รายงานโครงการวิจัยฉบับสมบูรณ์

โครงการ ผลิตภัณฑ์เมตาโบลิกและอนุกรมวิธานของแบคทีเรียกรดแลคติกจากไส้กรอกเปรี้ยว

Metabolic Products and Systematic of Lactic Acid Bacteria from Fermented Sausages

โดย จันทรพร ผลากรกุล และคณะ

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ABSTRACT

Lactic acid bacteria from traditional Thai fermented sausages were characterized. The fermented sausages were mainly produced from minced pork/beef and sliced pork/beef skin. One hundred and twenty-four strains were isolated from 16 samples collected from the central and northeastern parts of Thailand. Certain isolates were selected for taxonomic determination (65 out of 124). The strains were identified by conventional morphological, cultural, physiological and biochemical tests as well as 16S rDNA sequence analysis. Some of these species have not been previously isolated from Thai fermented sausages. The isolates were identified as *Weissella cibaria/kimchii* (5), *W. confusa* (3), *Pediococcus pentosaceus* (20), *P. acidilactici* (2), *Lactobacillus fermentum* (3), *L. brevis* (4), *L. farciminis* (4), *L. plantarum* (23), and *L. sakei* (1). Tested strains of each group produced DL-lactic acid from D-glucose, except those identified as *L. farciminis* which produced L-lactic acid. The distribution of these bacteria in fermented sausages in Thailand and their capabilities of producing metabolic compounds with antimicrobial property, i.e., diacetyl and bacteriocin, were discussed.

งานวิจัยนี้ศึกษาคุณลักษณะของแบคทีเรียกรดแลคติกแยกจากไส้กรอกหมักท้องถิ่นของไทย (ไส้กรอกเปรี้ยวและหม่ำ) ซึ่งมีส่วนผสมหลักคือเนื้อและหนังของหมูหรือเนื้อวัวคลุกกับเครื่องเทศ เชื้อกรดแลคติกจำนวน 124 ไอโซเลทถูกแยกจาก 16 ตัวอย่างจากภาคกลางและตะวันออกเฉียงเหนือของประเทศ เชื้อจำนวน 65 ไอโซเลทถูกคัดเลือกมาศึกษาจัดอนุกรมวิธานตามลักษณะทางสัณฐานวิทยาและคุณสมบัติด้านสรีรวิทยาและชีวเคมี และผลการวิเคราะห์ลำดับยีนสิบหกเอส ผลการศึกษาพบเชื้อที่ไม่เคยถูกคัดแยกได้จากไส้กรอกหมัก (ไส้กรอกเปรี้ยวและหม่ำ) มาก่อน โดยพบสายพันธุ์ที่จัดอยู่ในสกุล *Weissella cibaria/kimchii* (5ไอโซเลท) สกุล *W. confusa* (3 ไอโซเลท) สกุล *Pediococcus pentosaceus* (20ไอโซเลท) สกุล *P. acidilactici* (2ไอโซเลท) สกุล *Lactobacillus fermentum* (3 ไอโซเลท) สกุล *L. brevis* (4 ไอโซเลท) สกุล *L. farciminis* (4 ไอโซเลท) สกุล *L. plantarum* (23 ไอโซเลท) และสกุล *L. sakei* (1 ไอโซเลท) สายพันธุ์ที่คัดแยกผลิตกรดแลคติกชนิดดีแอลยกเว้นสายพันธุ์สกุล *L. farciminis* ซึ่งผลิตกรดแลคติกชนิดแอล นอกจากนี้รายงานการศึกษาได้อภิปรายลักษณะการกระจายของสกุลและความสามารถในการสร้างสารยับยั้งเชื้อจุลินทรีย์ประเภทไดอะซีติลและแบคทีรีโอซินของสายพันธุ์ที่คัดแยกได้

Key Words- fermented sausage; lactic acid bacteria; *Weissella*; *Lactobacillus*; *Pediococcus;* 16S DNA; diacetyl; bacteriocin.

EXECUTIVE SUMMARY

This study first reported the isolation of *W. cibaria/kimchii*, *W. confusa*, *L. brevis*, and *L. farciminis*, from Sai-krog-prieo, and *P. pentosaceus*, *P. acidilactici*, *L. fermentum*, *L. brevis*, and *L. sakei* from mum. It was noted that *L. plantarum* was distributed in at least 8 (of 12) samples and *P. pentosaceus* in 5 (of 12) samples used in this study. On the other hand, *Weissella* sp., *P. acidilactici*, *L. fermentum*, *L. brevis*, *L. farciminis* and *L. sakei* were isolated from much fewer samples. Tested strains of each group produced DL-lactic acid from D-glucose, except those identified as *L. farciminis* which produced L-lactic acid. Four strains, i.e., CP2-3A, CP2-11, CP3-16 and CP3-11, produced diacetyl, while the other isolates did not. The inhibition tests against *Escherichia coli, Bacillus subtillis, Salmonella spp.* and *Staphylococcus aureus* showed that 5 from 65 isolates could inhibit the growth of *B. subtillis* (CP1-15, CP2-11, CP3-1, CP7-3, CP10-3) and *S. aureus* (CP1-15, CP7-3) and they were identified as *W. confusa* (CP3-1), *P. acidilactici* (CP7-3), *L. plantarum* (CP1-15, CP2-11, CP10-3). Using Broth dilution test method, the MIC was found to be 1 ml filtrate/ml total volume. Percentages of survival of *B. subtilis* at MIC by CP1-15, CP2-11, CP3-1, CP7-3 and CP10-3 were 1.08, 0.44, 0.16, 1.28 and 0.23 BU/ml, respectively and those of *S. aureus* at MIC by CP1-15 and CP7-3 were 1.18 and 1.02 BU/ml, respectively.

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CHAPTER 1
INTRODUCTION

1.1 Motivation

Diversity of microbial forms in nature allows us to discover the microorganisms that produce varieties of bioactive compounds, or that have useful characteristics for specific purposes. For example, some microorganisms produce metabolites, which promote animal and plant growth. Some have impact on environment control. Some can tolerate harsh environment e.g. low pH, high temperature, and high ethanol concentration environment. Asian countries possess numerous potential sources of novel microbial species due to their different environment and geography. However, "research activities in this science have only recently been embarked upon by microbiologists from Asian countries as collaborative ventures" (Nga et al., 1999), and more researches have to be initiated in order to discover, isolate, and characterize novel strains.

Lactic acid bacteria (LAB) are interesting species because of their industrial importance. They are used all over the world in a large variety of industrial food fermentation. They possess lactic acid formation capacity, which results in rapid acidification of the food raw material, a critical parameter in the preservation of food products (Kleerebezem et al., 2002). LAB also contribute to other product characteristics like flavor, aroma, and texture, and various product characteristics may be obtained from different type strains. LAB also have agricultural importance. They have the ability to suppress *Fusarium* propagation, which is a harmful microorganism causing disease problems in continuous cropping. Moreover, lactic acid bacteria enhance the breakdown of organic matter such as lignin and cellulose, and ferment these materials without causing harmful influences to undecomposed organic matter (http://www.agriton.nl/apnanman.html).

LAB have been used for the production of lactic acid at a commercial scale. Lactic acid is the smallest natural molecule to exhibit optical activity. It exists in two isomeric forms, D(-)-lactic acid and L(+)-lactic acid (Demirci and Pometto, 1995).

Both isomeric forms and their mixtures (D,L-lactic acid) are used in copolymerization of polylactic acid, a biodegradable plastic. Adjustment of the composition of the copolymer enables bioadsorbability to be regulated. Lactic acid produced from LAB is also used as the starting material for the production of other organic acids such as polyproplyeneglycol, ethanol, and acetylaldehyde. In addition, with the arrival of genomics and other high throughput technologies, metabolic engineering of LAB that can reroute carbons to the end-products other than lactic acid is promising. These end-products include diacetyl, alanine, exopolysaccharides, and folic acid (Kleerebezem et al., 2002).

Thailand possesses many kinds of fermented food, an abundant source of LAB which play an important role for the ripening process. A popular traditional Thai fermented food is raw fermented sausages (local name: *Sai-krork-prieo* or *Sai-krork-ee-saan*) and related products such as *mam* (fermented beef or pork sausage). Sai-krork-prieo is a traditional Thai fermented sausage, consumed all around the country and produced from minced pork, sliced pork skin, garlic, pepper, spices, salt and sugar. Mum is a product similar to Sai-krork-prieo, but can be made from beef as well as pork, and is popular only in the Northeastern of Thailand. The chemical composition of the food products is shown in Table 1-1 (Phithakpol et al., 1995). However, the production of fermented sausages in Thailand has utilized the naturally occurring LAB, resulting in various and inconstant products. Instead, the use of well-studied starter cultures would result in more constant and food-safe products.

The aim of this study is the isolation and taxonomic determination of lactic acid bacteria from Thai fermented sausages by systematic molecular and physiology studies. In addition, their capability of producing L-lactic acid and metabolic compounds with antimicrobial properties, i.e, diacetyl and bacteriocin will be examined. The systematic studies include morphological, cultural, biochemical, physiological characterizations, characterizations of cell wall compositions, and characterizations of lactic acid isomers and the other metabolic products. The strains found in this study will have a potential use for the establishing of the so-called “functional foods” (Leroy et al., 2002; Reid, 1999).By isolating and identifying our

own bacteria, Thailand can save a lot of money spent in buying foreign bacteria used in industrial or medical applications.

Table 1-1. The chemical composition of Thai fermented sausages (Phithakpol et al., 1995)

Moisture (%)	Protein (%)	Fat (%)	Fibre (%)	Ash (%)	NaCl (%)	Total inv. sugar (mg %)	Acidity as lactic acid (%)	pH	a_w
31.4-38.5	11.8-14.0	43.0-43.5	0.1-0.8	2.0	1.1-1.9	trace-3.6	0.7-1.7	4.4[a]-5.8	0.96

[a]The lower value of 4.2 was found in this study.

1.2 Objectives

1.2.1 To isolate LAB strains from fermented sausage (as known by local name as *Sai-krork-prieo* or *Sai-krork-ee-saan*) and related products such as *mam* (fermented beef or pork sausages);

1.2.2 To characterize the strains based on their phenotypic and chemotaxonomic characterizations, i.e., morphological and cultural characteristics, biochemical and physiological characteristics, peptidoglycan type of cell wall, and 16S rRNA sequencing similarity;

1.2.3 To characterize their metabolic products e.g. food flavor, diacetyl, bacteriocin, and L- or D- lactic acid.

1.3 Significances and Contribution

This project will make a contribution to cultural collections in Thailand. Culture collections are needed as the depository and for further studies and applications of the cultures. The application of the cultures in turn will help reduce the expenses of the country on buying foreign bacteria. This project will provide the information that will be useful fundamental knowledge for subsequent strain utilization such as food flavor starter, producer of pure lactic acid isomer, or other useful metabolites e.g. diacetyl, and bacteriocin. Isolation and identification of LAB with high ratio of pure lactic acid isomers are crucial because in some applications,

only D- or L-lactic acids can be used. For example, in copolymerization of polylactic acid, certain ratios of D- and L-lactic acid are required because the composition of the copolymer regulates bioabsorbability property. In food application, only L-lactic acid is acceptable, while D-lactic acid is known to be toxic. The separation step for pure isomer can be costly, and the mixture of both isomers will lower the product yield. Therefore, the strains that produce pure isomers can be advantageous. The strains found in this study will have a potential use for the establishing of the so-called "functional foods".

CHAPTER 2
LITERATURE REVIEW

2.1 Lactic Acid Bacteria

The lactic acid bacteria (LAB) are a group of Gram-positive rod- and coccus-shaped organisms that have less than 55% mol G+C content in their DNA. They are non-sporeforming, non-motile and produce lactic acid as their major end product during the fermentation of carbohydrates. The taxonomy of LAB has changed considerably during the last few years and, at present, this group comprises the following genera: *Aerococcus*, *Alloicoccus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella, Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson 1998; Stiles and Holzapfel, 1997). Fig. 2.1 illustrates major phylogenetic groups of lactic acid bacteria and related gram-positive bacteria (Stiles et al., 1997)

LAB are widely distributed in the nature. They could be isolated from soils, waters, plants, silages, waste products, and also from the intestinal tract of animals and humans. The lactic acid fermentation, which these bacteria perform has long been known and applied by the humans for making different foodstuffs. It plays an essential role in the production of all dairy products and is involved in the production of many other foods and drinks – sausages, pickles, sorghum, maize, and millet beers in Africa etc. (Haggblade and Holzapfel, 1989). Since decades by these processes the application of sell-studied starter cultures was established in many countries. They should possess stable fermentation characteristics and should be resistant to bacteriophages (Lee, 1996). However, the local fermented products, the species composition of lactic acid bacteria is more various and inconstant when compared with those of the trade products (Tserovska et al., 2002). The “wild” strains, in biotechnological aspect are perspective as bacteriocin producers (Leisner et al., 1999; Vaughan et al., 2001) and probiotics (Holzapfel et al., 2001; Kitazawa et al., 2001).

2.2 The genera *Lactobaciilus* and *Pediococcus* (Excerpt from Stiles et al., 1997)

2.2.1 The genus *Lactobacillus*

The classical divisions of the lactobacilli was based on their fermentative characteristics: (1) obligately homofermentative; (2) facultatively heterofermentative; and (3) obligately heterofermentative. This division suited the interests of food microbiologists. Several lactobacilli of groups 1 and 2 and some of the heterofermentative group 3 lactobacilli are either used in fermented foods, but group 3 are also commonly associated with food spoilage. In Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986) the genus *Lactobacillus* was described with a heterogeneous group of 'regular non-sporing gram-positive rods.' Four of the seven genera are facultative anaerobes, of which *Lactobacillus* and *Erysipelothrix* are catalase negative and *Brochothrix* and *Listeria* catalase positive. The four genera are important in foods, but they represent unrelated groups of bacteria, including those associated with: (1) food fermentation, *Lactobacillus*; (2) food spoilage, *Brochothrix* and *Lactobacillus*; and (3) potential or established foodborne infections, *Erysipelothrix* and *Listeria*. The genus *Lactobacillus* is heterogeneous with 33-55 mol% G+C content in the DNA (Collins et al., 1991; Hammes and Vogel, 1995). Generally, it is suggested that not more than 10% range in G+C content should exist in a well defined genus (Vandamme et al., 1996).

With the development of phylogenetic analysis in the 1980s there have been many changes to the genus *Lactobacillus*. *Lb. catenaformis*, *Lb. rogosae* and *Lb. vitulinus* (Stackebrandt and Teuber, 1988) and several other former species are no longer members of the genus. *Lb. minutus*, *Lb. uli* and *Lb. rimae* are related to each other and to *S. parvulus*, justifying their classification in a new genus *Atopobium* that is more closely related to the bifidobacteria than the lactic acid bacteria (Collins and Wallbanks, 1992). *Lb. maltaromicus* is now *C. piscicola* and currently proposed as *C. maltaromicus* (Collins et al., 1991) and *Lb. carnis* and *Lb. divergens* were transferred to the new genus *Carnobacterium* (Collins et al., 1987). Pot et al. (1994) ably documented the LAB and their valid descriptions, including the names and synonyms for the lactobacilli. Homologies within the genus have resulted in the following synonyms being recognized and incorporated into the nomenclature of the lactobacilli: *Lb. murinus* as a synonym for *Lb. animalis* (Dent and Williams, 1982),

Lb. sake and *Lb. bavaricus* (Kagermeier-Callaway and Lauer, 1995), *Lb. helveticus* for *Lb. jugurti* (Dellaglio et al., 1973), *Lb. mali* for *Lb. yamanashiensis* (Carr and Davies, 1970), *Lb. fermentum* for *Lb. cellobiosus* (Vescovo et al., 1979) and *Lb. fructivorans* for *Lb. trichodes* (Weiss et al., 1983a). New species (*Lb. kefirgranum* and *Lb. parakefir*) from kefir grains (Takizawa et al., 1994) and sourdough bread (*Lb. pontis* and *Lb. panis*) (Vogel et al., 1994; Wiese et al., 1996) have been described since the listing by Pol et al. (1994). The present number of *Lactobacillus spp*. remains greater than 50, despite the transfer of five heterofermentative species of *Lactobacillus* to the new genus *Weissella* by Collins et al. (1993).

