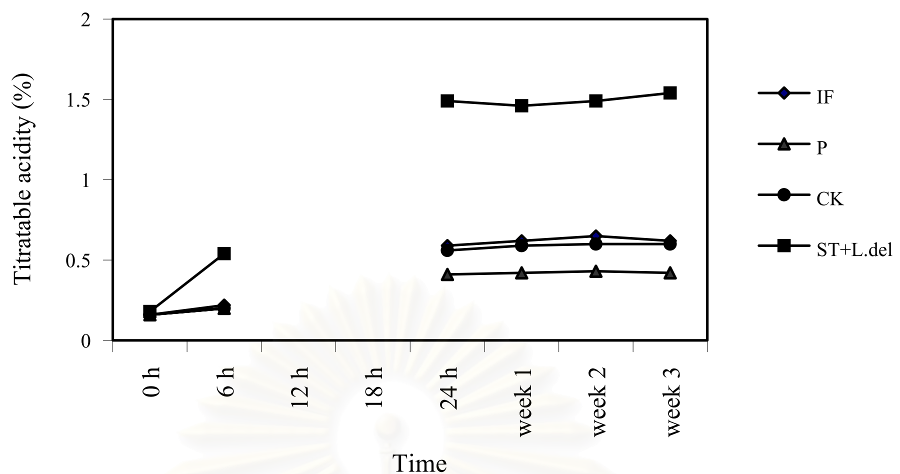
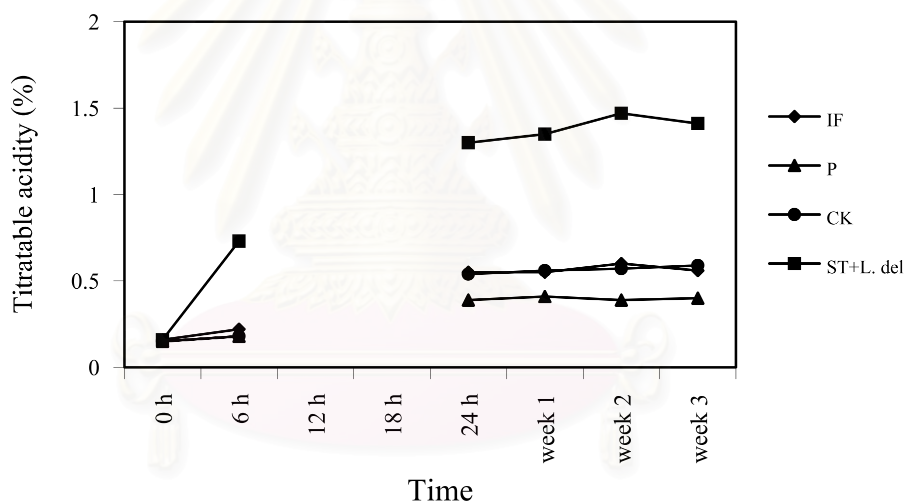


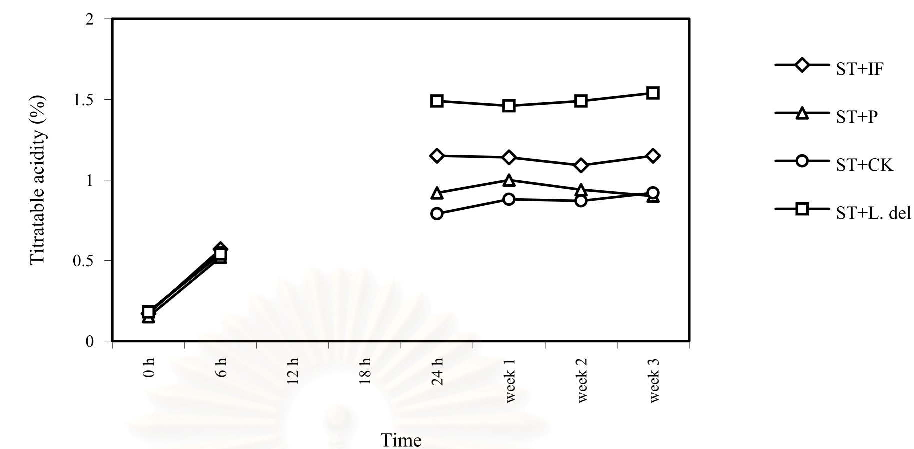
(A) 5% sucrose



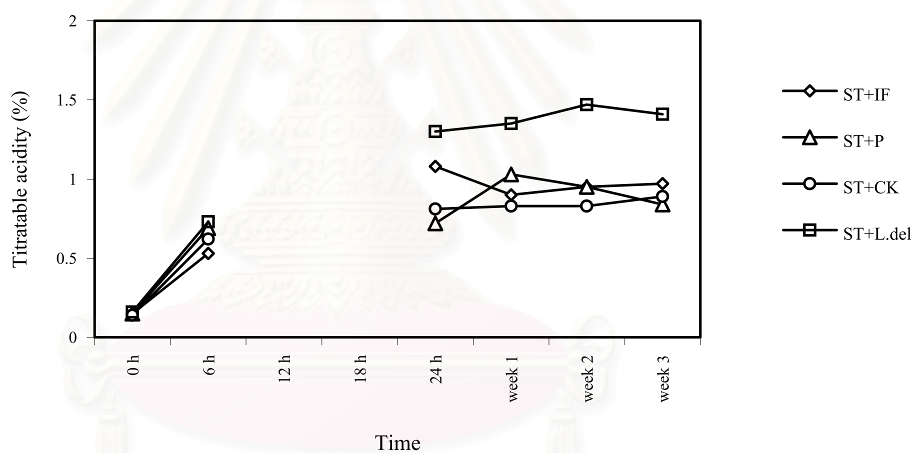
(B) 10 % sucrose

Fig. 4.6 Changes in titratable acidity of pure probiotic culture product at (A) 5% and (B) 10% sucrose added, during fermentation (24 h) and storage for 3 wee