The lactobacilli are strictly fermentative and have complex nutritional requirements. They grow in and are associated with many different habitats (Table 2-1). They are aciduric or acidophilic, producing pH 4.0 in foods containing a fermentable carbohydrate. As a result, they often suppress growth or kill other bacteria. It is generally accepted that lactobacilli grow up to a maximum pH of 7.2, although exceptions with respect to substrate and strain exist. Lactobacillus are used as starter cultures for several varieties of cheese, fermented plant foods, fermented meats, in wine and beer production, sourdough bread and silage. Unlike the coccus-shaped lactics, the lactobacilli were not separated into different genera based on their homo- or heterofermentation of hexoses. The current taxonomic status of the lactobacilli based on the classical phenotypic subdivision shown in Table 2-2 was derived from the reviews by Hammes et al. (1991). Pot et al. (1994) and Vandamme et al. (1996).

Group I includes the obligatory homofermentative lactobacilli that ferment glucose exclusively to lactic acid and do not ferment pentoses or gluconate. It represents Orla-Jensen's thermobacteria and the important food associated species: *Lb. acidophilus*, *Lb. delbrueckii* and *Lb. helveticus*, as well as *Lb. farciminis* and *Lb. kefiranofaciens.*

Lb. delbrueckii forms an important complex of bacteria that previously had species status as *Lb. delbrueckii*, *Lb. bulgaricus*, *Lb. lactis* and *Lb. leichmanii.* They have 80% DNA homology and have therefore been reclassified as *Lb. delbrueckii* subspp. *delbrueckii*, *bulgaricus* and *lactis* (Weiss et al., 1983b). *Lb. leichmanii* has

been incorporated with *Lb. delbrueckii* subsp. *lactis*. All of these organisms are associated with plant and dairy foods that are fermented at high temperatures of 45-50^{o}C. *Lb. delbrueckii* subsp. *bulgaricus* is a starter strain for yoghurt manufacture (see

Table 2-1 Habitats of the genus *Lactobacillus*

Humans
Oral cavity
Intestinal tract
Vagina
Other habitats
Plants and plant materials
Soil, water, sewage and manure
Food fermentations (milk, meat and vegetable)
Cereal products
Silage
Food spoilage
Beer
Fruit and grain mashes
Marinated fish
Sugar processing
Milk
Meat and meat products
Fermented beverages

Table 2-2 Major divisions within the genus *Lactobacillus* based on phenotypic characteristics

Group 1	Group 2	Group 3
Obligate homofermenters	Facultative heterofermenters	Obligate heterofermenters
Lb. acidophilus	***Lb. acetotolerans***	***Lb. brevis***
Lb. amylophilus	*Lb. agilis*	***Lb. buchneri***
Lb. amylovorus	***Lb. alimentarius***	***Lb. collinoides***
Lb. aviaries	***Lb. bifermentans***	***Lb. fermentum***
subsp. *araffinosus*	***Lb. casei***	***Lb. fructivorans***
subsp. *aviaries*	*Lb. coryniformis*	*Lb. fructosus* [b]
Lb. crispatus	subsp. *coryniformis*	*Lb. hilgardii*
Lb. delbrueckii	subsp. *torquens*	*Lb. kefir*
subsp. ***bulgaricus***	***Lb. curvatus***	***Lb. malefermentans***
subsp. ***delbrueckii***	*Lb. graminis*	*Lb. oris*
subsp. ***lactis***	*Lb. hamsteri*	***Lb. panis***[a]
Lb. farciminis	***Lb. homohiochii***	***Lb. parabuchneri***
Lb. gallinarum	*Lb. intestinalis*	***Lb. parakefir*** [a]
Lb. gasseri	*Lb. murinus*	***Lb. pontis*** [a]
Lb. helveticus	***Lb. paracasei***	***Lb. reuteri***
Lb. jensenii	subsp. ***paracasei***	***Lb. sanfrancisco***
Lb. johnsonii	subsp. ***tolerans***	***Lb. suebicus***
Lb. kefiranofaciens	***Lb. paraplantarum***[a]	***Lb. vaccinostercus***
Lb. kefirgranum[a]	***Lb. pentosus***	***Lb. vaginalis***
Lb. mali	***Lb. plantarum***	
Lb. ruminis	***Lb. rhamnosus***	
Lb. salivarius	***Lb. sake***	
subsp. *salicinus*		
subsp. *salivarius*		
Lb. sharpeae		

Data collected by Hammes and Vogel (1995). Pot et al. (1994) and Vandamme et al. (1996). Bold face, lactobacilli of importance in foods and as probiotics.

[a] Species added since the review by Pot et al. (1994).

[b] *Lb. fructosus* classified with the *Leuconostoc* group of lactic acid bacteria.

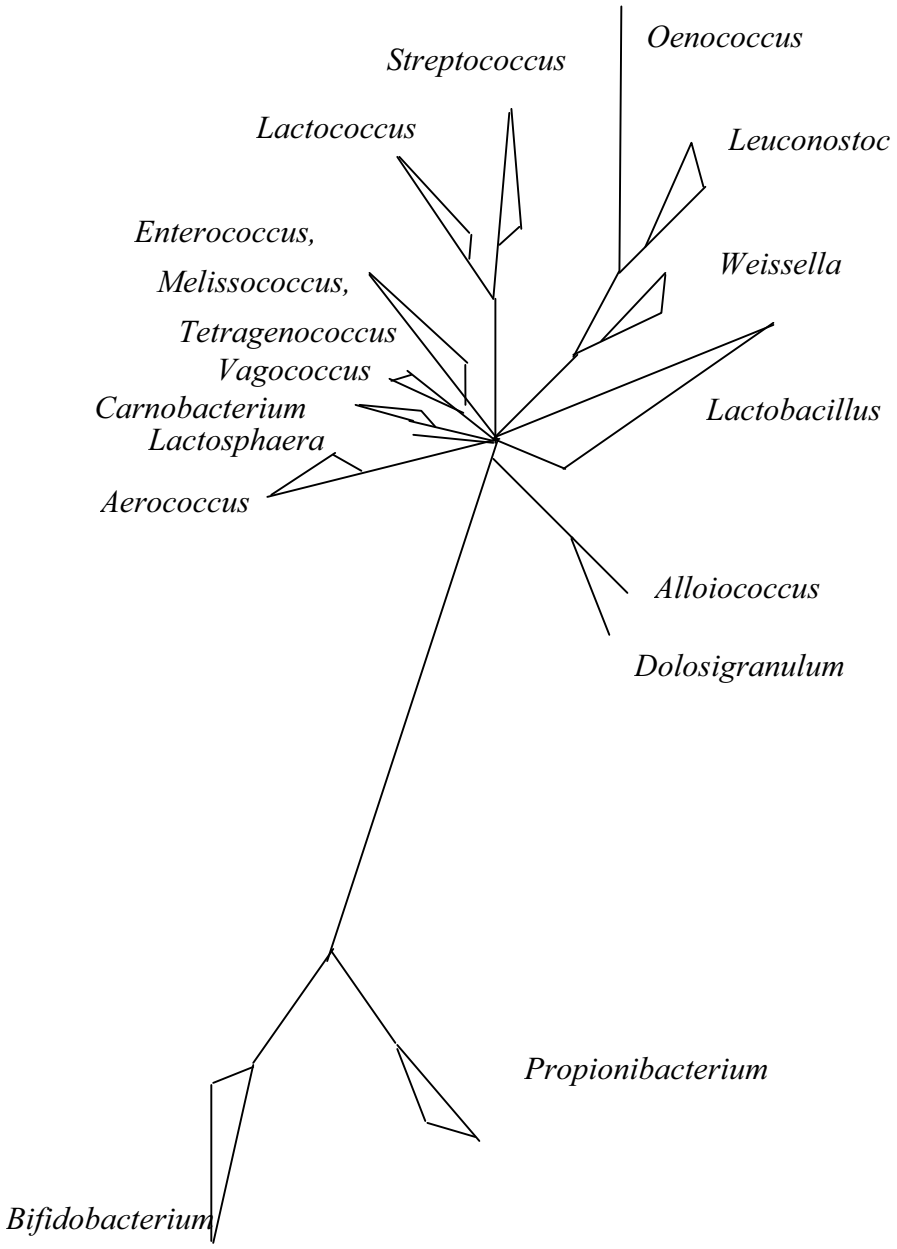

Figure 2.1. Major phylogenetic groups of lactic acid bacteria and related gram-positive bacteria with low (upper part) and high (lower part) mol% G+C in the DNA (Stiles et al., 1997)

S. thermophilus earlier). *Lb. delbrueckii* subsp. *bulgaricus* is also used in association with *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus* and *S. thermophilus* as starter cultures for Swiss-type Emmental and Gruyére chesses and Italian-type Gorgonzola, Mozzarella, Provolone and Caciocavallo cheeses (Hammes et al., 1991). *Lb. delbrueckii* subsp. *delbrueckii* may be associated with the fermentation of sourdough bread.

The original isolation of *Lb. acidophilus* in 1900 was from infant faces but strains reported as *Lb. acidophilus* based on biochemical characteristics were shown to be heterogeneous on the basis of DNA-DNA homology (Lauer et al., 1980). They were divided into two main genotypic groups designated A and B (Johnson et al., 1980), or 1 and 2 (Lauer et al., 1980) Altogether six homology groups within the *Lb. acidophilus*-group have been proposed as valid species. These species cannot be differentiated by simple phenotypic tests, although a number of growth features may be useful for their characterization (Mitsuoka, 1992). *Lb. acidophilus* and *Lb. gasseri* can be differentiated by 23S rRNA oligonucleotide probes (Pot et al., 1993). *Lb. acidophilus* is used in the production of acidophilus milk. It is considered as important representative of probiotic bacteria, although these are not restricted to *Lb. acidophilus sensu stricto* (Gasser and Mandel, 1968) or other species of the *Lb. acidophilus* homology group (Johnson et al., 1980; Lauer et al., 1980; Cato et al., 1983). *Lb. acidophilus* BG2 FO4 that was studied by Kleeman and Klaenhammer (1982) has been identified as *Lb. johnsonii* (Fujisawa et al., 1992). This strain adheres to human foetal intestinal cells *in vitro*, indicating its ability of adherence to epithelial cells *in vivo*. Especially, *Lb. johnsonii* appears to be typically used in novel type yoghurts with claims on their healthfulness (Holzapfel et al., 1996). This species and other of the *Lb. acidophilus*-group are currently being studied for their typical 'probiotic' properties. To avoid the probiotic potential of these bacteria falling once again into disrepute, reliable basic information on microbial antagonism, survival of the stomach passage, growth and metabolic behavior in the small and large intestines and attachment to the epithelial cells of the gut is required.

Lb. helveticus is considered closely related to *Lb. acidophilus* (Yang and Woese, 1989; Collins et al., 1991) and was established as synonymous with *Lb. jugurti* (Pot et al., 1994). *Lb. helveticus* is distinct from *Lb. delbrueckii* and is an

important organism in Swiss- and Italian-type cheeses. *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. acidophilus* and *Lb. farciminis* are obligately homofermentative lactobacilli associated with the fermentation of sourdough bread. *Lb. keforanofaciens* is an important organism associated with kefir grains. It produces the kefiran polymer that forms the matrix of the kefir grains. Other bacteria associated with kefir are *Lb. acidophilus*, the obligately heterofermentative *Lb. kefir* and the yeast *Candida kefir* (Toba et al., 1986). A study of *Lactobacillus* isolates from kefir grains (Takizawa et al., 1994) resulted in the description of a new heterofermentative species *Lb. parakefir*.

Group 2 includes the facultatively heterofermentative lactobacilli that ferment hexoses to lactic acid and may produce gas from gluconate but not from glucose. They also ferment pentoses by an inducible phosphoketolase to produce lactic and acetic acids. Important food-associated species in this group include *Lb. casei* and *Lb. plantarum.*

Lb. casei is associated with many habitants, including dairy products, silage, the human mouth and intestine and sewage. It is specifically associated with sourdough bread and some brined cheese fermentations and it can cause spoilage of cheeses y fermentation of citrate to release carbon dioxide (Hammes et al., 1991). The species was poorly defined and contained five subspecies based on phenotypic characteristics. Studies of DNA homology by Collins et al. (1989) indicated that the majority of organisms designated *Lb. casei* subsp. *casei*, together with *Lb. casei* subsp. *alactosus*, *pseudoplantarum* and *tolorans*, have high levels of DNA relatedness but they are reported to be distinct form the type strain of *Lb. casei* subsp. *casei*. Collins et al. (1989) proposed that this homology group be given species status as *Lb. paracasei* with subspecies *paracasei* to contain all of the subspecies, except subspecies *tolerans* which was proposed as *Lb. paracasei* subsp. *tolerans*. Furthermore, it was proposed that *Lb. casei* subsp. *rhamnosus* should be elevated to species rank as *Lb. rhamnosus*. The close relationship between there strains was confirmed by 16S RNA sequence homology (Collins et al., 1991); however, the species name *Lb. paracasei* was questioned by Dellaglio et al. (1991), since the type strain (ATCC 393) of *Lb. casei* ssp. *casei* shows only low levels of DNA homology with other strains of *Lb. casei* ssp. *casei* and of *Lb. paracasei* ssp. *paracasei*. On the

other hand, strain ATCC 393 exhibits high DNA homology with *Lb. rhamnosus* ATCC 15820, which was the original 'type strain' of *Lb. zeae*, Kuznetsov, 1959, isolated from corn steep liquod. After initial rejection of their request, Dicks et al. (1996) now finally recommended the reclassification of *Lb. casei* spp. *casei* ATCC 393 and *Lb. rhamnosus* ATCC 15820 as *Lb. zeae* and strain ATCC 344 as the neotype strain of *Lb. casei* ssp. *casei*. They also recommended the rejection of the species name *Lb. paracasei*.

Lb. plantarum is used as a starter organism in some fermented sausages and cereal products; it is a part of the adventitious LAB growing in fermented vegetable and meat products and it is a spoilage organism in citrus juice, wine and some cheeses (Hammes et al., 1991). It was considered an important organism in the natural fermentation of meats, although this assumption may be based on the false classification of atypical streptobacteria. In North America its use as a meat starter culture has been superseded by *P. acidilactici* and in Europe mainly by *Lb. curvatus* and *Lb. sake*. *Lb. plantarum* was classified with Orla-Jensen's streptobacteria because the strains studied were not able to grow at 45^{o}C. Although some strains have been reported to grow at 45^{o}C, the general ability to grow at 15^{o}C serves as confirmation for the allocation of *Lb. plantarum* to the streptobacteria. It has been a 'catch-all' species for atypical lactobacilli. The strains classified as *Lb. plantarum* include two DNA homology groups. *Lb. plantarum* and *Lb. pentosus* have long been considered synonymous, but Zanoni et al. (1987) revived the presently valid species name *Lb. pentosus*. Collins et al. (1991) reported high 16S rRNA sequence similarity between these species, confirming that they are closely related. Facultatively heterofermentative lactobacilli with similar physiological characteristics to *Lb. plantarum* isolated as beer contaminants and from faeces were tested for their relatedness to *Lb. plantarum* and *Lb. pentosus*. Based on the lack of DNA relatedness, a new species *Lb. paraplantarum* has been proposed (Curk et al., 1996).

A third subgroup of the group 2 lactobacilli includes *Lb. curvatus* and *Lb. sake* which have important associations with foods. Most strains of *Lb. bavaricus* that produce L(+)-lactate show high DNA homology with *Lb. sake* and some with *Lb. curvatus*, and they are considered racemase-defective variants of these bacteria. Because of heterogeneity within these two species, two subgroups for each have been

suggested (Klein et al., 1996). These bacteria are an important part of the adventitious microflora of modified atmosphere and vacuum packaged meats and meat products that are stored at refrigeration temperatures (<5^{o}C) and they are beneficial in sauerkraut, brined fruit and vegetable fermentations. *Lb. curvatus* and *Lb. sake* are important starter cultures in fermented meat products (Hammes et al., 1991; Vogel et al., 1993) and typically dominate the microbial population of these products. Sulphide-producing strains of *Lb. sake* have been implicated in spoilage of chill stored, vacuum packaged meat (Egan et al., 1989) Co-culture of bacteriocinogenic *Leuc. gelidum* and sulphide-producing *Lb. sake* on chill stored, vacuum packaged beef resulted in inhibition of sulfide spoilage (Leisner et al., 1996). *Lb. sake* (comprising ca. 60%) and Lb. curvatus (with ca. 20%) were found to dominate the spoilage association of vacuum packaged Frankfurter, Vienna and related sausage types (German, 'Brühwurst'), causing sliminess, exudation, gas-production and off-flavours (Holzapfel and Gerber, 1986). They were also shown to be the major cause of ropiness in Finnish ring sausages (Korkeala et al., 1988; Mäkelä et al., 1992). The type strain of *Lb. homohiochii* that is part of the LAB microflora of sourdough bread is closely related to *Lb. sake* (Dellaglio et al., 1975). DNA:DNA hybridization studies of the species in this subgroup have shown that they are closely related, but their species status has been maintained. Particular atypical streptobacteria, designated non-acidulic lactobacilli, were described by Shaw and Harding (1984) as an important group of bacteria growing in vacuum packaged meats (see genus *Carnobacterium*).

Group 3 includes the obligatory heterofermentative lactobacilli that ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. The production of gas from glucose is a characteristic feature of these bacteria. The most important obligate heterofermentative *Lactobacillus* associated with food fermentations is *Lb. sanfrancisco* which converts maltose to lactic and acetic acids and various flavor compounds in sourdough bread. New species of lactobacilli associated with the rye sourdough fermentation have been described as *Lb. pontis* (Vogel et al., 1994) and *Lb. panis* (Wiese et al., 1996). *Lb. brevis* and *Lb. fermentum* are also associated with lactic fermentations of sourdough. *Lb. kefir* is one of the bacteria involved with kefir grains (Hammes et al., 1991). Among the heterofermentative lactobacilli, *Lb. reuteri* has attracted considerable interest because of its antimicrobial metabolite 'reuterin' (3-hydroxypropionaldehyde) that is active against a broad spectrum of

microorganisms. A commercial milk product based on *L. reuteri* (BRA-milk) was introduced in Sweden in 1991 (Ståhl and Molin, 1994). *Lb. reuteri* and *Lb. fermentum* are difficult to distinguish by conventional physiological tests, but genetically they are not closely related, as indicated by the difference in mol% G+C of the DNA (40-42 and 52-54, respectively), and ornithine in the peptidoglycan of *Lb. fermentum* (Kandler and Weiss, 1986). Some lactobacilli, especially among the group III, cause spoilage of foods, such as *Lb. bifermentans* that causes cracking defects in Gouda and Edam cheeses due to gas production, *Lb. brevis* that causes spoilage of citrus fruit, wines and beer, *Lb. fructivorans*, *Lb. brevis* and *Lb. buchneri*, causing spoilage of mayonnaise dressings and acetic acid (vegetable) preserves (Baumgart, 1965; Dakin and Radwell, 1971) and *Lb. viridescens* (now *Weissella viridescens*) that causes greening of cured meat products (Hammes et al., 1991). The heterofermentative lactobacilli that lack aspartic acid or diaminopimelic acid in their peptidoglycan appear phylogenetically different from other lactobacilli and were grouped with *Leu. paramesenteroides* into a new genus *Weisella* (Collins et al., 1993).

2.2.2 The genus *Pediococcus*

As a result of their association with beer spoilage, the pediococci were among the first bacteria to be studied by Louis Pasteur. Tetrad formation and spherical shape served as key characteristics for their early recognition. They were the only LAB that divide in two planes to produce tetrads or pairs, but taxonomic changes have increased the number of tetrad forming genera to three. Pediococci are most likely to be confused with micrococci because of morphological similarities, pseudocatalase production and salt tolerance (Garvie, 1986) and also with the aerococci. The pediococci are homofermentative and, with the exception of *L. dextrinicus* which produces L(+)-lactic acid all species produce DL-lactate from glucose. The pediococci of beer and plant origin were included in one species as *P. cerevisiae*, but studies on isolates from these two sources were shown to be different and they were assigned to *P. damnosus* and *P. pentosaceus*, respectively (Raccach, 1987). Most of the strains designated *P. cerevisiae* that were used as meat starters have been reclassified as *P. acidilactici*. In Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986) eight species are recognized (Table 2-3). The genus *Pediococcus* was shown to be phylogenetically heterogeneous (Collins et al., 1990). The type strain *Pediococcus damnosus* forms a closely related group with *P. acidilactici*, *P. parvalus*

and *P. pentosaceus*. Despite their morphological distinctiveness, Collins et al. (1991) clearly demonstrated a relationship between the pediococci and lactobacilli of the *Lb. casei* group. *P. acidilactici*, *P. damnosus*, *P. parvulus* and *P. pentosaceus* form an evolutionary grouping with *Lb. pentosus*, *Lb. brevis* and the *Lb. buchneri* complex. *P. dextrinicus* is related but peripheral to other members of the genus *Pediococcus* and shares phylogenetic relatedness to *Lb. coryniformis* and *Lb. bifermentans* (Collins et al., 1990, 1991).

Pediococci are most often found in low numbers, together with leuconostocs and lactobacilli, on plant materials, in various foods and as spoilage agents in alcoholic beverages such as beer. The pediococci are important starter bacteria in fermented sausages of some regions. Some of the widely used starter strains such as *P. acidilactici* and *P. pentosaceus* produce bacteriocins. Although they are of economic importance as starter cultures, not all of the fermentation pathways utilized by pediococci are clear. They require a fermentable carbohydrate for growth and grow poorly in milk because lactose is not readily utilized. Fermentation of glucose follows the Embden-Meyerhof pathway with DL- or L-(+)-lactate as the major end product under optimal conditions. Pyruvate can be diverted to other end products and diacetyl/acetoin is often produced by *P. damnosus*, while *P. pentosaceus* produces equimolar amounts of lactate and acetate from pentoses (Fukui et al., 1957).

Some species exhibit extreme tolerances to temperature, pH and NaCl. For example, *P. acidilactici* grows at 50^{o}C and is heat tolerant; *P. damnosus* and *P. parvulus* are acid and hop tolerant and grow at low temperatures but generally require more anaerobic growth conditions than other strains. Production of diaetyl is diagnostic for *P. damnosus*, but under growth limiting conditions acetoin and/or diacetyl can be produced. *P. pentosaceus* and *P. acidilactici* are closely related species that may not be clearly differentiated by phenotypic characteristics, but they are differentiated by DNA-DNA homology (Garvie, 1986b). *P. halophilus* grows in 18% NaCl, but it has been transferred to a new genus *Tetragenococcus* that is more closely related to the enterococci and carnobacteria than to the pediococci and lactobacilli (*vide supra*). Strains belonging to *P. urinaeequi* have been transferred to *Aerococcus*.

Table 2-3 Species of *Pediococcus* and related tetrad-forming bacteria and their common habitats

Bacterial name	Habitat
P. damonosus	Breweries (beer cloudiness); wine and cider
P. dextrinicus	Beer, silage
P. parvulus	Sauerkraut, silage
P. inopinatus	Sauerkraut, beer
P. pentosaceus	Vegetable material, fermented sausages, milk and dairy products
P. acidilactici	Vegetable material, milk and dairy products
Aerococcus urinae-equi[a]	Not associated with foods
Tetragenococcus halophilus[b]	Soy sauce, pickling brines (requires salt for growth)

[a] Previously *P. urinae-equi.*

[b] Previously *P. halophilus.*

2.3 Diacetyl

The diketone, diacetyl, is a major flavour metabolite produced by lactic acid bacteria family, including *Streptococcus*, *Leuconostoc*, *Lactobacillus*, *Pediococcus* and *Oenococcus*. The dikeone (CH_3-CO-CO-CH_3), diacetyl, or 2,3-butanedione, is sometimes referred as biacetyl. Diacetyl is a major flavour compound in dairy products and extensive research has been done in this topic. However, there are only few researches on diacetyl from LAB found in various fermented food products in Thailand. Pakdeeto (2002) and Pakdeeto et al. (2003) reported the isolation of diacetyl-producing LAB from pasteurized milk, raw cow's milk, and fermented foods, including fermented mango and guava, vegetables, noodles. Their results showed that 115 isolates out of 137 strains could produce diacetyl ranged from 0.01 to 6.49 mM. Since diacetyl is important not only because of its responsibility for the buttery aroma and flavour in many fermented foods and beverages, but also because of its

antimicrobial properties (Ray, 1992), the isolation of diacetyl-producing LAB from various fermented food products in Thailand should be further studied.

2.4 Bacteriocins (Excerpts from Paul Ross et al., 2002, and Rodríguez et al., 2003)

The term "bacteriocin" comprises a large and diverse group of ribosomally synthesized antimicrobial proteins or peptides some of which undergo post-translational modifications (Nes and Eijsink, 1999). Although bacteriocins may be found in numerous Gram-positive and Gram-negative bacteria, those produced by lactic acid bacteria (LAB) have received particular attention in recent years due to their potential application in the food industry as natural preservatives. This trend reflects the increasing consumer awareness of the risks derived not only from food-borne pathogens, but also from the artificial chemical preservatives used to control them (Abee et al., 1995). In contrast, the use of LAB and/or their metabolites for food preservation is generally accepted by consumers as something "natural" and "health-promoting" (Montville and Winkowski, 1997).

Most LAB bacteriocins are small (< 6 kDa), cationic, heat-stable, amphiphilic, membrane-permeabilizing peptides that may be divided into two main classes (Nissen-Meyer and Nes, 1997). Class I bacteriocins (lantibiotics) contain unusual amino acids, such as lanthiocine and β-methyllanthionine, as a result of extensive post-translational modification. Class II bacteriocins do not undergo post-translational changes, with the exception of cleavage of the leader or signal peptide and, in some cases, formation of disulfide bridges. A third class (Class III) has been proposed (Klaenhammer, 1993) which includes large (>30 kDa) heat-labile antimicrobial proteins produced by LAB. Within any class of bacteriocin, there may be amino acid sequence homologies not only within the mature peptide, but also in the N-terminal leader region and the associated proteins involved in bacteriocin secretion and processing.

2.4.1 Class I: the lantibiotic family

These are generally small bacteriocins composed of one or two peptides of approximately 3 kDa. An unusual feature of this group is that they are post-

translationally modified to contain lanthionine, β-methyllanthionine and dehydrated amino acids, while at least two members of the group also contain D-alanine (Skaugen et al., 1994; Ryan et al., 1999). This latter amino acid residue is derived from dehydroalanine resulting from the dehydration of a serine residue. Nisin and lacticin 3147 both belong to the antibiotic family and inhibit a broad range of Gram-positve bacteria (De Vuyst and Vandamme, 1994b; Ryan et al., 1996). The lantibiotics were originally subdivided into two groups, A and B (Jung, 1991; Sahl and Bierbaum, 1998). Type A includes the elongated flexible molecules that have a positive charge and act via membrane depolarization, such as nisin. Type B lantibiotics are globular in structure and interfere with cellular enzymatic reactions and examples include mersacidin and actagardine. In addition, some members of the lantibiotic family (e.g. lacticin 3147) require two separate peptides for activity. The continual discovery of extra members of this complex group of peptides has meant that their classification has to be periodically updated (Sahl and Bierbaum, 1998; Guder et al., 2000). On the basis of alignment of mature peptides, Twomey et al. subdivided the lantibiotic peptides from LAB into six subgroups (Twomey et al., 2002) as follows: nisin A and nisin Z make up a single group as they do not share significant homology to any of the other members. The second group is the lacticin 481 group and is made up of just two members, lacticin 481 (Piard et al., 1992; Rince et al., 1994) and lacticin J49 (Hout et al., 1996). The third group, called the mersacidin group, is composed of plantaricin C (Turner et al., 1999), LtnA1 (one component of the two-peptide lacticin 3147) (Ryan et al., 1999) and Plwα (Holo et al., 2001). A fourth group is characterized by LtnA2 (the second component of lacticin 3147) and is composed of itself and Plwβ (Holo et al., 2001). Finally, the two peptides, $CylL_l$ and $CylL_s$, from the two-peptide cytolysin (Gilmore et al., 1994), form a group of their own, while lactocin S (Mortvedt et al., 1991) is also grouped separately.

2.4.2 Class II: small non-modified peptides

These are generally small unmodified peptides of < 5 kDa, which are subdivided into two groupings. The first (Class IIa) are the *Listeria* active peptides which are characterized by a YGNGV N-terminus. They are synthesized with a leader peptide attached which is removed by proteolytic processing, usually after a double

glycine residue, with examples including bacteriocins such as pediocin PA-1 (Henderson et al., 1992) and sakacin A (Holck et al., 1992). However, some Class II bacteriocins (e.g. acidocin B, Leer et al., 1995) use the sec system for secretion. As in the lantibiotic family, some Class II bacteriocins are composed of two separate peptides and are referred to as the Class IIb type. These two-component non-modified bacteriocins include lacticin F (Muriana and Klaenhammer, 1991) and lactococcin G (Nissen-Meyer et al., 1992).

2.4.3 Class III: large heat-labile proteins

The class III bacteriocins are the least well-characterized group and consist of heat-labile proteins which are generally > 30 kDa. The group includes Helvetin J produced by *Lactobacillus helveticus* (Joerger and Klaenhammer, 1986) and enterolysin produced by *Enterococcus faecium* (Nilson, 1999)

CHAPTER 3
MATERIALS AND METHOD

3.1 Sample collection, bacterial cell counts, and isolation method

Sixteen fermented sausage samples of various brands were collected from factories and local markets. The samples pHs were represented by the pH of the suspension of a 5-gram portion in 10 ml deionized water. The cell numbers were counted by a plating method on MRS agar (2.0 g glucose, 0.5 g yeast extract, 1.0 g peptone, 1.0 g beef extract, 0.5 g sodium acetate, 0.2 g ammonium citrate, 0.1 g tween 80, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot 4H_2O$, 0.1 g K_2HPO_4, 1.5 g agar, and 100 ml deionized water, final pH of 6.2-6.4) at 30^oC after a 3- to 5-day incubation as described in De Man et al., 1960. Pure cultures were obtained by streaking cultured cells on MRS agar plates with 0.2% $CaCO_3$.

3.2 Phenotypic characteristics

Cell form, cell size, cell arrangement, Gram stain, spore formation, motility, and colony appearances were examined according to Hucker, and Conn (1923) and Whittenbury (1963). Growth at different temperature (30, 45, and 50^oC), at different starting pHs (3.5, 4.0, 4.5, 8.0, 8.5 and 9.6), and at different concentrations of NaCl (4, 6, 8, and 10%) were tested by using MRS broth (2.0 g glucose, 0.5 g yeast extract, 1.0 g peptone, 1.0 g beef extract, 0.5 g sodium acetate, 0.2 g ammonium citrate, 0.1 g tween 80, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot 4H_2O$, 0.1 g K_2HPO_4, and 100 ml distilled water, final pH of 6.2-6.4). Half strength MRS (MRSH; 1.0 g glucose, 1.0 g yeast extract,1.5 g peptone, 1.0 g beef extract, 0.5 g sodium acetate, 0.2 g ammonium citrate, 0.1 g tween 80, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot 4H_2O$, 0.1 g K_2HPO_4, and 100 ml deionized water, final pH of 6.2-6.4) broth was used for subculturing the isolates and for determining the final pH after a 5-day incubation.

3.2.1 The production of gas from D-glucose

The production of gas from D-glucose was examined by using MRS broth with a Durham tube. Each strain was cultivated in 3 ml MRS broth without

ammonium citrate in a test tube containing a Durham tube at 30°C for 3 days. The observation of gas bubbles was recorded as a positive result indicating the strain was heterofermentative. In contrast, if no gas bubble was observed, the result was negative, and the strain was homofermentative.

3.2.2 Acid production from carbohydrates

Acid production from carbohydrates was determined as described in Tanasupawat et al., 1998. Each strain was cultivated in 3 ml fermentable carbohydrate broth (0.5 g carbohydrate, 0.4 g yeast extract, 0.5 g peptone, 0.2 g beef extract, 0.025 g tween 80, 0.5 ml salt solution, and 100 ml deionized water, final pH of 6.8, sterilized at 110°C for 10 minutes; salt solution composed of 4.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g $MnSO_4 \cdot 4H_2O$, 0.2 g $FeSO_4 \cdot 7H_2O$, 0.2 g NaCl, and 100 ml deionized water) at 30°C for 3 days. The tested carbohydrates were D-amygdalin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, gluconate, glycerol, inulin, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, α-methyl-D-glucoside, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose, D-trehalose, and D-xylose). Acid production was tested by adding two drops of the mixed indicator (0.2 g bromthymol blue, 0.1 g neutral red, and 300 ml ethanol) and titration with 0.1 N NaOH. The amount of 0.1 N NaOH used to turn the broth from red to green was recorded.

3.2.3 Catalase production, nitrate reduction, hydrolysis of aesculin, arginine, slime formation and reactions in litmus milk

Catalase production, nitrate reduction, hydrolysis of aesculin, arginine, slime formation and reactions in litmus milk were investigated as described by Tanasupawat et al. 1992 and 1998. For catalase production test, few drops of H_2O_2 (3.0 g H_2O_2, and 100 ml distilled water) were applied on cell colonies of each strain on MRS agar plate inoculated at 30°C for 3 days. The observation of gas bubble was recorded as a positive result, indicating the strain produced catalase. In contrast, if no gas bubble was observed, the result was negative, and the strain could not produce catalase.