IF, fermented milk used IF2-8; P, fermented milk used P2-10; CK, fermented milk used CK8-1



(A) 5% sucrose



(B) 10% sucrose

Fig. 4.7 Changes in titratable acidity of mixed culture product at (A) 5% and (B)10% sucrose added, during fermentation (24 h) and storage for 3 week

ST+IF, fermented milk used *S. thermophilus* and IF2-8; ST+P, fermented milk used *S. thermo* and P2-10; ST+CK, fermented milk used *S. thermophilus* and CK8-11

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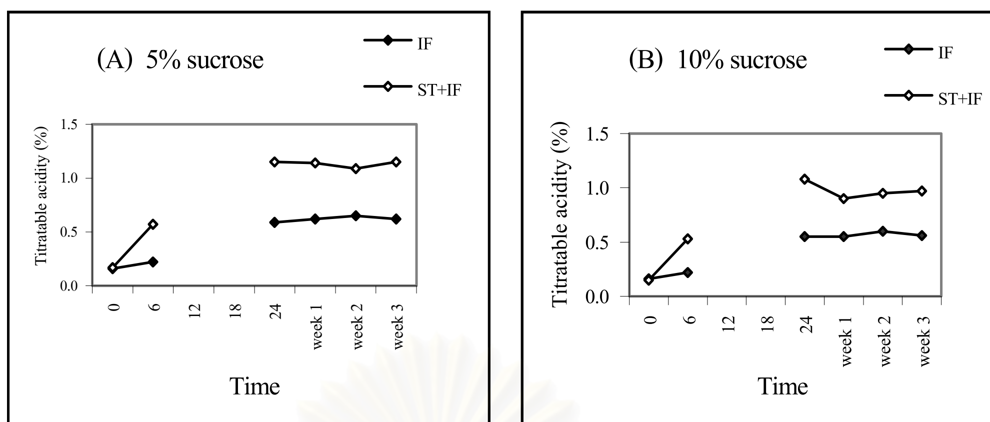

Fig. 4.8 Changes in titratable acidity of IF2-8 in pure probiotic culture and mixed culture products at (A) 5% and (B)10% sucrose added, during fermentation (24 h) and storage for 3 weeks.

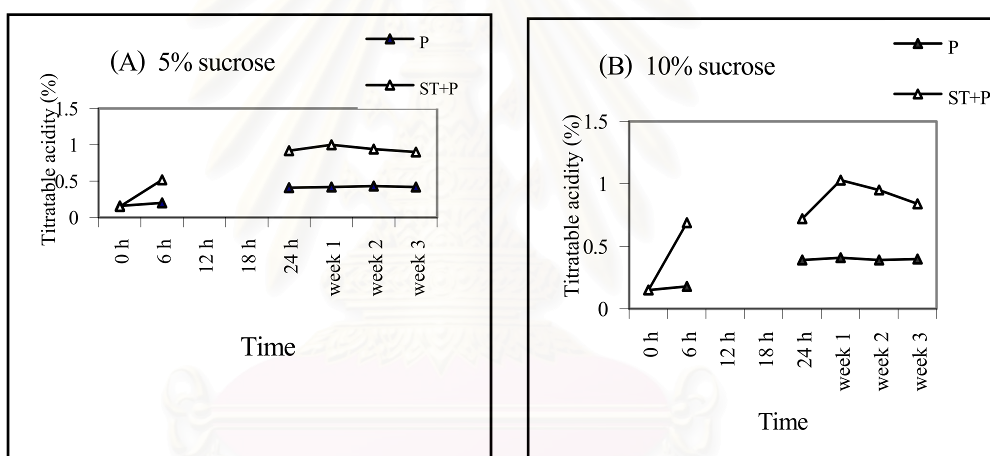

Fig. 4.9 Changes in titratable acidity of P2-10 in pure probiotic culture and mixed culture products at (A) 5% and (B)10% sucrose added, during fermentation (24 h) and storage for 3 weeks.