For nitrate reduction test, each strain was cultivated in nitrate broth (0.5 g yeast extract, 1.0 g peptone, 1.0 g NaCl, 0.1 g KNO_3, 0.1 g agar, and 100 ml

deionized water, final pH of 6.8) at 30°C for 7 days. One ml of the broth was transferred into a test tube, which was then added with 3 drops of solution A (0.8 g sulfanilic acid, and 100 ml 5 N acetic acid) and 2 drops of solution B (0.06 g dimethyl-naphthylamine, and 100 ml 5 N acetic acid). The solution was well mixed. The observation of red color was recorded as a positive result, indicating nitrate was reduced to nitrite. In contrast, if no red color was observed, the result was negative, and there was no nitrate reduction.

For aesculin hydrolysis test, each strain was cultivated in aesculin broth (1.0 g aesculin, 0.25 g glucose, 0.25 g ferric citrate, 0.5 g beef extract, 0.5 g yeast extract, 0.01 g $MnSO_4 \cdot 4H_2O$, 0.1 g tween 80, and 100 ml deionized water, final pH of 6.8) at 30°C for 7 days. The observation of color change of the broth to dark green and/or the formation of white crystals were recorded as a positive result, indicating aesculin was hydrolyzed to aesculetin. In contrast, if neither color change nor crystal formation was observed, the result was negative, and there was no aesculin hydrolysis.

For arginine hydrolysis test, each strain was cultivated in arginine broth (0.1 g peptone, 0.5 g NaCl, 0.03 g K_2HPO_4, 1.0 g L(+) arginine HCl, 0.001 g phenol, 0.3 g agar, and 100 ml deionized water, final pH of 6.8) at 30°C for 7 days. The observation of color change of the broth from orange to pink was recorded as a positive result, indicating arginine was hydrolyzed and NH_3 was formed. In contrast, if the color change was from orange to yellow, the result was negative, and there was no arginine hydrolysis.

Slime formation on sucrose (2%) in yeast extract peptone agar (10.0 g yeast extract, 10.0 g peptone, 15.0 g agar, and 1000 ml distilled water) was investigated after incubation at 30°C for 3 days.

For reactions in litmus milk test, each strain was cultivated in litmus milk (Difco Laboratories, Detroit, Michigan, USA) at 30°C for 7 and 14 days. The observation of color change from purple to white indicated the reduction of milk; from purple to pink indicated acidification. If litmus milk turned to be semi-solid, there was coagulation of protein milk. If litmus milk turned transparent, there was liquefaction of protein milk.

3.2.4 Peptidoglycan type of cell wall

Diaminopimelic acid in the cell wall was detected by hydrolysis of 3 mg dried cells grown in GYPB broth (glucose yeast extract peptone beef extract broth; 1.0 g glucose, 0.5 g yeast extract, 0.5 g peptone, 0.2 g beef extract, 0.3 g sodium acetate, 0.025 g tween 80, 0.5 ml salt solution, and 100 ml deionized water, final pH of 6.8) with 1 ml 6 N HCl at 100^{o}C for 18 h, and the hydrolysate was applied on cellulose TLC plate (Merck no. 5577). The TLC plate was developed with the solvent system of methanol-pyridine-12 N HCl-water (32: 4: 1: 7) (v/v) (Komagata and Suzuki, 1987). Spots were visualized by spraying with 0.5% ninhydrin solution in n-butanol followed by heating at 100^{o}C for a few minutes.

3.3 Sequencing and comparison of 16S rRNA gene

Template DNA for 16S rRNA gene amplification was prepared by the method modified from Nilsson et al. (2003). Two loops from an overnight MRS plate culture were transferred into 100-500 μL of TE buffer (10 mM Tris-Cl, pH 7.5, and 1 mM EDTA). Samples were boiled for 10-15 minutes. Then, the debris was pelleted by centrifugation 12000 rpm for 10 minutes, and 50-200 μL of supernatant was collected. 16S rRNA gene was amplified using the PCR method with a 1U *Taq* DNA polymerase (BioLab Ltd., Auckland, New Zealand) and primers UFUL (GCCTAACACATGCAAGTCGA) and URUL (CGTATTACCGCGGCTGCTGG) (Great American Gene Co., California, USA). These primers target two highly conserved regions known to be variable among bacterial species (De Rijk et al., 1992; Van de Peer et al., 1996). 16S rRNA gene was sequenced by using a BigDye v. 3.1 cycle sequencing kit (Applied Biosystems, California, USA), according to the manufacturer's protocol, with UFUL as the primer. The 16S rRNA gene sequences determined (ca. 450-500 bases) were aligned along with the sequences of type strains obtained from the GenBank by using the program CLUSTAL X (version 1.82) (Thompson et al., 1997). Distance matrices for aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with the program PHYLIP (version 3.64) available at http://evolution.genetics.washington.edu/phylip.html.

Confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1,000 samplings.

3.4 Reference strains

Weissella confusa ATCC10881^T, *W. cibaria/kimchii* LMG17699^T, *Pediococcus pentosaceus* ATCC33316^T, *P. acidilactici* DSM20284^T, *Lactobacillus fermentum* ATCC14931^T, *L. brevis* ATCC14869^T, *L. farciminis* ATCC29644^T, *L. plantarum* ATCC14917^T and *L. sakei* ATCC15521^T were used as reference strains.

3.5 Isomers of lactic acid

The strains tested were cultivated in GYP-mineral salt broth (glucose yeast extract peptone beef extract broth; 1.0 g glucose, 0.5 g yeast extract, 0.5 g peptone, 0.2 g beef extract, 0.025 g tween 80, 0.5 ml salt solution, and 100 ml deionized water, final pH of 6.8) for 3 to 5 days. Lactic acid was analyzed enzymatically according to Okada et al., 1978 using D- and L- lactate dehydrogenase (Boehringer, Germany).

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3.6 Metabolic product determination

3.6.1 Screening for diacetyl formation

Diacetyl formation was screened by the method modified from Mattessich and Cooper (1989) and Phalip et al. (1994), which yielded qualitative results. Cells were grown in MMRS broth (100 ml consisted of 0.18 gram glucose, 0.5 gram yeast extract, 1.0 gram peptone, 1.0 gram beef extract, 0.5 gram sodium acetate, 1.29 gram trisodium citrate·$2H_2O$, 0.15 gram tween 80, 0.02g $MgSO_4 \cdot 7H_2O$, 0.005 gram $MnSO_4 \cdot 4H_2O$, and 0.2 gram K_2HPO_4) at 35^oC for 3-5 days. One ml of each test solution (0.5% creatine solution and 7.5% α- naphthol in 2.5 N NaOH) was added into each 3 ml of culture medium. The degree of change in culture medium color was recorded.

3.6.2 Screening for bacteriocin production

The screening method was modified from Yin et al. (2003). Each LAB strain was grown on Trypticase soy agar (without glucose) with a 2.0% yeast extract supplement (TSAYE) slant at 37°C for 2 days. The cultured LAB cells were streaked on TSAYE plate. Working pathogen (*Bacillus cereus* TISTR 037, *Clostridium sporogenes* TISTR 1481, *Staphylococcus aureus* TISTR 029, and *Escherichia coli* TISTR 073) were cultured in nutrient agar (NA) slant at 37°C for 2 days. The cultured pathogen cells were transferred to 0.85% saline solution to obtain cell density of 10^5-10^6 colony-forming units (CFU) per ml. One ml of the suspended pathogen cells was mixed with 15 ml of melted Trypticase soy agar (TSA) at 50°C. The melted TSA was then overlaid on the TSAYE plate growing LAB. The plate with the overlay was incubated anaerobically at 37°C for 2 days. The control TSAYE plate with pathogen overlay but without cultured LAB was incubated in parallel for comparison. Colonies revealed inhibition zones were recorded.

The broth dilution method modified from Waterworth (1978) was employed to determine the minimum inhibitory concentration (MIC). Each LAB strain was grown anaerobically on Trypticase soy broth (without glucose) with a 2.0% yeast extract supplement (TSAYE) slant at 37°C for 24 hours. The broth was centrifuged at 5000 rpm for 15 minutes and then filtered through a membrane (0.45 μm, no. 4654, Gelman) to remove the cells. The filtrate was diluted with Mueller Hinton Broth (MHB) at 1-, ½-, ¼-, 1/8-, 1/16-, and 1/32 fold to get a final volume of 2.5 ml. Working pathogen (*Bacillus cereus* TISTR 037, *Clostridium sporogenes* TISTR 1481, *Staphylococcus aureus* TISTR 029, and *Escherichia coli* TISTR 073) were cultured in nutrient agar (NA) slant at 37°C for 2 days. The cultured pathogen cells were transferred to 0.85% saline solution to obtain cell density of 10^5-10^6 colony-forming units (CFU) per ml. Two and a half milliliter of pathogen cell suspension was transferred to each dilution after which was incubated at 37°C for 24 hours. Optical density at 600 nm of each dilution was measured and compared with a control and the difference was reported as percent survival. One bacterial unit (B.U.) was defined as the amount of bacteriocin that inhibited growth of the indicator microorganism by 50%, when comparing with control culture without bacteriocin (Cuozzo et al., 2001).

CHAPTER 4
RESULTS

Sausage samples contained $1.4x10^{11}$-$5.5x10^{13}$ bacterial cells/g, and showed a pH between 4.2-5.0 (Table 4-1). All isolates were Gram-positive, non-motile, and non-sporing. Their colonies on MRS agar plates were circular, low convex with entire margin, and non-pigmented. The characteristics are shown in Table 4-2. The cells of sphere-shaped strains were 0.8 to 1.0 μm in size and appeared in pairs or in tetrads. The cells of rod-shaped strains were 0.8-1.0x1.5-5.0 μm in cell size, and appeared singly, in pairs, or in chains. Table 4-3 shows the characteristics in producing acid from carbohydrates.

Certain strains were selected for the determination of lactic acid isomer (L-, D-, or DL-), the presence of diaminopimelic acid in the cell wall, sequencing of 16S rRNA gene, and the results were shown in Table 4-4, Figure 4.1 and Table 4-5, respectively. Tables 4-6 and 4-7 report the results of diacetyl and bacteriocin screening against the tested pathogens, i.e. *B. cereus* TISTR 037, *C. sporogenes* TISTR 1481, *S. aureus* TISTR 029, and *E. coli* TISTR 073, respectively. Figures 4.2-4.13 demonstrate the inhibition against the tested pathogens. Figures 4.14 - 4.21 presented percentages of survival of *B. cereus* at each dilution of LAB filtrates (ml filtrate/ml total volume). Figure 4.22 presented percentage of survival of *S. aureus* at each dilution of LAB filtrate (ml filtrate/ml total volume). Table 4-8 presented the bacteriocin unit (BU) of isolated strains that inhibited tested pathogens.

Table 4-1 Bacterial cell counts and sausage characteristics

No.	Sausage names and province where collected	Days of fermen-tation	pH	Bacterial counts (cells/g)	No. of isolates
CP1	Sai-krork-prieo Bangkok	4	4.2	$3.7x10^{11}$	CP1-5, CP1-8, CP1-13, CP1-14, CP1-15, CP1-17, CP1-18, CP1-19, CP1-20
CP2	Sai-krork-prieo Pathumthani	5	4.2	$2.5x10^{12}$	CP2-3A, CP2-3B, CP2-10, CP2-11, CP2-15, CP2-16, CP2-17, CP2-19
CP3	Sai-krork-prieo Pathumthani	4	4.9	$1.4x10^{11}$	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP3-15, CP3-16, CP3-17
CP4	Sai-krork-prieo Pathumthani	4	4.8	$1.9x10^{12}$	CP4-5, CP4-6, CP4-11, CP4-12, CP4-16, CP4-17, CP4-18
CP5	Sai-krork-prieo Bangkok	2	4.3	$4.0x10^{13}$	CP5-2, CP5-4, CP5-6, CP5-9
CP6	Mum (beef) Chaiyaphoom	3	4.5	$3.0x10^{13}$	CP6-2, CP6-3, CP6-4, CP6-5, CP6-6, CP6-7, CP6-8, CP6-9, CP6-10, CP6-11, CP6-12
CP7	Mum (beef) Chaiyaphoom	3	4.4	$5.5x10^{13}$	CP7-2, CP7-3, CP7-4, CP7-5, CP7-7, CP7-8, CP7-9, CP7-10, CP7-11, CP7-12, CP7-13
CP8	Mum (beef) Konkean	4	4.6	$1.5x10^{12}$	CP8-1, CP8-2, CP8-4
CP9	Mum (beef) Chaiyaphoom	4	4.6	$2.5x10^{11}$	CP9-1, CP9-2, CP9-3, CP9-6
CP10	Mum (pork) Chaiyaphoom	4	5.0	$8.0x10^{11}$	CP10-1, CP10-2, CP10-3, CP10-4
CP11	Sai-krork-prieo Chaiyaphoom	4	4.6	$1.0x10^{13}$	CP11-2, CP11-3, CP11-5, CP11-6, CP11-7, CP11-8, CP11-9, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15
CP12	Mum (pork) Chaiyaphoom	4	5.0	$2.0x10^{13}$	CP12-4, CP12-8, CP12-9, CP12-10, CP12-11, CP12-14, CP12-15
CP13	Sai-krork-prieo Chaiyaphoom	4	4.8	$8.5x10^{12}$	CP13-1, CP13-2, CP13-3, CP13-4, CP13-7, CP13-9, CP13-11, CP13-12
CP14	Mum (pork) Chaiyaphoom	4	4.7	$6.5x10^{12}$	CP14-1, CP14-2, CP14-3, CP14-4, CP14-5, CP14-6, CP14-7, CP14-8, CP14-9, CP14-10, CP14-11, CP14-12
CP15	Sai-krork-prieo Chaiyaphoom	4	5.0	$5.0x10^{12}$	CP15-1, CP15-2, CP15-5, CP15-6, CP15-7, CP15-8, CP15-9, CP15-10, CP15-12
CP16	Mum (pork) Chaiyaphoom	4	4.8	$7.0x10^{12}$	CP16-1, CP16-2, CP16-3, CP16-4, CP16-5, CP16-8, CP16-9
				Total isolated strains	124

Table 4-2. Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP1-5	CP1-8	CP1-13	CP1-14	CP1-15	CP1-17	CP1-18	CP1-19	CP1-20	CP2-3A	CP2-3B	CP2-10
Cell form	cocci	cocci	rod	rod	rod	rod	cocci	rod	rod	rod	rod	rod
Cell arrangement	In pairs or tetrads	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	+	+	+	-	-	-	+	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	-	-	+	+	-	-	+	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	-	-	-	-	+	+	-	-	+	+	+	+
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	+	-	-	-	-	-	+	-	-	-	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	-	-	+	-	+	+	-	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	-	-	+	+	+	-	+	+	+	+
8.5	-	+	-	-	-	-	-	-	-	-	-	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	-	+	-	-	-	-	+	-	+	+	+	+
8%	-	+	-	-	-	-	-	-	+	-	+	-
Slime formation on sucrose	-	+	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP2-11	CP2-15	CP2-16	CP2-17	CP2-19	CP3-1	CP3-8	CP3-9	CP3-10	CP3-11	CP3-15	CP3-16
Cell form	rod	rod	rod	rod	rod	cocci	cocci	cocci	cocci	cocci	rod	rod
Cell arrangement	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	in pairs or tetrads	in pairs or tetrads	in pairs or tetrads	in pairs or tetrads	in pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	-	-	-	+	+	+	+	+	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	+	-	+	+	+	+	+	+	+	+	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	+	+	+	+	-	-	-	+	-	-	+	-
Coagulation	-	-	W	-	-	-	-	+	-	-	+	+
Reduction	-	-	-	-	-	-	-	-	-	-	-	+
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	-	+	+	+	+	-	-	+	-	-	-	-
4.0	+	+	+	+	+	W	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	-	+	+	+	+	+	+	+
8.5	-	-	+	+	-	+	-	-	-	+	-	-
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	-	+	+	+	+	+	+	+	+	+	+	+
8%	-	+	-	-	-	+	+	+	+	+	-	-
Slime formation on sucrose	-	-	-	-	-	+	+	+	+	+	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP3-17	CP4-5	CP4-6	CP4-11	CP4-12	CP4-16	CP4-17	CP4-18	CP5-2	CP5-4	CP5-6	CP5-9
Cell form	rod	rod	cocci	cocci	rod	rod	rod	rod	rod	rod	rod	rod
Cell arrangement	Singly, in pairs or in chains	Singly, in pairs or in chains	In pairs or tetrads	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	+	+	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	-	+	+	-	-	-	-	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	W	W	+	-	-	+	+	+	+	-	+	+
Coagulation	-	-	W	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	W	+	-	-	+	+	+	+	-	-	+	-
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	-	-	-	-	-	-	-	+	-	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	-	+	+	-	-	-	+	+	+	+	+
Slime formation on sucrose	-	-	+	+	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP6-2	CP6-3	CP6-4	CP6-5	CP 6-6	CP6-7	CP6-8	CP6-9	CP6-10	CP6-11	CP6-12	CP7-2
Cell form	cocci	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Cell arrangement	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	-	-	-	-	+	-	-	-	-	+
Catalase produciton	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	+
Arginine hydrolysis	+	-	+	+	+	+	-	+	-	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	-
Reaction in litmus milk												
Acidification	-	-	+	W	+	+	-	+	W	+	-	-
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	+	-	-	-	-	-	-	-	-	-	-	+
Growth at 50°C	+	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	+	+	-	+	+	+	+	+	+	-	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	-	+	+	+	-	-
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	-	+	+	+	-	+
8%	+	+	+	+	+	+	-	+	+	+	-	-
Slime formation on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP7-3	CP7-4	CP7-5	CP7-7,	CP7-8	CP7-9	CP7-10	CP7-11	CP7-12	CP7-13	CP8-1	CP8-2
Cell form	cocci	cocci	rod	rod	cocci	rod	rod	rod	rod	rod	rod	rod
Cell arrangement	In pairs or tetrads	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	In pairs or tetrads	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	-	-	-	+	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	-	-	+	+	-	+	-	-	+	+
Esculin hydrolysis	+	+	+	+	+	-	+	+	+	+	-	+
Reaction in litmus milk												
Acidification	-	-	-	+	-	-	W	-	+	+	-	+
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	+	+	-	-	+	+	-	-	-	-	-	-
Growth at 50°C	+	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	-	+	W	+	+	+	W	-	+	+	-	-
4.0	+	+	+	+	+	+	+	+	+	+	-	+
4.5	+	+	+	+	+	+	+	+	+	+	W	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	-	+	+	+	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	+	-	+	+	-	+	+	+	+	-	+
Slime formation on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP8-4	CP9-1	CP9-2	CP9-3	CP9-6	CP10-1	CP10-2	CP10-3	CP10-4	CP11-2	CP11-3	CP11-5
Cell form	rod	rod	rod	rod	rod	rod	rod	rod	rod	cocci	cocci	cocci
Cell arrangement	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	-	+	+	+	+	-	-	+	+	+	+
Esculin hydrolysis	+	-	+	+	+	+	+	+	-	+	+	+
Reaction in litmus milk												
Acidification	W	-	W	-	-	-	-	W	-	-	-	-
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	-	-	-	-	-	-	-	-	-	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	-	+	-	-	-	+	+	-	+	+	+
4.0	+	W	+	+	-	+	+	+	-	+	+	+
4.5	+	+	+	+	-	+	+	+	W	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+
9.6	-	-	-	-	+	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	+	+	-	+	+	+	+	-	+	+	+
Slime formation on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP11-6	CP11-7	CP11-8	CP11-9	CP11-10	CP11-11	CP11-13	CP11-14	CP11-15	CP12-4	CP12-8	CP12-9
Cell form	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Cell arrangement	in pairs or tetrads	In pairs or tetrads	In pairs or tetrads	in pairs or tetrads	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	in pairs or tetrads	in pairs or tetrads
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	-	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	+	-	-	+	-	-	-	-	-	-	-	-
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	-	+	+	-	+	+	+	+	+	+	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	+	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	+	+	+	+	+	+	+	+	+	+	+
Slime formation on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP12-10	CP12-11	CP12-14	CP12-15	CP13-1	CP13-2	CP13-3	CP13-4	CP13-7	CP13-9	CP13-11	CP13-12
Cell form	cocci	cocci	cocci	cocci	rod	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Cell arrangement	in pairs or tetrads	in pairs or tetrads	in pairs or tetrads	in pairs or tetrads	Singly, in pairs or in chains	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	In pairs or tetrads	in pairs or tetrads	In pairs or tetrads	in pairs or tetrads
Gas from glucose	-	-	-	-	+	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	-	-	-	+	+	+	+	+	-	+	-
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	+	+	+	+	+	-	-	-	-	+	-	+
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 45°C	-	-	-	-	+	+	+	+	+	-	+	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	+	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	+	+	+	+	+	+	+	+	+	+	+
Slime formation on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP14-1	CP14-2	CP14-3	CP14-4	CP14-5	CP14-6	CP14-7	CP14-8	CP14-9	CP14-10	CP14-11	CP14-12
Cell form	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Cell arrangement	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	-	+
Reaction in litmus milk												
Acidification	+	-	-	+	+	+	+	+	-	+	+	+
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	-	+	+	-	-	-	-	-	+	-	-	-
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	+	+	+	+	+	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+	+	+	+
4.5	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+
8.5	+	+	+	+	+	+	+	+	+	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	+	+	+	+	+	+	+	+	+	+	+
6%	+	+	+	+	+	+	+	+	+	+	+	+
8%	+	-	+	+	+	+	+	+	+	+	+	+
Slime production on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP15-1	CP15-2	CP15-5	CP15-6	CP15-7	CP15-8	CP15-9	CP15-10	CP15-12	CP16-1	CP16-2	CP16-3
Cell form	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Cell arrangement	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains	Singly, in pairs or in chains
Gas from glucose	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	+	-	-	-	-	-
Arginine hydrolysis	+	+	-	+	+	-	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+
Reaction in litmus milk												
Acidification	-	+	-	+	+	-	+	-	-	+	-	+
Coagulation	-	-	-	-	-	-	-	-	-	-	-	-
Reduction	+	-	+	-	-	+	-	+	+	-	+	-
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-	-
Growth at pH												
3.5	+	+	+	+	+	+	+	+	+	-	+	-
4.0	+	-	+	+	+	+	+	+	+	+	+	+
4.5	+	-	+	+	+	+	+	+	+	+	+	+
8.0	+	-	+	+	+	+	+	+	+	+	+	-
8.5	+	-	+	+	+	+	+	+	+	+	+	-
9.6	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl												
4%	+	-	+	+	+	+	+	+	+	+	+	+
6%	+	-	+	+	+	+	+	+	+	+	+	+
8%	+	-	+	+	+	+	+	+	+	+	+	+
Slime production on sucrose	-	-	-	-	-	-	-	-	-	-	-	-

Table 4-2 (continued). Morphological, cultural, physiological, and biochemical characteristics

	Isolated No.											
Characteristics	CP16-4	CP16-5	CP16-8	CP16-9								
Cell form												
Cell arrangement												
Gas from glucose												
Catalase production	-	-	-	-								
Nitrate reduction	-	-	-	-								
Arginine hydrolysis	+	+	+	+								
Esculin hydrolysis	+	+	+	+								
Reaction in litmus milk												
Acidification	+	-	-	+								
Coagulation	-	-	-	-								
Reduction	-	+	+	-								
Growth at 45°C	-	-	-	-								
Growth at 50°C	-	-	-	-								
Growth at pH												
3.5	-	-	-	-								
4.0	+	+	+	+								
4.5	+	+	+	+								
8.0	-	+	+	+								
8.5	-	+	+	+								
9.6	-	-	-	-								
Growth in NaCl												
4%	+	+	+	+								
6%	+	+	+	+								
8%	+	+	+	+								
Slime production on sucrose	-	-	-	-								

Table 4-3. Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP1-5	CP1-8	CP1-13	CP1-14	CP1-15	CP1-17	CP1-18	CP1-19	CP1-20	CP2-3A	CP2-3B	CP2-10
D-Amygdalin	+	+	-	-	+	+	+	-	+	+	+	+
L-Arabinose	-	+	+	+	+	+	+	+	+	+	-	+
D-Cellebiose	+	+	-	-	+	+	+	-	+	+	+	+
D-Fructose	+	+	W	W	+	+	+	W	+	+	+	+
D-Galactose	+	W	-	W	+	+	+	-	+	+	+	+
D-Glucose	+	+	W	W	+	+	+	W	+	+	+	+
D-Gluconate	-	W	-	-	-	-	-	-	-	+	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	+	-	-	-	-	-	-	-	-	+	W	-
Lactose	+	+	-	-	+	+	+	-	+	+	+	+
Maltose	+	+	W	W	+	+	+	W	+	+	-	+
D-Mannitol	-	W	-	-	+	W	-	-	+	W	-	+
D-Mannose	+	+	-	-	+	+	+	-	+	+	+	+
D-Melibiose	-	-	W	-	+	+	+	-	+	+	-	+
D-Melezitose	-	-	-	-	+	+	-	-	+	+	-	+
α-Methyl-D-glucoside	-	-	W	W	W	-	-	W	-	-	-	-
Raffinose	-	-	-	-	+	+	-	-	+	+	-	+
L-Rhamnose	-	W	-	-	-	-	-	-	-	+	-	W
D-Ribose	+	W	+	+	+	+	+	+	+	+	-	+
Salicin	+	+	-	-	+	+	+	-	+	+	+	+
D-Sorbitol	-	-	-	-	+	-	-	-	+	W	-	+
Sucrose	+	+	-	-	+	+	+	-	+	+	+	+
D-Trehalose	+	-	-	-	+	+	+	-	+	+	+	+
D-Xylose	-	+	+	+	-	-	+	+	-	-	-	-

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP2-11	CP2-15	CP2-16	CP2-17	CP2-19	CP3-1	CP3-8	CP3-9	CP3-10	CP3-11	CP3-15	CP3-16
D-Amygdalin	+	+	+	+	-	W	W	W	-	W	+	W
L-Arabinose	+	-	+	-	+	+	W	W	+	W	-	-
D-Cellebiose	+	+	+	+	-	+	W	W	W	W	+	W
D-Fructose	+	+	+	+	W	W	W	W	W	W	+	+
D-Galactose	+	+	+	+	-	W	W	W	-	+	+	+
D-Glucose	+	+	+	+	-	W	W	W	W	W	+	+
D-Gluconate	-	-	-	-	-	W	-	-	-	W	-	W
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-
Inulin	+	-	+	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	-	-	-	-	-	-	+	-
Maltose	+	W	+	+	W	W	W	W	W	W	+	W
D-Mannitol	+	-	+	-	-	-	-	-	-	-	-	+
D-Mannose	+	+	+	+	-	W	W	W	W	W	+	+
D-Melibiose	+	-	+	-	-	-	-	-	-	-	-	-
D-Melezitose	+	-	+	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	W	-	-	-	-	-	-	-	-	-	-	-
Raffinose	+	-	+	-	-	-	-	-	-	-	-	-
L-Rhamnose	W	-	W	-	-	-	-	-	-	-	-	-
D-Ribose	+	-	+	-	-	-	-	-	-	-	-	+
Salicin	+	+	+	+	-	W	W	W	W	W	W	+
D-Sorbitol	+	-	W	-	-	-	-	-	-	-	-	W
Sucrose	+	+	+	+	-	W	W	W	W	W	+	+
D-Trehalose	+	+	+	+	-	-	-	-	-	-	+	+
D-Xylose	-	-	-	-	-	W	W	W	W	W	-	-

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP3-17	CP4-5	CP4-6	CP4-11	CP4-12	CP4-16	CP4-17	CP4-18	CP5-2	CP5-4	CP5-6	CP5-9
D-Amygdalin	+	+	W	W	+	+	+	+	-	-	+	-
L-Arabinose	-	+	W	W	+	+	+	+	-	-	-	-
D-Cellebiose	+	+	W	W	+	+	+	+	+	+	+	+
D-Fructose	+	+	W	W	+	+	+	+	+	+	+	+
D-Galactose	+	+	-	-	+	+	+	+	W	+	+	+
D-Glucose	+	+	W	W	+	+	+	+	+	+	+	+
D-Gluconate	-	-	-	-	W	-	W	-	-	-	-	-
Glycerol	-	-	-	-	W	-	-	-	-	-	-	-
Inulin	-	W	-	-	W	-	-	-	-	-	-	-
Lactose	+	+	-	-	+	+	W	+	+	+	+	+
Maltose	-	+	W	W	+	+	+	+	-	-	+	+
D-Mannitol	-	+	-	-	W	+	W	+	-	-	-	-
D-Mannose	+	+	W	W	+	+	+	+	+	+	+	+
D-Melibiose	-	+	-	-	+	+	+	+	-	-	-	-
D-Melezitose	-	+	-	-	+	+	+	+	-	-	-	-
α-Methyl-D-glucoside	-	+	-	-	+	-	-	-	-	-	-	-
Raffinose	-	+	-	-	+	+	+	+	-	-	-	-
L-Rhamnose	-	W	-	-	W	-	-	-	-	-	-	-
D-Ribose	-	+	-	-	+	+	+	+	-	-	-	-
Salicin	+	+	W	W	+	+	+	+	+	+	+	+
D-Sorbitol	-	+	-	-	W	-	-	-	-	-	-	-
Sucrose	+	+	W	W	+	+	+	+	+	+	+	+
D-Trehalose	+	+	-	-	W	+	+	+	-	+	+	-
D-Xylose	-	-	W	W	-	W	-	W	-	-	-	-

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP6-2	CP6-3	CP6-4	CP6-5	CP6-6	CP6-7	CP6-8	CP6-9	CP6-10	CP6-11	CP6-12	CP7-2
D-Amygdalin	+	+	+	+	+	+	-	+	+	+	-	-
L-Arabinose	-	+	-	W	-	-	+	-	+	-	+	-
D-Cellebiose	+	+	+	+	+	+	-	+	+	+	-	-
D-Fructose	+	+	+	+	+	+	W	+	+	+	W	W
D-Galactose	+	+	+	+	+	+	W	+	+	+	W	W
D-Glucose	+	+	+	+	+	+	+	+	+	+	W	W
D-Gluconate	-	+	-	-	-	-	-	-	-	-	-	-
Glycerol	-	W	-	-	-	-	-	-	-	-	-	-
Inulin	-	W	-	-	-	-	-	-	-	-	-	-
Lactose	-	+	+	+	+	+	-	+	+	+	-	W
Maltose	-	+	-	+	+	-	W	-	+	-	W	W
D-Mannitol	-	W	-	-	-	-	-	-	+	-	-	-
D-Mannose	+	W	+	+	+	+	-	+	+	+	-	W
D-Melibiose	-	+	-	-	-	-	-	-	+	-	-	W
D-Melezitose	-	+	-	-	-	-	-	-	+	-	-	-
α-Methyl-D-glucoside	-	W	-	-	-	-	W	-	+	-	W	-
Raffinose	-	+	-	-	-	-	-	-	+	-	-	W
L-Rhamnose	+	W	-	-	-	-	-	-	W	-	-	-
D-Ribose	+	+	-	-	-	-	+	-	+	-	+	+
Salicin	+	+	+	+	+	+	-	+	+	+	-	-
D-Sorbitol	-	W	-	-	-	-	-	-	+	-	-	-
Sucrose	W	+	+	+	+	+	-	+	+	+	-	W
D-Trehalose	+	+	+	+	+	+	-	+	+	+	-	W
D-Xylose	+	W	-	-	-	-	+	-	+	-	+	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP7-3	CP7-4	CP7-5	CP7-7	CP7-8	CP7-9	CP7-10	CP7-11	CP7-12	CP7-13	CP8-1	CP8-2
D-Amygdalin	+	+	+	+	+	-	+	+	+	+	-	W
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	-
D-Cellebiose	+	+	+	+	+	-	+	+	+	+	W	+
D-Fructose	+	+	+	+	+	W	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	W	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	W	+	+	+	+	+	+
D-Gluconate	-	-	+	W	-	-	W	-	W	W	W	-
Glycerol	-	-	-	W	-	-	W	+	-	-	W	-
Inulin	-	-	-	+	-	-	+	-	+	+	-	-
Lactose	-	-	+	+	-	-	+	W	+	+	+	+
Maltose	-	-	+	+	-	W	+	+	+	+	-	-
D-Mannitol	-	-	W	+	-	-	+	-	W	+	-	-
D-Mannose	+	+	+	+	+	-	+	+	+	+	+	+
D-Melibiose	-	-	+	+	-	W	+	-	+	+	+	-
D-Melezitose	-	-	+	+	-	-	+	-	+	+	-	-
α-Methyl-D-glucoside	-	-	W	-	-	-	-	-	-	-	-	-
Raffinose	-	-	W	+	-	W	+	-	+	+	-	-
L-Rhamnose	-	W	W	-	-	-	W	-	-	W	-	-
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	-
Salicin	+	W	+	+	W	-	+	+	+	+	W	+
D-Sorbitol	-	-	W	+	-	-	+	-	+	+	-	-
Sucrose	+	+	+	+	+	W	+	-	+	+	+	+
D-Trehalose	-	-	+	+	-	W	+	+	+	+	+	+
D-Xylose	+	+	+	-	+	+	-	+	-	W	W	-