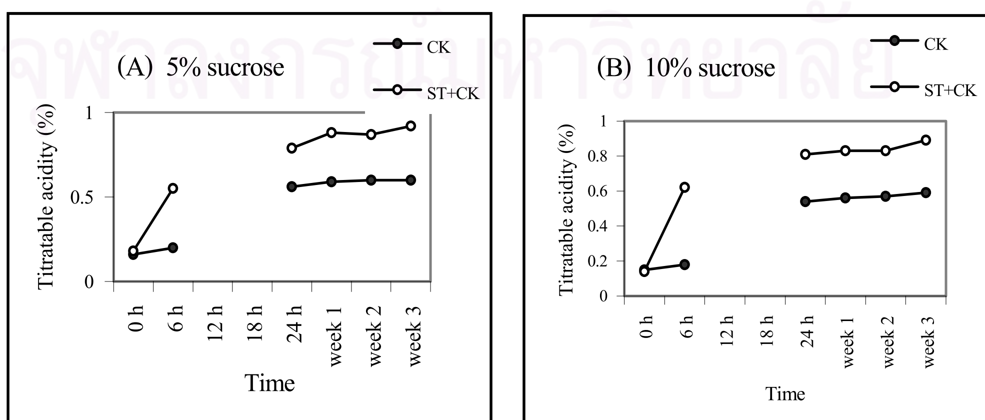

Fig. 4.10 Changes in titratable acidity of CK8-11 in pure probiotic culture and mixed culture products at (A) 5% and (B)10% sucrose added, during fermentation (24 h) and storage for 3 weeks.

In pure culture product, IF2-8 and CK8-11 produced more acid than P2-10 (Table 4.21). This may indicate that during fermentation, P2-10 was slowly grew. The acid production from all of 3 isolates (0.40-0.62%) were much lower than the control (1.41-1.54%). Therefore all of 3 isolates could not be used as starter culture alone in fermented milk.

In typical yoghurt production, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were used as starter culture. Although they can grow independently, the rate of acid production is much higher when used together than either one alone. *S. thermophilus* grows faster and produces both acid and CO_2. The formate and CO_2 produced stimulates *L. delbrueckii* growth. On the other hand, the proteolytic activity of *L. delbrueckii* produces stimulatory peptides and amino acids for use by *S. thermophilus*. The *L. delbrueckii* is the responsible for the further decrease of the pH to approximately 4, and the end products has a total acidity of 0.85 to 0.9% (Goff, 2006).

The growth association between the 2 organisms of the yoghurt starter culture is termed a symbiosis. In this study, the group of mixed culture products produced acid higher than the pure culture products. This results indicated that *S. thermophilus* and probiotic isolate may exhibit a symbiotic relationship during the process of fermented milk.

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4.4.4 Viscosity

The results of viscosity, summarized in Table 4.23, and comparison among products are detailed as follows;

4.4.4.1 The comparison among fermented milk products in set 1, viscosity of product IF was similar to product CK (Table 4.23 and Fig. 4.11). Product P had lower viscosity than those products and the control had the highest viscosity as shown in Fig. 4.11.

4.4.4.2 The comparison among fermented milk products in set 2, product ST+ IF had the highest viscosity (Table 4.23 and Fig. 4.12) and higher than the control, whereas the viscosity of product ST+ P was similar to product ST+ CK and these products had lower viscosity than product ST+ IF.

4.4.4.3 The comparison between fermented milk products in set 1 and set 2, the viscosity of products in set 1were lower than mixed culture products about 2-fold (Table 4.23 and Fig. 4.13).

4.4.4.4 The comparison between 5 and 10% sucrose fermented milk (Fig. 4.13), the viscosity of them did not differ significantly ($P>0.05$).

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Table 4.23 Viscosity (cPs, mean[a] ± SD) of pure probiotic culture and mixed culture (probiotic + *S. thermophilus*) products at 5 and 10% sucrose fermented milk during the fermentation process at 42 °C for 24 h and storage at 5 °C for 3 weeks

Set no.	Fermented milk	Viscosity (cPs, mean[a] ± SD)			
		5% sucrose		10% sucrose	
		24 h	Week 3	24 h	Week 3
1	IF	462 ± 82	255 ± 37	436 ± 56	271 ± 58
	P	204 ± 45	173 ± 42	194 ± 20	171 ± 8
	CK	572 ± 99	276 ± 71	472 ± 17	265 ± 28
2	ST + IF	617 ± 61	601 ± 55	481 ± 69	562 ± 3
	ST + P	492 ± 45	470 ± 113	492 ± 25	358 ± 59
	ST + CK	497 ± 44	428 ± 45	473 ± 18	460 ± 11
3 (control)	ST + L.del	519 ± 27	488 ± 79	479 ± 21	377 ± 44

[a] Means are average from two independent trials

IF, fermented milk used IF2-8 culture; P, fermented milk used P2-10 culture; CK, fermented milk used CK8-11 culture

ST+ IF, fermented milk used *S. thermophilus* and IF2-8 culture; ST+ P, fermented milk used *S. thermophilus* and P2-10 culture; ST+ CK, fermented milk used *S. thermophilus* and CK8-11 culture; ST+ L. del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* culture

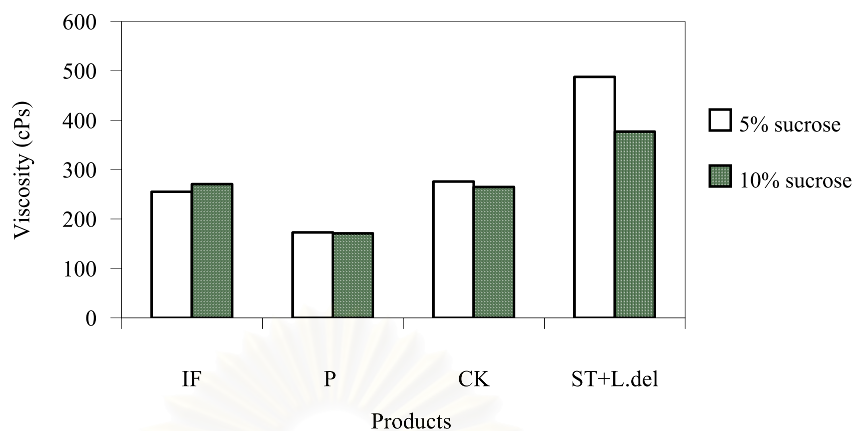

Fig. 4.11 Viscosity of product IF, P and CK, compare with the control (product ST+L.del) at 2 concentrations of sucrose fermented milk products (5 and 10%)

IF, fermented milk used IF2-8; P, fermented milk used P2-10; CK, fermented milk used CK8-11; ST+L.del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*

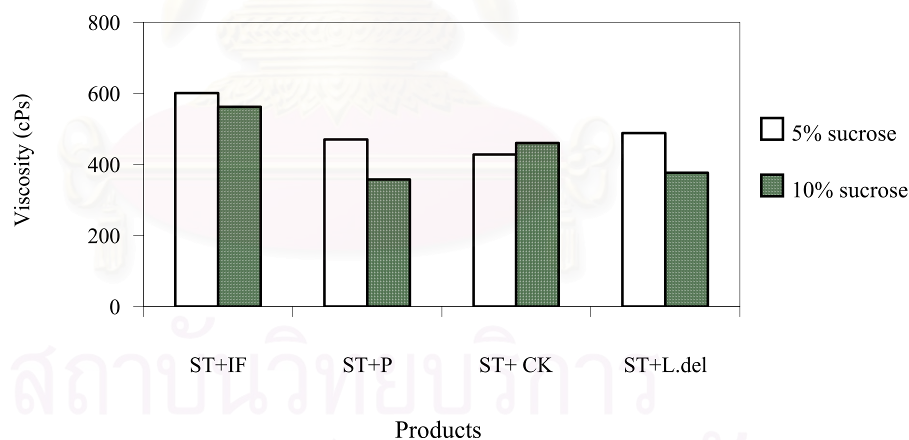

Fig. 4.12 Viscosity of product ST+IF, ST+P and ST+CK, compare with the control (product ST+L.del) at 2 concentrations of sucrose fermented milk product (5 and 10%)

ST+IF, fermented milk used *S. thermophilus* and IF2-8; ST+P, fermented milk used *S. thermophilus and* P2-10; ST+CK, fermented milk used *S. thermophilus* and CK8-11; ST+L.del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*

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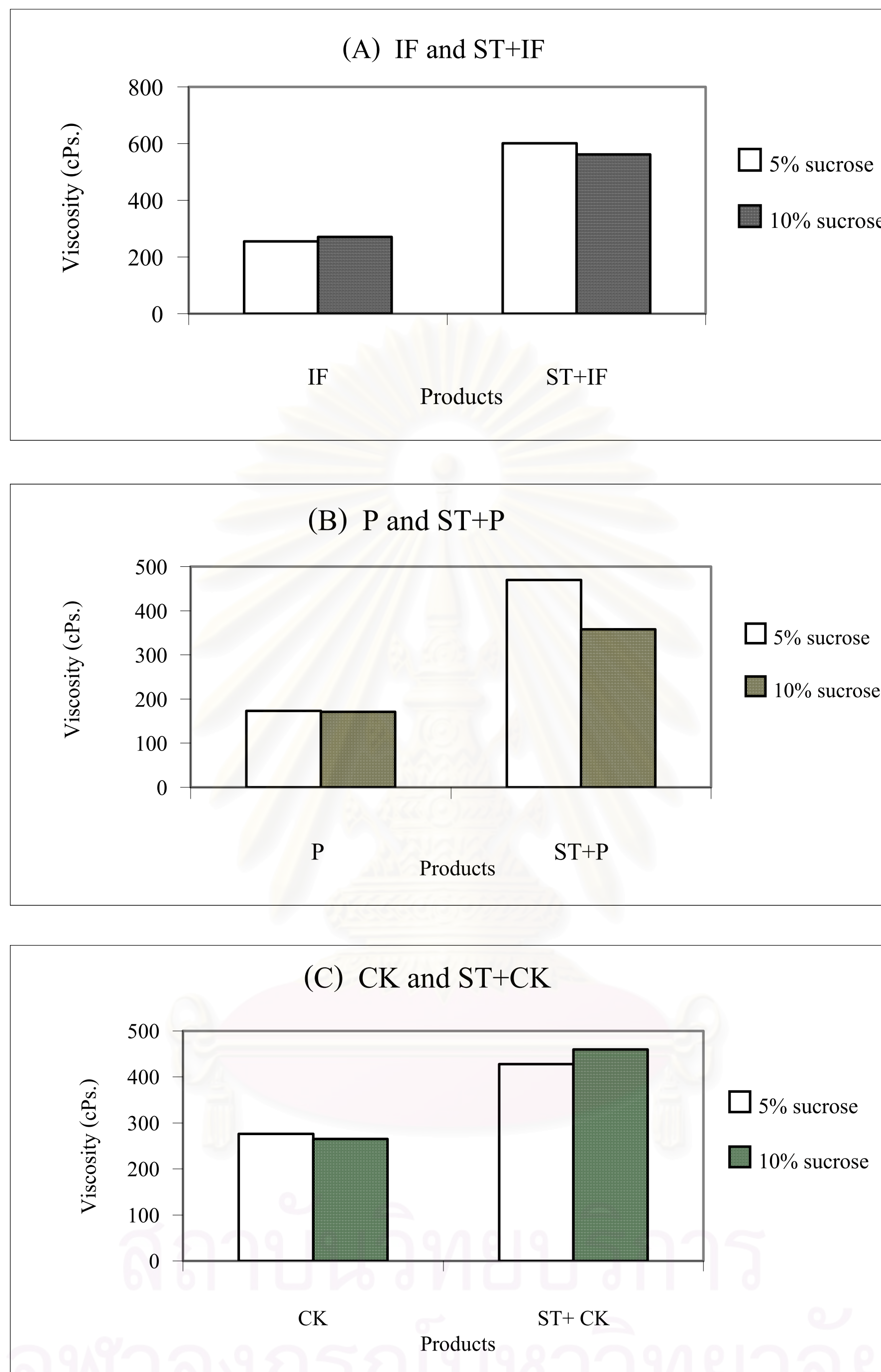