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP8-4	CP9-1	CP9-2	CP9-3	CP9-6	CP10-1	CP10-2	CP10-3	CP10-4	CP11-2	CP11-3	CP11-5
D-Amygdalin	+	W	+	W	W	-	+	+	+	+	+	+
L-Arabinose	+	+	-	-	-	-	-	+	+	+	+	+
D-Cellebiose	+	+	+	+	W	+	+	+	+	+	+	+
D-Fructose	+	+	+	W	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	-	W	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	W	+	+	+	+	+	+	+
D-Gluconate	W	+	-	+	-	-	W	-	-	-	-	-
Glycerol	-	-	-	-	W	-	-	-	-	-	-	-
Inulin	+	-	-	-	W	-	+	-	-	-	-	W
Lactose	+	+	+	W	W	+	+	+	-	-	-	-
Maltose	+	+	+	+	W	-	+	+	-	-	-	-
D-Mannitol	+	-	-	-	W	-	+	+	-	-	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	-	W	W	-	+	+	+	-	-	-
D-Melezitose	+	-	-	W	-	-	-	+	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	W	-	-	-	-	-	-	-
Raffinose	+	-	-	W	W	-	+	+	-	-	-	-
L-Rhamnose	-	-	-	W	W	-	-	-	+	W	+	W
D-Ribose	W	+	-	W	+	-	+	+	+	+	+	+
Salicin	+	W	+	W	W	-	+	+	+	+	+	+
D-Sorbitol	+	W	-	W	-	-	W	+	-	-	-	-
Sucrose	+	+	+	+	W	+	+	+	+	+	+	+
D-Trehalose	+	+	+	-	W	+	+	+	+	-	-	-
D-Xylose	-	-	-	+	W	-	-	-	-	+	+	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP11-6	CP11-7	CP11-8	CP11-9	CP11-10	CP11-11	CP11-13	CP11-14	CP11-15	CP12-4	CP12-8	CP12-9
D-Amygdalin	+	+	+	+	+	+	+	+	+	+	+	W
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+
D-Cellebiose	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	W
Inulin	-	-	-	-	W	-	W	-	-	-	-	-
Lactose	+	-	-	+	-	-	-	-	-	-	+	-
Maltose	+	-	-	+	-	-	-	-	-	-	+	+
D-Mannitol	+	-	-	+	-	-	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	-	-	+	-	-	-	-	-	-	+	+
D-Melezitose	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	+	+
L-Rhamnose	-	W	+	-	+	W	+	W	W	W	-	-
D-Ribose	-	+	+	-	+	+	+	+	+	+	+	+
Salicin	-	+	+	+	+	+	+	+	+	+	W	+
D-Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	+	-	+	+	+	+	+	+	+	+
D-Trehalose	+	-	-	+	+	-	-	-	-	-	+	+
D-Xylose	-	+	+	-	+	+	+	+	+	+	-	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP12-10	CP12-11	CP12-14	CP12-15	CP13-1	CP13-2	CP13-3	CP13-4	CP13-7	CP13-9	CP13-11	CP13-12
D-Amygdalin	W	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	W	+	+
D-Cellebiose	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	W	W	+	+	-	-	-	-	-	W	-	-
Glycerol	W	-	-	-	-	-	-	-	-	W	-	W
Inulin	+	+	+	+	+	-	-	-	-	W	-	W
Lactose	+	+	+	+	+	W	W	W	W	+	W	+
Maltose	+	+	+	+	+	-	-	-	-	+	-	+
D-Mannitol	W	+	+	+	+	-	-	-	-	+	-	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	-	-	-	-	+	-	+
D-Melezitose	+	+	+	+	+	-	-	-	-	+	-	+
α-Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	+
Raffinose	+	+	+	+	+	-	-	-	-	+	-	-
L-Rhamnose	-	-	-	-	+	W	+	+	+	-	+	+
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	-	+	+	+	+	+	+	+	-
D-Sorbitol	W	+	+	+	+	-	-	-	-	+	-	+
Sucrose	+	+	+	+	+	-	-	-	-	+	-	-
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	+	+	+	+	+	+	+	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP14-1	CP14-2	CP14-3	CP14-4	CP14-5	CP14-6	CP14-7	CP14-8	CP14-9	CP14-10	CP14-11	CP14-12
D-Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	-	-	W	+	-	+	+	-	-	-	+
D-Cellebiose	-	-	-	-	-	-	-	-	-	-	-	-
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	W	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	-	-	+	-	-	-	+	-	+	-
Maltose	-	+	-	-	-	-	-	-	+	-	+	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannose	W	-	+	+	+	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	-	-	-	+
α-Methyl-D-glucoside	-	-	-	-	-	-	-	-	-	-	-	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	+	-	-	+	+	+	+	W	-	+	-	-
D-Ribose	+	+	-	+	+	+	-	-	-	+	-	-
Salicin	+	-	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	+	+	-	-	-	-
Sucrose	+	-	+	+	+	+	+	+	+	+	+	+
D-Trehalose	-	-	+	+	-	+	-	-	+	+	+	-
D-Xylose	+	W	-	+	+	+	+	+	-	+	-	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP15-1	CP15-2	CP15-5	CP15-6	CP15-7	CP15-8	CP15-9	CP15-10	CP15-12	CP16-1	CP16-2	CP16-3
D-Amygdalin	+	+	+	+	+	+	+	W	+	+	+	+
L-Arabinose	-	-	+	-	-	-	+	-	+	-	+	-
D-Cellebiose	-	-	-	-	-	-	-	-	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	W	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	W	W	W	W
Inulin	-	-	+	-	-	-	+	-	+	-	W	W
Lactose	+	-	+	-	+	+	+	+	+	-	-	-
Maltose	+	-	+	-	+	+	+	+	+	-	-	-
D-Mannitol	-	-	+	+	-	-	+	-	+	-	-	-
D-Mannose	+	-	+	-	+	+	+	+	+	+	+	+
D-Melibiose	-	-	+	+	-	-	+	-	+	-	-	-
D-Melezitose	-	-	-	-	-	-	-	-	+	-	-	-
α-Methyl-D-glucoside	-	-	+	-	-	-	+	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	+	-	-	-
L-Rhamnose	-	-	+	-	-	-	+	-	W	+	+	+
D-Ribose	-	-	+	-	-	-	+	W	+	+	+	+
Salicin	+	-	W	+	+	+	+	+	+	+	+	+
D-Sorbitol	-	-	+	-	-	-	+	-	+	-	-	-
Sucrose	+	-	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	-	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	+	W	+	+	+

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-3 (continued). Acid production from carbohydrates by isolated strains

Carbohydrates	Isolated No.											
	CP16-4	CP16-5	CP16-8	CP16-9								
D-Amygdalin	W	+	-	+								
L-Arabinose	-	-	-	-								
D-Cellebiose	+	+	+	+								
D-Fructose	+	+	+	+								
D-Galactose	+	+	+	+								
D-Glucose	+	+	+	+								
D-Gluconate	-	-	-	-								
Glycerol	-	-	-	+								
Inulin	-	+	-	-								
Lactose	-	+	-	-								
Maltose	-	-	-	+								
D-Mannitol	+	-	+	-								
D-Mannose	-	+	+	+								
D-Melibiose	-	-	-	-								
D-Melezitose	-	-	-	-								
α-Methyl-D-glucoside	-	-	+	-								
Raffinose	+	-	-	-								
L-Rhamnose	+	-	-	+								
D-Ribose	+	-	-	+								
Salicin	-	+	+	+								
D-Sorbitol	+	+	-	-								
Sucrose	+	-	+	+								
D-Trehalose	+	+	+	+								
D-Xylose	+	-	-	+								

+: Strongly produced acid; W: Weakly produced acid; - Very weakly or not produced acid.

Table 4-4. Types of lactic acid isomer of selected isolates

Type of lactic acid isomer	Isolated No.
D	-
L	CP2-3B, CP5-2, CP5-6, and CP5-9
DL	CP1-5, CP1-8, CP1-13, CP1-14, CP1-15, CP1-17, CP1-18, CP1-19, CP1-20, CP2-3A, CP2-10, CP2-11, CP2-16, CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP3-16, CP4-5, CP4-6, CP4-11, CP4-12 CP4-16, CP4-17, CP4-18, CP6-2, CP6-3, CP6-8, CP6-10, CP7-2, CP7-3, CP7-4, CP7-5, CP7-7, CP7-8, CP7-9, CP7-10, CP7-12, CP7-13, CP8-1, CP8-4, CP10-2, CP10-3, CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15, CP12-4, CP13-1, CP13-2, CP13-3, CP13-4, CP13-7, and CP13-11

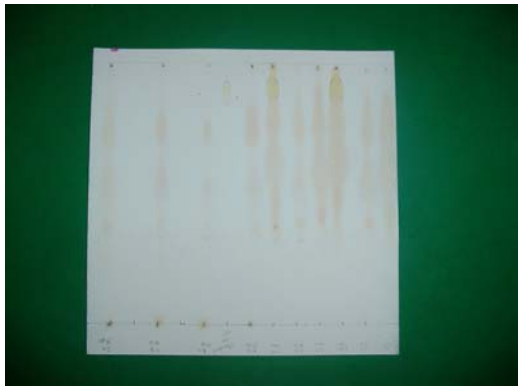

Figure 4.1. Spots on cellulose TLC plate (Merck no. 5716) visualized by spraying with 0.5% ninhydrin solution in n-butanol

Table 4-5. 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP1-5	NGCCTTAACNAGCGCGACCCTATCTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTA GGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATG GACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACT GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAA GGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAAGCCACGGCT AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTTAAGTCTA ATGTGAANCCCTTCGGCTCACCNNAAAAAGTGCATTGGAAACTGGGANACTTGAGTGCANAAAAGACAGTGGAACTCCNTGTGTAGCGGTGAA ATGCGTAAATNTTTGGAANAACCCCAGTGGCGAAGGCGGCTNTNTGGCCTGCANTNAACCTNAGCTCCAAACNTGGTTAGCNAANNGATTAAA NCCCTGGAGCCNTGCNNAANAATATTATAANGTNGGAGGTTNCCCCNTCAGGCTGCGCTAACCATTAANNANCCCNGGGGAACCNCCCCAGTT TAACNNAAAAATTNCGGGNCCCCAANGGGGGCCTGGTTTNTNNANCTCNGAAACTTCCGGTTGACNNTTGAAATAAAAANANNTCCTNGNANA AANANGGGCTTTTNCCC
CP1-8	GTGGCCACCTGTATTTGAAGAGCTTGCTCAGNATATGACGATGGACATTGCNAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCT CTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTNCTTAAAAGATGGTTCTGCTATCACTA AGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGNAATGGCTCCCCAAGACGATGATGCNTANCCGAGTTGAGAGACTGANCGGCCACAA TGGGANTGAGACACGGCCCATACTCNTACGGGAGGCNGCAGTAGGGAATCTTCCACAATGGGCGAAAGCNNGATGGNGCACCNCCGCGTGTG TNATGAAGGNTTTCGGCTCNTAAAACACTTTTTGTAAGANAAGAATGACNTTGAGAGTANCTGTCCAATGTGTGANGGTATCTTACCAGAAAG GANCGGCTAAAATACGTGCCANCGCNGGGGGTTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP1-15	TTGNTTGGTGCATTGCATCATGAATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACA CCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTNGGCTATCACTTTTGGATGGTCCCGCGG CGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGG CCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC TCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCC AGCAGCGCGNGGTTAATACGAA
CP1-18	TGCTGCACNAGCGCACCCTATCTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGG GGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTGAAAGATGGCTCTGCTATCACTTCTGGATGGA CCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGA GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGG GTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAAC TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAANGCGAGCGCAGGCGGTCTTTTAAGTCTAATGT GAAAGCCTTCGGCTCANCCGAAAANGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAAT GCGTAGATATNTGGAANANCNCCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGCTCGAAAGCATGGTAGCGAACAGGATTANA TACCNTGGTAGTCCATGCCGTAANCGATGATTCTANGGTTGGAGGGTTCCCCCCTNCAGTGCTGCACTAACCATTAGTATCCCCCTGGGGGTNC ACCNCAGNTNAAACTCAAANATTNANGGGCCCCCNAACCGGGGACNTNTGTTTATNCANCTCNGAAAACTTNCGGNNTGACTTTTGANNCTAA AAAAAAGTCCCNCGGNAAANAAGNGGCATGTTNCCNCNCGCG