Fig. 4.13 Viscosity of pure probiotic culture products and mixed culture products at 5 and 10% sucrose fermented milk products

IF, fermented milk used IF2-8; P, fermented milk used P2-10; CK, fermented milk used CK8-11; ST+L.del, fermented milk used *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* ST+IF, fermented milk used *S. thermophilus* and IF2-8; ST+P, fermented milk used *S. thermophilus* and P2-10; ST+CK, fermented milk used *S. thermophilus* and CK8-11;

Apart from flavour, quality of fermented milk is largely depended on its viscosity. Viscosity is one of the rheological properties and defined as a group of physical properties of fermented milk influenced by its structural elements and perceived by human senses (Tamime and Muir,1997). In this study, fermented milk have a higher viscosity when *S. thermophilus* was present especially product ST+IF has the highest viscosity and higher than the control group. The increasing of viscosity indicated that the products with *S.thermophilus* produced high amount of acid. Afterthat, when the pH of the fermenting milk reaches 4.6-4.7 the micelles of casein (the major protein of milk) aggregate, leading to the formation of a continuous gel, in which all the components are entrapped. Consequently, little or no wheying off takes place (Lucey, 2002).

CHAPTER V

CONCLUSION

In the screening of probiotic lactic acid bacteria, 379 isolates were isolated from 11 samples of infant faeces, 13 of pig intestine, and 15 of chicken intestine. Based on their phenotypic characteristics, 216 isolates were belonged to *Lactobacillus*, 122 isolates were *Enterococcus* and the other 41 isolates were unidentified. Twenty-nine isolates were acid- and bile-resistant at pH 2.5 and 0.3% bile salt, respectively. The 3 potentially probiotic isolates, IF2-8, P2-10 and CK8-11 were selected based on acid- and bile-tolerant and antibacterial activity. All of 3 isolates were able to grow in 0.3% bile salt, IF2-8 was the best acid-tolerant isolate at the survival rate 96.4%, P2-10 and CK8-11 had lower tolerance at 88.5 and 66.2%, respectively. Strain IF2-8 could inhibit *E. faecium*, *Y. enterocolitica* and *B. cereus* while P2-10 and CK8-11 could inhibit only *E. faecium*.

On the basis of their phenotypic characteristics and 16S rDNA sequence analysis, IF2-8, P2-10, and CK8-11 were closely related to *L. gasseri*, *L. johnsonii*, and *L. salivarius*, with 99.91, 99.53 and 99.91% similarity, respectively. Furthermore the other strains P12-3, IF7-5, and IF8-1 were closely related to *L. amylovorus, E. raffinosus*, and *E. faecalis* with 99.53, 99.75 and 99.83% of 16S rDNA similarity, respectively.

In milk fermentation, IF2-8 had greatest survival (73.0-88.4%) in both fermented milk used only probiotic and fermented milk used probiotic and *S. thermophilus*. Furthermore, fermented milk mixed with IF2-8 and *S. thermophilus* resulted increasing in viability of IF2-8 (2 log cycles) compared to fermented milk used only IF2-8. For acid production, the control product, only IF2-8 product, and IF2-8 mixed with *S. thermophilus* product were 1.54,0.62, and 1.15%, respectively. Their viscosities were 488, 256 and 601 cPs, respectively. Acid production and viscosity of fermented milk used only IF2-8 was much lower than the control, whereas acid production from mixed culture of IF2-8 and *S. thermophilus* produced was not different from the control. Therefore, this results

indicated that IF2-8 should be applied along with *S. thermophilus* in fermented milk but it could not be used as starter culture alone in fermented milk.

Furthermore, the strains were selected according to the basis of their probiotic potential. After the initial fermented milk trials, strain IF2-8 (identified as *L. gasseri*) emerged as the best candidate for probiotic fermented milk production, mainly because of the very good survival properties of the manufactured fermented milk. *L. gasseri* is indigenous intestinal flora in man and animal and thus can be considered safe. In addition, it has been found to be resistant in condition simulating the condition in GI tract. With this isolate as an adjunct, high-quality fermented milk was produced with population level satisfying to international recommendations and guidelines for probiotic and starter culture bacteria.

There are several desirable characteristics for organisms to be used as dietary adjuncts. Organisms should be a normal inhabitant of the human intestinal tract, non pathogenic, non toxic and be capable of surviving passage through the gastrointestinal tract. Within the gut, it must produce the desired effects. Furthermore, it must maintain viability and activity in the carrier food before consumption. It is also important to know the number of organisms needed to colonise human subjects to estimate the effective therapeutic dose.

Further investigations are required, especially for the use of micro-encapsulated bacteria in fermented milks. The scope of this work has been achieved producing fermented milk with a potential probiotic strains. Subsequent *in vivo* trials and clinical studies should be performed before such a product can be marketed, in order to verify any potential health benefits claimed.

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APPENDIX A

Culture media

All media were dispensed and steriled in the autoclave for 15 min at 15 pounds/inch^2 pressure (121 $^{\circ}$C) except for acid from carbon sources test which was sterilized at 10 pounds/inch^2 (110 $^{\circ}$C) for 10 min.

1. MRS medium

Peptone	10	g
Beef extract	10	g
Yeast extract	5	g
Glucose	10	g
Tween 80	1	ml
K_2HPO_4	2	g
Sodium acetate	2	g
Diammnium citrate	0.2	g
$MgSO_4 . 7H_2O$	0.2	g
$MnSO_4 . 4 H_2O$	0.05	g
Distilled water	1	L

Final pH 6.5 ± 0.2

2. Lee's agar

Tryptone	10	g
Yeast extract	10	g
Lactose	5	g
Sucrose	5	g
K_2HPO_4	0.5	g
Agar	15	g
Distilled water	1	L

Final pH 6.5 ± 0.2

3. Phosphate buffer for lyophylize

Monosodium glutamate	3	g
Adonitol	1.5	g
Cystein-hydrochloride	0.05	g
0.1 M phosphate buffer pH7.0	100	ml

4. Tomoto juice agar

Tomoto juice	400	ml
Yeast extract	2	g
Agar	15	g
Distilled water	600	ml
Final pH 6.5 ± 0.2		

5. Reduced physiological salt solution (RPS)

Buffered peptone water	1	g
Cystein- HCl	0.5	g
Tween-80	1	g
Distilled water	1	L
Final pH 6.5 ± 0.2		

6. Salt solution

$FeSO_4 . 7H_2O$	0.2	g
$MgSO4 . 7H_2O$	4	g
$MnSO4 . 4H_2O$	0.2	g
NaCl	0.2	g
Distilled water	1	L

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7. Glucose yeast extract peptone beef extract (GYPB) medium

Glucose	10	g
Yeast extract	5	g
Peptone	5	g
Beef extract	2	g
Sodium acetate	3	g
Tween-80	0.5	ml
Salt solution	5	ml
Distilled water	1	L

Final pH 6.5 ± 0.2

8. Modified GYPB medium (for isomer of lactic acid analysis)

Glucose	20	g
Yeast extract	10	g
Peptone	10	g
Beef extract	4	g
Tween-80	0.5	ml
Salt solution	5	ml
Distilled water	1	L

Final pH 6.5 ± 0.2

9. Trypticase phytone yeast extract (TPY) medium

Trypticase	10	g
Soytone	10	g
Glucose	5	g
Yeast extract	2.5	g
Tween-80	1	ml
Cystein-HCl	0.5	g
K_2HPO_4	2	g

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$MgCl_2 \cdot 6H_2O$	0.5	g
$ZnSO_4 \cdot 7H_2O$	0.25	g
$CaCl_2$	0.15	g
$FeCl_3$	0.001	g
Agar	15	g
Distilled water	1	L

Final pH 6.5 ± 0.2

10. Carbohydrate fermentation broth (for carbon sources test)

Carbohydrate	0.5	g
Yeast extract	0.5	g
Peptone	0.5	g
Beef extract	0.2	g
Tween-80	0.025	ml
Salt solution	0.5	ml
Distilled water	100	ml

Final pH 6.8 ± 0.1

11. Arginine agar

Peptone	0.1	g
Yeast extract	0.3	g
NaCl	0.5	g
K_2HPO_4	0.03	g
L(+) arginine HCl	0.5	g
Phenol red	0.001	g
Tween-80	0.1	ml
Agar	0.3	g
Distilled water	100	ml

Final pH 7.2 ± 0.2

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12. Nitrate broth

Yeast extract	0.5	g
Peptone	1	g
NaCl	1	g
KNO_3	0.1	g
Agar	0.1	g
Distilled water	100	ml

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APPENDIX B

Reagents for DNA extraction and purification, and DNA-DNA hybridization

1. Saline-EDTA (0.15 M NaCl + 0.1 M EDTA)

NaCl	8.76	g
EDTA	37.22	g

NaCl and EDTA were dissolved in 1 L ultra pure water and adjusted the pH 8.0 by adding 6 N HCl and then steriled by autoclaving at 121 $^{\circ}$C, 15 pounds/inch^2 pressure, for 15 min.