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP1-19	
UFUL	GGTGATGNGTNCTTGCACTCGTTTNGACAATGTAANCCAGCGGTCTNCNGCGNTGTAACACNTGCTAGATCTGCNCNGNNNCCNGGCNNAACA CTTGNTAANAGGTNCTGNTANCGCATACTTTNNAAATCCNNNTGNCNTTNNTNNGNNNNGTGNNTTCNGNTNTCACTTCTGGATGATCNNGCN NACGNATCTAGTTNGNTGNGGAGNNNNANGCNNACCNAGANNATNNTNCNTACCCNAGCTGANATNCGAATCGGCCACATTGGGACTGAGAC ACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTT CGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTAACTACG TGCCAGCAGCGCCGGGTTAATACGAA
URUL	CGGNTNNGCGTTAGGTGGTTCCCACTCTAGCTGGCGNGTGTGTNTCACTCGGTACNNAGGCNTGNCCTNTCTTNANANCGCCTAGNNNGGTCAC CATCTNATCAAAAGNGCTGACCTNGANNAANCATANTNGGNGCCCNNGNNGGNNACTGCNTGNNNNNGANATGCGCTGCTNANNNGNCAGAN NGTTGTTTTAANTNTNCGTNNGTATTCGNGNGTNCCNNATCATCATAAGCNACTCGACTTGCATGTGTTAGGCAAACTAACTAATACGCCGCGG GATCATCCAGAAGTGATAGCCGAAGCCACCTTTCAAACAAAATCCATGCGGATTTTGTTGTTATACGGNATTAGCACCGNGTTTCCAAGTGTTA TCCCCTGCTTCTGGGCAGATTTCCCACGTGTTACTCACCAGTTCGCCACTCGCTTCATTGTTGAAAATCAGTGCAAGCNCNTCATTCAACGGAAG CTCGTTCGACTTGCANGTGNTANGCA
CP1-20	GCTTGGTGCATTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCT GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGT ATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCC AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCG TAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTTAACCAGAAAGCCACGGCTAACTACGTGCCAG CAGCGCCGGGTTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP2-3A	GATTGGTGCTTGCATCATGATTTCATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGG AAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTAT TAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAA ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTA AAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAAGCCACGGCTAACTACGTGCCAGC AGCGCGGGNTAATACGAA
CP2-17	GATGCNTGCTGTNTTGCANTCAGATTTGAGTGAGTGGCGGACGGGTGAGTAACACGNGGGTAACCTGCCCAAAAGTGGGGGATAACATCTGGA AACAGGTGCTAATACCGCATAACAACTACTTTCACNTGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCGCGGCGTATTA GCTAGTTGGTGAGGTAACGGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGCACTGAGACACGGCCCAAAC TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGANTGAAGAAGGTTTTCGGATCNTAAA ACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAANTGTTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAAANTACGTGCCANCN GCCGCGGNGTAATACGA
CP3-1	CGNTTCCGCCGCCGGGTTTNTANNGNTTGCTCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTAC CTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCNGCNTTAAAAGATGGTTCTGCTATCA CTAAGAGATGGTCCCGCGGTGCTTTTAGNTTAGTTGGNGNGGAAATGGCTCCCCAGGACGATGNTGCTTACCCGATTTGAGANNNTGANCGNC CNCAANGGNNNTGAAAACNCGCCCCANACNCTTNCGGAAGGNGNCAGTNGGAAATNTNCCACATNGGGCGAAANCTGGATGNNCCACCCCCG CGTTGTNTAATGAAGGGTTTTGGNCTCNAAAANCCTTTTTTNTAAGNAAANNATNANCTTNGAGAANTACCTNTTCAATGGGGGGGNGGNTNT CTNCCCNAAAAAGGNAAGNGGCTAAAATACGNGCCAGCCNNGGGGGNTAAAAAANGGAAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP3-8	GNTTGTGGNTCACCTGATTTGAAGAGCTTGCTCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTA CCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGTTCTGCTATCA CTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCNTAGCCGAGTTGAGAGACTGATCGGCCA CAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGT GTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAGAATGACNTTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAA AGGAACGGCTAAATACGTGCCAGCACCCGGGGNTANTACGAA
CP3-9	GTGGCCACCTGATTTGAAGAGCTTGCTCAGAATATGACGATGGACATTGCNAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTC TTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGTTCTGCTATCACTAA GAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAAT GGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTG ATGAAGGGTTTCGGCTCNTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAA CGGCTAAATACGTGCCAGCACCGGGGGNTAATACGAA
CP3-11	CNNNTCCGCCGCNNGGATTTGNAGAGCTTGCTCAGATATGACGATGGACATTGCNAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTA CCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTNCTTAAAAGATGGTTCTGCTATCA CTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGNGAGGTAATGGCTCCCCAAGNCGATGATGCTTACCCGAGTTGAGAGACTGATCGNCCA CAATGGNCACTGAGACACGGCCCATACTCCTACGGAAGGCNGCAGTAGGNAATCTTCCACATTGGGCGAAANCTNGATGNNCCACCCCCGCGT GTGTCATGAAGGTTTTCGGCTCTTAAANCTNTTTTGTAAGAAAAAANTNACNTTGAGAATANCTGTCAATGTGTGANGGTTTCTCNCCAAAAGG NACGGCTAAATACGTGCCACCCCNGGGNNTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP3-16	CGGTCCCGCGGCCNCGTGGNNNNTTNGNCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGG GGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTCTGGAT GGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGAC TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGA AGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCT AACTACGTGCCAGCAGCCGCNGGTAATACGAA
CP4-6	CGNTTTGTGGTNCACCTGNATTTGAAGAGCTTGCTCAGNATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAA CCTACCTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGTTCTGCT ATCACTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCACCAAGACGATGATGCNTAGCCGAGTTGAGAGACTGATCG GCCACAATGGGACTGAGACACGGCCCATACTCCTACGGNAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACNCC GCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAGAATGACNTTGAGAGTAACTGTCCAATGTGTGACGGTATCTTACCA GAAAGGAACGGCTAAATACGTGCCAGCNGCCCCGGNTANTACGAA
CP4-11	GTGGNNCACCTGGATTTGAAGAGCTTGCTTCAGAATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTAC CTCTTAGCAGGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGTTCTGCTATCAC TAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCNCCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGNCCAC AATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCNGCAGTAGGGAATCTTCCACAATGGGCGAAAGCNTGATGGAGCANCNCCGCGTG TGTGATGANGGNTTTCGNCTCNTAAANCACTGTTGTAAGAGAAGAATGACNTTGAGAGTANCTGTNCANTGTGTGACGGTATCTTACCAGAAA GGGACCGGCTAAATACGTGCNACACNGGGGGNTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP5-2	GAGATTGAAGCTTGCTTTCATGAATTCAGACCTTGGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAAAGTGGGGGATAACA TTTGGAAACAAGTGCTAATACCGCATAACAACTACTTTCACATGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCGCGGC GTATTAGCTAGTTGGTGAGGTAATAGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGC CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGAT CGTAAAACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAACTGTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAACTACGTGCC AGCAGCCCNGGGTTAATACGAA
CP5-9	CCTGAAGATTGAAGCTTTGCTTTCATGATTCAGACCTTGGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAAAGTGGGGGAT AACATTTGGAAACAAGTGCTAATACCGCATAACAACTACTTTCACATGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCG CGGCGTATTAGCTAGTTGGTGAGGTAATAGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTC GGATCGTAAAACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAACTGTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAACTACG TGCCAGCACGCNNGGGTTAATACGAA
CP6-2	GTTATTGATTATGACGTGCTTGCACTGAATGAGATTTTAACACGAAGTGAAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAG CAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGA TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGA ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCANCNCCGCGTGAGTGAA GAAGGTTTTCGGCTCGTAAAGCTCTGTTGTTAAAAAAAAACGTGGGTGAAAGTACTGTTCNCCCAGTGACGGTATTNACCAGAAAGCCCGGCT AACTACGTGCCAGCAGCGNGGGTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP6-4	AGGATTGAAGCTTGCTTTCATGATTCAGATTTTGGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCNAAAGTGGGGGATAACAT TTGGAAACAAGTGCTAATACCGCATAACAACTACTTTCACATGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCGCGGCG TATTAGCTAGTTGGTGAGGTAATAGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCC CAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGATC GTAAAACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAACTGTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAACTACGTGCCA GCACGNNGGGTTAATACGAA
CP6-8	CNGNTCCGTTGGATGCGTGCATTCGNATGAATTTCAACAATGAAGCGAGTGGCGAACTGGTGAGTAACACGTGGGAAATCTGCCCAGAAGCAG GGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATCCGCATGGATTTTGTTTGAAAGGTGGCTTCGGCTATCACTTCTGGATG ATCCCGCGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACT GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAA GGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGCTA ACTACGTGCCAGCAGCCGCGGGTAATACGAA
CP6-10	GGCTGGTGCGTTGGACNATGNTTTACATTTGAGTGAGTGGCGAACTGGTGAGNAACACGTGGGANACCTGCCCAGAAGCGGGGGATAACACCT GGNAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCNNAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGNCGT ATTAGCTAGATGGTGNGGTAACGGCTCACCATGNCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCC ANACTCCTACGGGACGCAGCAGTCAGNNAATCTTCCACAATGNACGAAAGTCTGATGGAGCAACGCNNCNTGANTNAAGAAGGATTTCGGCTC NTAAAACTCTGTTGTTAAAGANGANNATATCTGANAGTAACTGTTCAGGTATTGACGNTATTTAACCAGAAAGCCACNNCTAACTACGTGNCA NCNNCCGCGGTTATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP7-2	GGCCCAATTGATTGATGGTGCCTTGCACCTTGAATTGATTTTGGTNGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGA AGCGGGGGACAACATTTGGAAACAGATGCTAATACCGCATAACAGCGTTGTTCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTC TGGATGGACCTGCGGTGCATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAAT GGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCNTGATGGAGCAACNCCGCGTGAGT GAAGANGGTTTCGGCTCGTAAAGCTCTGTTGTNAAAAAAAACCNCGTATGAGAGTAACTGTNCATACGTTGACGGNTNTTTANCCAGAANGTC ACGGCTAACTACGTGCCAGAGCGGGGGTNANTACGAA
CP7-3	GTTATTGATTATGACGTGCTTGCANTGAAATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAG CAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGA TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGA CTGAGACACGGCCCAGACTCCTACGGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTTGATGGAGCANCNCCGCGTGAGTGAA GAAGGNTTTCGGCTCGTAAAGCTCTTTTGTNAAGAAAAACGTGGGTGANAGTACTGTTCNCCCAGTGACGGTNTTAACCAGAAGCACGGCTAN TACGTGCAGCGCGGGGGTAATACGAA
CP7-4	AATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAGATGC TAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGCATTAGCTAGTTGG TGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGNACTGAGACACGGCCCAGACTCCTACGGG AGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCANCNCNGCGTGATNAAAGAAGGTTTTCGNCTCGTAAAGCTCTGTTG TTAAAGAAGAACGTGGGTGAGAGTACTGTNCNCCAGTGACGGTNTTTACCAGAAAGCCACGGTAAATTACGTGCCAGCAGCGGGGTATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP7-7	GATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTG GAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTA TTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCA AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGT AAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGC ACGNCNGGGTNAATACGAA
CP7-9	TTGATTGATGGTGCTTGCACCTGAATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTNACCTGCCCAGAAGCGGGGG ACAACATTTGGAAACAGATGCTAATACCGCATAATAACGTTGTTCGCATGAACAATGCTTAAAAGATGGCTTCTCGCTATCACTTCTGGATGGA CCTGCGGTGCATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGA GACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGTGAAGAAGG GTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTAACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAAC TACGTGCCAGCACNNNGGGGNTNAATACGAA
CP8-1	GNNTGATTGAAGGAGCTTGCTCCTGAATTGATAAACATTTGAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTAAAGTGGGG GATAACATTTGGAAACAGATGCTAATACCGCATAAAACCTAACACCGCATGNTGTAGGGTTGAAAGATGGTTTCGGCTATCACTTTAGGATGG ACCCGCGGTGCATTAGTTAGTTGGTGAGGTAAAGGCTCACCAAGACCGTGATGCATAGCCGACCTGAGAGGGTAATCGGCCACACTGGGACTG AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAG GTTTTCGGATCGTAAAACTCTGTTGTTGGAGAAGAATGTATCTGATAGTAACTGATCAGGTAGTGACGGTATCCAACCAGAAAGCCACGGCTAA CTACGTGCCAGCAGCGCNGGNTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP8-4	GATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGANACCTGCCCAGAAGCGGGGGATAACACCTG GAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCNAGCTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTA TTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCA AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGNTTTCGGCTCGT AAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGC ACGNNNGGNTAATACGAA
CP9-1	CNNTNCGGCCGCGGGAGGANNTNGNTCCTGATTGATAAACATTTGAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCTAAAGT GGGGGATAACATTTGGAAACAGATGCTAATACCGCATAAAACCTAACACCGCATGGTGTAGGGTTGAAAGATGGTTTCGGCTATCACTTTAGG ATGGACCCGCGGTGCATTAGTTAGTTGGTGAGGTAAAGGCTCACCAAGACCGTGATGCATAGCCGACCTGAGAGGGTAATCGGCCACACTGGG ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAA GAAGGTTTTCGGATCGTAAAACTCTGTTGTTGNAGAAGAATGTATCTGATAGTAACTGATCAGGTAGTGACGGGTATCCAACCAGAAAGCCAC GGCTAACTACGTGCCAGCAGCNCCGGGTTAATACGAA
CP9-2	CNGCAGATGNAGCTTGCTTCATGATTCAGATTTTGGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCNAAAGTGGGGGATAACA TTTGGAAACAAGTGCTAATACCGCATAACAACTACTTTCACATGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCGCGGC GTATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGC CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGAT CGTAAAACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAACTGTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAACTACGTGCC ANCAGCNNNGGGNTAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP10-1	GATGATTGAAGCTTGCNTCATGAAATCAGATTTGAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAAAAGTGGGGGATAACA TTTGGAAACAAGTGCTAATACCGCATAACAACTACTTTCACATGATCGTAGCTTGAAAGATGGCTCTGCTATCGCTTTTGGATGGACCCGCGGC GTATTAGCTAGTTGGTGAGGTAATAGCTCACCAAGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGC CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGTTTTCGGAT CGTAAAACTCTGTTGTTGAAGAAGAACATGCGTGAGAGTAACTGTTCACGTACTGACGGTATTCAACCAGAAAGCCACGGCTAACTACGTGCC AGCACCGGGGGGNTTAATACGAA
CP10-3	TGGATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGANACCTGCCCAGAAGCGGGGGATAA CACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCNAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGC GGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACAC GGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGNTTTC GGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGT GCCAGCAGCGNNGGNTAATACGAA
CP11-2	NGACCCTNAACNAGCGCGACCCTTAANCTGNATTGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAG AAGCAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCT GGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTG AAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACCCAGTGACGGTATTTAACCAGAAAGCCA CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAGGCGTTATCCGGATTTNTTGGGCGTAAAGCGAGCGCAGNGGGTCTTTTAA GTCTAATGTGAAAGCCTTCGGCTCANCCGAANANGTGCATTGGAAACTGGGANACTTGAGTGCAAAANAGGACAGTGGAACTCCNTNTGTNGC GGGGAAATGCGTNNATTNTGNAANAACNCCAGTGGGNAAGNGGCTNTCTGGTCTTTAACTAACNCTGAGCTCAAAGNTNGGTTAGCGANAGG ATTAAAACCCTGGTNGNCNTGCNGTAANNATTTNATANTNTGGNGGNTTCCCCNTCAGGGTGCNCTAACCNNAANNANCCCNGGGGNNTCNNC CNAGTTNACNNCAAAAATTNCGGGCCCCCANGNGNGAACTTGTTTTTTNGAGTCCGGNAACTTCCGGNTTTATNTNCGCACNNAAAATAGCTN CTCGGAAAAAAAAAGGGGCANTTNCCCCCCGCNGAATGT

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP11-6	TGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCT GGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGT ATTAGCTAGATGGTGNGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCC AAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCG TAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA CNCGGGGNTANTACGAA
CP11-9	NTGCTGCACGAGCGCNACCCANAATNTTTCNTTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAAC ACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGNTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCG GCGTATTAGCTAGATGGTGNGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACG GCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCG GCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAG CCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAG ATATATGGAAGANCNCCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACNCTGAGCTCGAAAGTNTGGNTAGCAAACNGGATNAAATNCCC TGGTAGNCCTACCGTAANCGATGAATGCTANGGTTGGAGGGTTNCCCCCTTCAGGCTGCAGCTANCCATTAACATCCCCCTGGGGAAACGCCCC ANGNTAANCCNAANAATNACGGGGCCCCNAANCGGGGGANCNTTGNTTTATTNGANCTCCGANAACNTTCCGGTNTNGAANTTTCAATTAAAA NAAACTCCCTCGGAANTGAANAGGGGGCAGTTTCCCCCCNGCNGGNTTTTTTTTTNCCCCCCNNCCC
CP11-11	TGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGNTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAGATGCTA ATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGCATTAGCTAGTTGGTG AGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAAACACGGCCCAGACTCCTACGGGA GGCAGCAGTAGGGAATCTTCCACANTGGACGCAAGNCTGATGGAGCAACGCCGCGTGAGTNAAGANGGTTTTCGGCTCGTAAAGCTCTGTTGT TAAANAANAACGTGGGTGAGAGTAACTGTCCNCCAGTGACGGTNTTTAACCAGAAGCCACGGNTAACTACGTGCCANCAGCCGGGNTAATACG AAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP12-8	TNGACNANCNCGNCCNTATCTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGG ATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACC CGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGA CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGT TTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTA CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTG AAGCCTTCGGCTCACCNGAAAAGTGCATNGGAAACTGGGANACTTGAGTGCAGAANAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCG TAAATNTTTGGAAAACCCCAGTGGCGAAGCGGCTGTCTGGTCTGCAACTGACNCTGAGCTCGAANCNTGGNTGCGAAAGGATTANATNCCTGG TAGCCATGCCGTAACNATGATNCTAAGGNTGGAGGNTTCCCCCNTCAGNCTGCAGCTANNNNTAAATATCCCCTGGGGATNCCCCNCAGNTGA ACNCAAAAATNGCGGGCCCCCCANNCGGGGACNTGGTTNATNNANCTCCGNAAACTTNNGGCTNNANNTNNNANCTNAAAATAAGNNCNTNG GNAAAAANNGGGNCAGTTTCCCCCNCGCGNANTGTTTTTNCCCCCCCNN
CP12-9	TGCTGCCTGCAACGAGCGCAACCCTAATCTGNTTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAG AAGTAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCT GGATGGACCCGCGGCGTATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTG AAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTTAACCAGAAAGCC ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTA NGTCTAATGTGNAAGCCTTCGCTCACCGAAGANGTGCATTGGAAACTGGGANACTNGAGTGCAGAANAGNACGGTGGAACTCCATGTGTAGCG GGAAATGNGTANATTTTGGNAAAACCCNGTGGCGAANGNGGCTNTNTTNTCTGCAATTNNCCTTAGCTCNAAANCNNTGGTAGCGAAAAGNAT NANANCCCTGGGAGCCNTGCNGNAANAANAANTNCTAAGGGTGGNGGNTTCCCCCNNCANGCTGCGCTAACNATNAANANCCCCNTGGGGAA NCCCCCCAGGTNAANNNNAAAATTTGCNGGGNCCCCCANCGGGGNACTTGTNTTNTTNTGNNCCCNGNNAAACTNCCGGGTTNAANTTTNAAA ATTAAAANAANNNNCTNGGNAAAANNNAAGGGNTCTTTTTTCC