2. Phosphate-buffer saline (PBS)

NaCl	8.00	g
KCl	0.20	g
KH_2PO_4	0.12	g
Na_2HPO_4 (anhydrous)	0.91	g
Distilled water	1	L

Steriled by autoclaving at 121 $^{\circ}$C, 15 pounds/inch^2 pressure, for 15 minutes

3. 20× SSC (20× standard saline citrate)

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	1	L

Adjusted pH to 7.0 and steriled by autoclaving at 121 $^{\circ}$C, 15 pounds/inch^2 pressure, for 15 minutes

4. 100× Denhardt solution

Bovine serum albumin (Fraction V)	2	g
Polyvinylpyrrolidone	2	g
Ficoll 400	2	ml

All ingredients were dissolved in 100 ml ultra pure water and stored at 4 °C until used.

5. Salmon sperm

Salmon sperm DNA	10	mg/ml

Salmon sperm DNA 10 mg/ml was dissolved in 1 ml of 10 mM Tris + EDTA buffer pH7.6 , boiled for 10 min and then immediately cooled in ice. Salmon sperm DNA solution was sonicated for 3 min and storaged at 4 °C until used.

6. Prehybridization solution

100× Denhardt solution	2	ml
10 mg/ml Salmon sperm DNA	1	ml
20× SSC	10	ml
Formamide	50	ml
Distilled water	34	ml

All of ingredients were dissolved in ultra pure water, steriled and kept at 4 °C

7. Solution 1

bovine serum albumin (Fraction V)	0.25	g
triton X – 100	50	μl
PBS	50	ml

All of ingredients were mixed and kept at 4 °C

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9. Solution 2

Streptovidin	1	μl
Solution 1	4	ml

Streptovidin – POD conjugate was dissolved in solution 1 before used. The solution 2 was freshly prepared.

10. Solution 3

3,3', 5,5' Tretramethylbenzidine (TMB)		
(10 mg/ml in DMSO)	100	ml
0.3% H_2O_2	100	ml
0.1 M citric + 0.2 M Na_2HPO_4 buffer		
pH6.2 in 10% DMSO	5	ml

All of ingredients were mixed before used. The solution 3 was freshly prepared.

11. Nuclease P1 solution

Nuclease P1 0.1 mg or 40 units/ml was dissolved in 40 mM CH_3COONa + 12 mM $ZnSO_4$ pH 5.3 then stored at 4 $^\circ$C until used.

12. Alkaline phosphatase solution

Alkaline phosphatase solution 2.4 units/ml of 0.1 M Tris-HCl, pH 8.1

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APPENDIX C

16S rDNA nucleotide sequences of the isolates from infant (IF), pig (P), and chicken (CK).

IF2-8

ACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGATGAATTTGGTGCTTGCACCAAATG
AAACTAGATACAAGCGAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAGAGACTGGGATAACACCTG
GAAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTAAAAGATGGTTCTGCTATCACTCTTGG
ATGGACcTGCGCTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAATGATGCATAGCCGAGTTGAGAGACT
GATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
CAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTAGTGAAGAAAGAT
AGAGGTAGTAACTGGCCTTTATTTGACGGTAATTACTTAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGTGCAGGCGGTTCAATAAGTCTGATGTGAAA
GCCTTCGGCTCAACCGGAGAATTGCATCAGAAACTGTTGAACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTG
TAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGG
CTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGTGCTAAGTGTTGGG
AGGTTTCCGCCTCTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT
CTTGACATCCAGTGCAAACCTAAGAGATTAGGAGTTCCCTTCGGGGACGCTGAGACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTAAGTTGGG
CACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGACGGTACAACGAGAAGCGAACCTGCGAAGGCAAGCGGATCTCTGAAAGCCGTTC
TCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTCTGTAACACCCAAAGCCGGTGGGATAAC
CTTTATAGGAGTCAGCCGTCTAAGGTAGGACAGATGA

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IF7-5

TTGAGTTTGTCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTTTTtTTTCACCGGA
GCTTGCTCCACCGAAAGAAAAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATA
ACACTTGGAAACAGGTGCTAATACCGTATAACAATAGAAACCGCATGGTTTCTATTTGAAAGGCGCTTTTGCGTCAC
TGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTG
AGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCA
ATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAA
GAACAAGGATGAGAGTAGAATGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTG
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TTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTG
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AGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGT
TGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCA
TTTAGTTGGGCACTCTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGGAAGTACAACGAGTCGCGAAGTCGCGAGGCTAAGCTAATCTCTT
AAAGCTTCTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCA
CGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGT
GAGGTAACC

IF8-1

TCAGGACGAACGCAGGCGGCGTGCCTAATACATGCAAGTCGAACGCTTCTTTCCTCCCGAGTGCTTGCACTCAATTG
GAAAGAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAACACTTGGAAACAGG
TGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGACC
CGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTC
TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTT
AGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCC
GGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTT
TCCGCCCTTCAGTGTTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGG
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ACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTNGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTTAGTTGGGCACT
CTAGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
ACACACGTGCTACAATGGGAAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAAATCTCTTAAAGCTTCTCTCAGT
TCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACTCCGCCCGTGCACACCACGAGAGTTTGTAACACCCGAAGTGCGGTGAGGTAACCTTT
TTGGAGCCAGCCGCCTAAGGT

P2-10

TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTTGCCTAGATGATTTTGGTGCT
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GATAACACCTGGAAACAGATGCTAATACCGGATAACAACACTAGACGCATGTCTAGAGTTTGAAAGATGGTTCTGC
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CCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGGTA
GTGAAGAAAGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATTACTTAGAAAGTCACGGCTAACTACGTGCCA
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GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCC
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TCTTAAAGCCGTTCTCAGTTCGGACTGTAGGCTGCAACTCGCCTACACGAAGCTGGAATCGCTAGTAATCGCGGATC
AGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTCTGTAACACCCAAAG
CCGGTGGGATAACCTTTATAGGAGTCAGCCGTCTAAGGTAGGACAGATGATTAGGGT

P12-3

CCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCGGAACCAACAGATTTACTTCGG
TAATGACGTTGGGAAAGCGAGCGGCGGATGGGTGAGTAACACGTGGGGAACCTGCCCCTAAGTCTGGGATACCATT
TGGAAACAGGTGCTAATACCGGATAATAAAGCAGATCGCATGATCAGCTTTTGAAAGGCGGCGTAAGCTGTCGCTA
AGGGATGGCCCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGAGTTGAG
AGACTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAAT
GGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGA
AGGATAGAGGTAGTAACTGGCCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCG
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AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCTAGTGCAATCTGTAGAGATACGGAGTTCCCTTCGGGGACGCTAAGACAGGTGGTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTATTAGTTGCCAGCATTAA
GTTGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTAT
GACCTGGGCTACACACGTGCTACAATGGGCAGTACAACGAGAAGCAAGCCTGCGAAGGCAAGCGAATCTCTGAAA
GCTGTTCTCAGTTCGGACTGCAGTCTGCAACTCGACTGCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGC
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTCTGCAATGCCCAAAGCCGGTGG
CCTAACCTTCGGGAAGGAGCCGTCTAAGGCAGGGCAGATGACT

CK8-11

CTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAAACTTTCTTACACCGAATGCTTGCATTCAC
CGTAAGAAGTTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTAAAAGAAGGGGATAACACTTGGAAA
CAGGTGCTAATACCGTATATCTCTAAGGATCGCATGATCCTCAGATGAAAGATGGTTCTGCTATCGCTTTTAGATGG
ACCCGCGGCGTATTAACTAGTTGGTGGGGTAACGGCCTACCAAGGTGATGATACGTAGCCGAACTGAGAGGTTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAA
GTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACACGAGT
GAGAGTAACTGTTCATTCGATGACGGTATCTAACCAGCAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGGGAACGCAGGCGGTCTTTTAAGTCTGATGTGAAAGC
CTTCGGCTTAACCGGAGTAGTGCATTGGAAACTGGAAGACTTGAGTGCAGAAGAGGAGAGTGGAACTCCATGTGTA
GCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTT
CGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGTTGGAGG
GTTTCCGCCCTTCAGTGCCGCAGCTAACGCAATAAGCATTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCTTTGACCACCTAAGAGATTAGGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTCAGTTGCCAGCATTAAGTTGGGC
ACTCTGGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGACGGTACAACGAGTCGCAAGACCGCGAGGTTTAGCTAATCTCTTAAAGCCGTTCTC
AGTTCGGATTGTAGGCTGCAACTCGCCTACANGAAGTCGGAATCGCTAGTAATCGCGAATCAGCATGTCGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCGC
AAGGAGCCAGCCGTCTAAGGTGGGACAGATGA

APPENDIX D

Preparation of McFarland Standards

Principle

A chemical induced precipitation reaction can be used to evaluate the turbidity of a bacterial suspension.

Method

1. Set up 10 test tubes or ampoules of equal size and of good quality. Use new tubes that have been thoroughly cleaned and rinsed.
2. Prepare 1% chemically pure sulfuric acid.
3. Prepare a 1.175% aqueous solution of barium chloride ($BaCl_2 \cdot 2H_2O$)
4. Slowly, and with constant agitation, add the designated amount of the two solutions in Table 1D to make a total of 10 ml per tube.
5. Measure absorbance at 660 nm and plot points on graph.
6. Plot standard graph use a linear regression to draw the best fitting line between the points.

Table 1D McFarland Standards

Tube number											
	0.5	1	2	3	4	5	6	7	8	9	10
Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approx. cell density ($x10^8$/ml)	1.5	3	6	9	12	15	18	21	24	27	30

Preservation

1. Seal the tubes or ampoules. The suspended barium sulfate precipitation corresponds approximate to homogenous *E. coli* cell densities per ml throughout the range of standard as shown in table 1D.
2. Store the McFarland standard tubes in the dark at room temperature. They are stable for 6 months.

Note : The turbidity standards should be vigorously agitated on a vortex mixer or manually done before use and inspected for a uniform turbid appearance. If large particles appear, the standard should be replaced.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

Miss Nittaya Methawanitpong was born on December 12, 1970. She obtained a Bachelor of Science Degree in Microbiology from Prince of Songkla University, Songkla, Thailand, in 1994.

Poster presentation :

1. Nittaya Methawanitpong, Romanee Sanguandeekul, Somboon Tanasupawat and Suttisak Suknaisilp. 2005. Screening of probiotic bacteria as a supplemented in fermented milk product. 3rd Biothailand. November, 2-5, 2005. Queen Sirikit international convention center.