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP13-1	GGTGCTTGCCCCTGATTGATTTTGGTCGCCAACGAGTGGCGGACGGGTGAGTAACACGTAGGTAACCTGCCCAGAAGCGGGGGACAACATTTG GAAACAGATGCTAATACCGCATAACAGCGTTGTTCGCATGAACAACGCTTAAAAGATGGCTTCTCGCTATCACTTCTGGATGGACCTGCGGTGC ATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCC ATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCNTGATGGAGCAACNCCGCGTGAGTGAAGAAGGNTTTCGGCTCG TAAAGCTCTGTTGTNAAAGANAACCCGTATGAGAGTAACTGTTCATACGTNGACGGTNTTTAACCAGAAAGTCACGGCTAACTACGTGCCAGC AGCCGGGGTTAATACGAA
CP13-2	CTGCTGCNACGAGCGCNACCCTTAANCTGNNTGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAA GCAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGG ATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGG ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAA GAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAACTGTTCACCCAGTGACGGTATTTAACCAGAAAGCCACG GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTANGTC TAATGTGAAAGCCTTCGGCTCANCCGAANANGTGCATTGGAAACTGGNANACTTGAGTGCAGAAAAAGGACAGTGGAACTCCATGTGTAGCGG TGAAATGCGTAATATNTGGAANAACNCCAGTGGCGAAGCGGCTGTCTGGTCTGTAACTGACNCTGAGCTCGAAGCNTGGTAGCGAACAGGATT AAATCCCTGGTAGNCCTGCCGTAACGANGATTCTAAGGTTGGAGGGTTCCNCCTTCAGGCTGCACTAACNATTANTANCCNCNTGGGGATCCNC NCAGNTGAACCAAAAATTGCGGGCCCCCAACGGGGACTTGGNTTATNGACTCNGNAACTTCNGCTNNANTTNGCACNAAAATAGNTCCCTNGA AAANAAGGGGCANNTTCCNCNTNN
CP13-7	TGAATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGCAGGGGATAACACCTGGAAACAGAT GCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGCATTAGCTAGTT GGTGAGGTAACGGCTCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCANCNCCGCGTGAGTGAAGAAGGNTTTCGGCTCGTAAAGCTCTGT TGTTAAAAAAAAACGTGGGGTGANAGTAACTGTTCNCCCAGTGACGGTATTTANCCAGAAAGCCACGGCTAACTACGTGCCAGCAGCGGGGGN TAATACGAA

Table 4-5 (continued). 16S rRNA gene sequences of selected strains

Isolated No.	16S rRNA gene sequences
CP13-9	NNAATTGNATTGAGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGAT AACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGA TGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGA CTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAG TGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCC ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGG NTTTTTAAGTCTGATGTGAAAGCCTTCGNCTCACCGAAAAAGTGCATCGNAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT GTGTAGCGGTGAAATGCGTANATATNTGGAANAACNCCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACNCTGAGCTCGAAAGT TTGGGTGCAACNGGATTANANACCCTGGTAGTCCATCCGTAAACNATGAATGCTAAGGTNGGAGGTTTCCCCCCTNANGGCTGCANNTANCCN TTAACATTCCCCNGGGGATACCGCCNCAGCTNAAACTCAAAGAATTGCGGGGCCCCCAAGCGGGGGACNTGGNTTNNTCGNAANNCC GNAAAACTNNNNGCNTGAATTTGNAANTTAAAANTAACCTCCCNN
CP13-12	CTTGAACGAGCGCTNCCCNTAGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAAGCGGGGGATAACAC CTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGNTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGC GTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGC CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCT CGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCA GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGNTTTTTTAAGTCTGATGTGAAAGCCT TCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGAT ATATGGAANAACNCCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACNCTGAGCTCGAAGTNTGGGTAGCAACAGGATNAGATACCTGGTA GNCNNACNGTAACATGATGCTAGTGTGGAGGNTTCCNCCTTCAGTCTGCGCTANCCATTAGCNTCCCCTGGGAGTACGCCCCAGCTNAACTCAA GNATTACGGGGCCCCCCAACGGNGGACATGGNTTTTTCNAGCTCCNGANACTTNNGGCTNGNATCTTGNAANTAANATAAACTNCCTNGGAAT GANNNGGGGCTGTTNCCCCCCCCCGAANTNGTANCCCCCCNCNCNN

Table 4-6. The result of diacetyl screening

Level of change in medium color from the original to red	Isolate No.
-	CP1-8, CP1-13, CP1-14, CP1-15, CP1-17, CP1-19, CP1-20, CP3-8, CP3-9, CP3-10, CP4-6, CP4-11, CP4-18, CP6-2, CP6-8, CP7-2, CP7-3, CP7-4, CP7-8, CP7-9, CP8-4, CP10-2, CP10-3, CP11-2, CP11-3, CP11-5, CP11-6, CP11-7, CP11-8, CP11-9, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15, CP12-4, CP12-8, CP12-9, CP12-10, CP12-11, CP12-14, CP12-15, CP13-1, CP13-2, CP13-3, CP13-4, CP13-7, CP13-9, CP13-11, CP13-12
+	CP1-5, CP1-18, CP2-3B, CP2-10, CP2-16, CP3-1, CP4-5, CP4-12, CP4-16, CP4-17, CP5-2, CP5-6, CP5-9, CP6-3, CP6-10, CP7-5, CP7-7, CP8-1
++	CP2-11, CP3-11, CP3-16,
+++	CP2-3A, CP11-6

-, no change; +, slight change; ++, moderate change; and +++, strong change.

Table 4-7. The result of bacteriocin screening

Pathogens	*B. cereus* TISTR 037	*C. sporogenes* TISTR 1481	*S. aureus* TISTR 029	*E. coli* TISTR 073
Isolate No.	CP1-15, CP2-11, CP3-1, CP7-3, CP10-3, CP11-6, CP14-1, CP14-4	To be determined	CP1-15, CP7-3, CP14-2, CP14-3	-

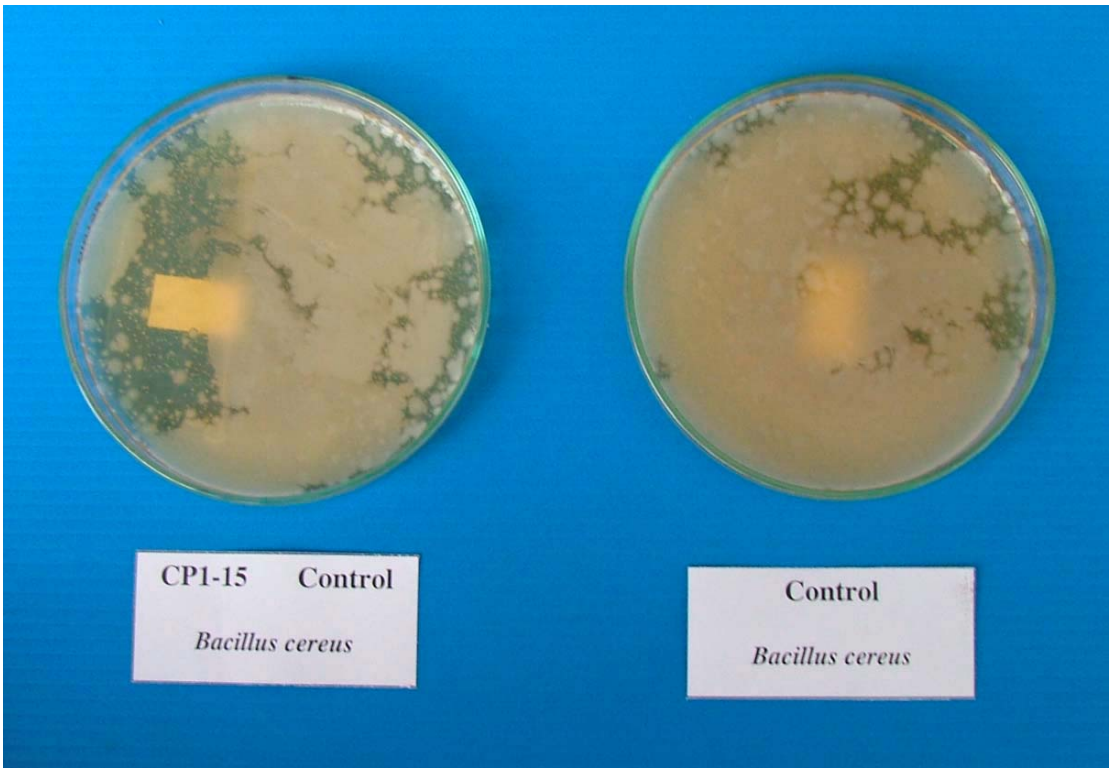

Figure 4.2. Inhibition zone against *B. cereus* by CP1-15

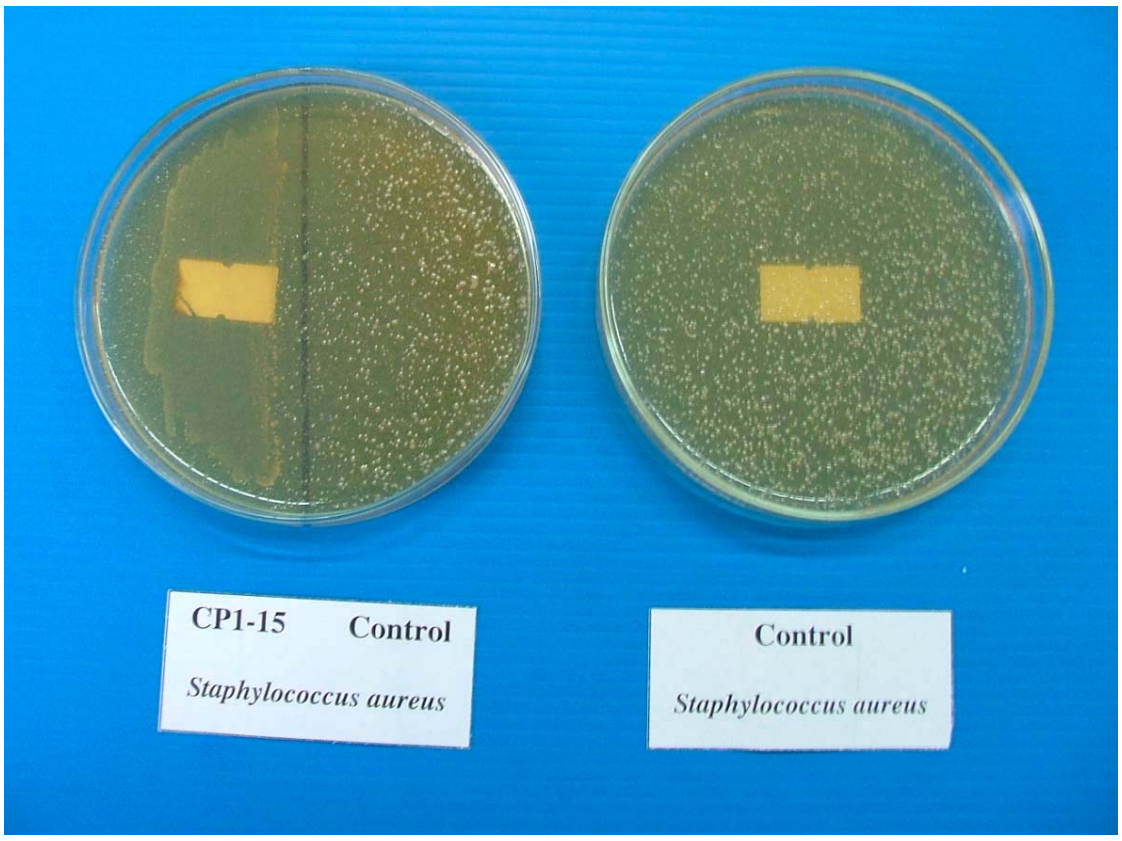

Figure 4.3. Inhibition zone against *S. aureus* by CP1-15

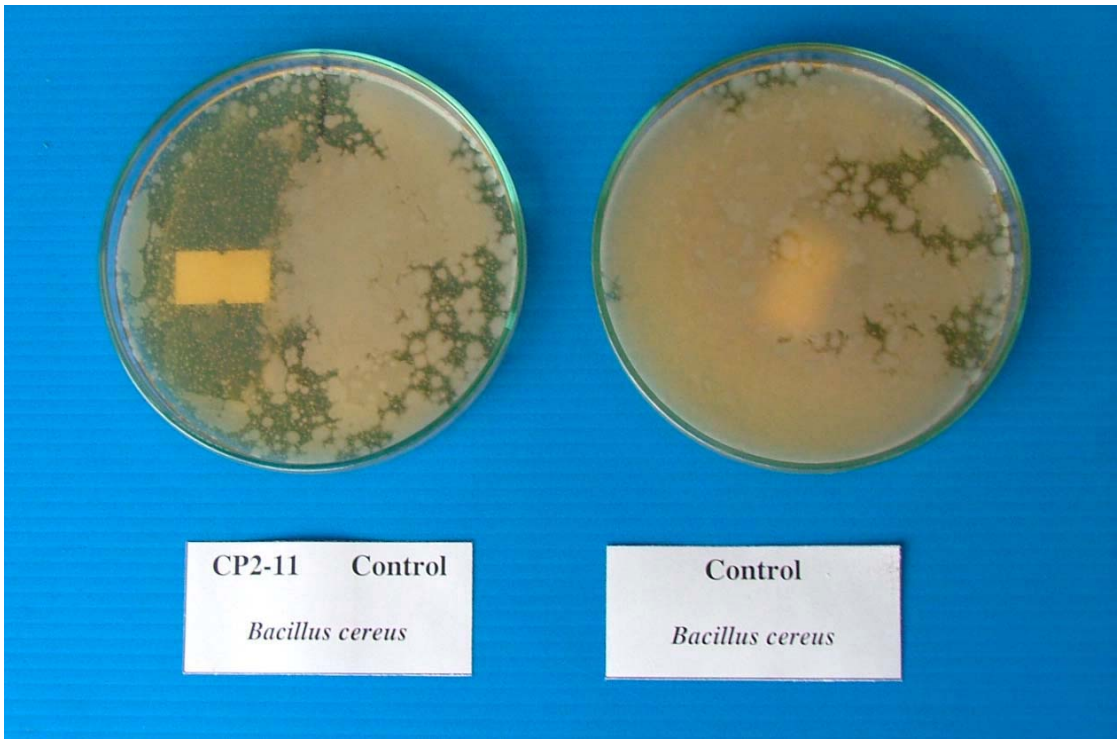

Figure 4.4. Inhibition zone against *B. cereus* by CP2-11

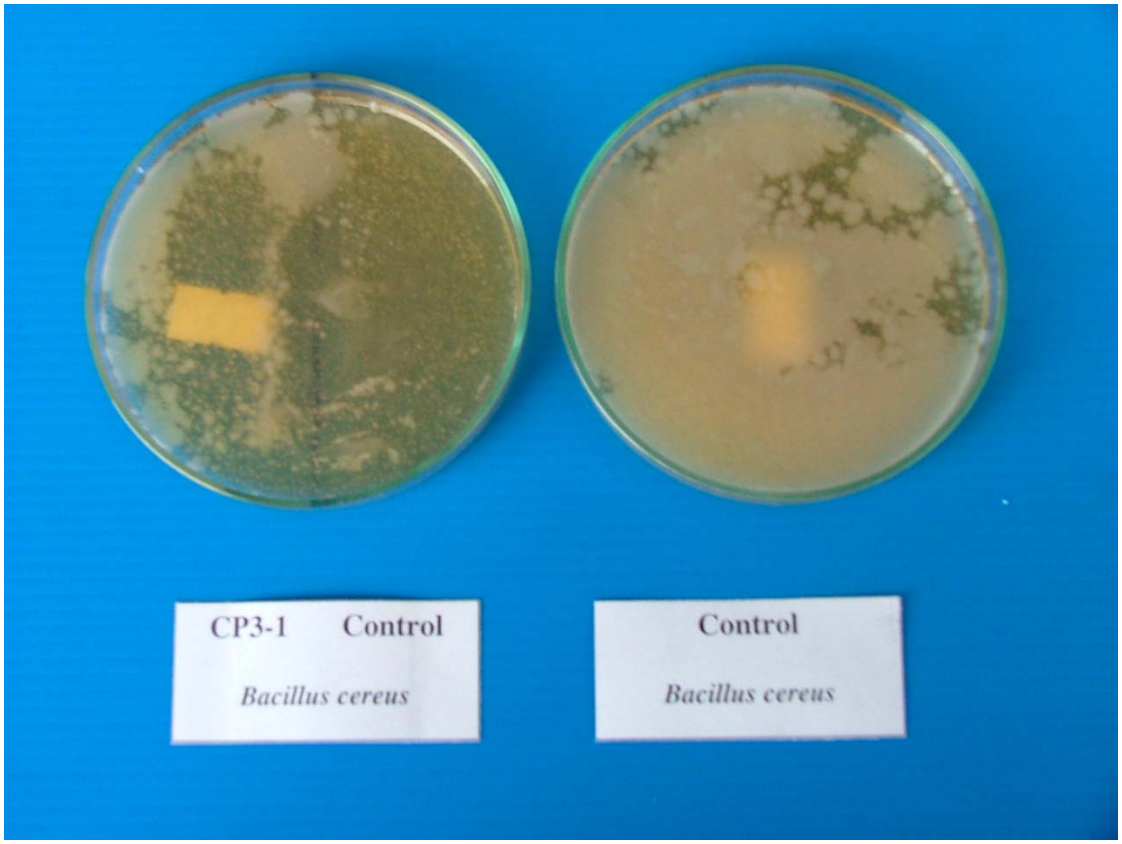

Figure 4.5. Inhibition zone against *B. cereus* by CP3-1

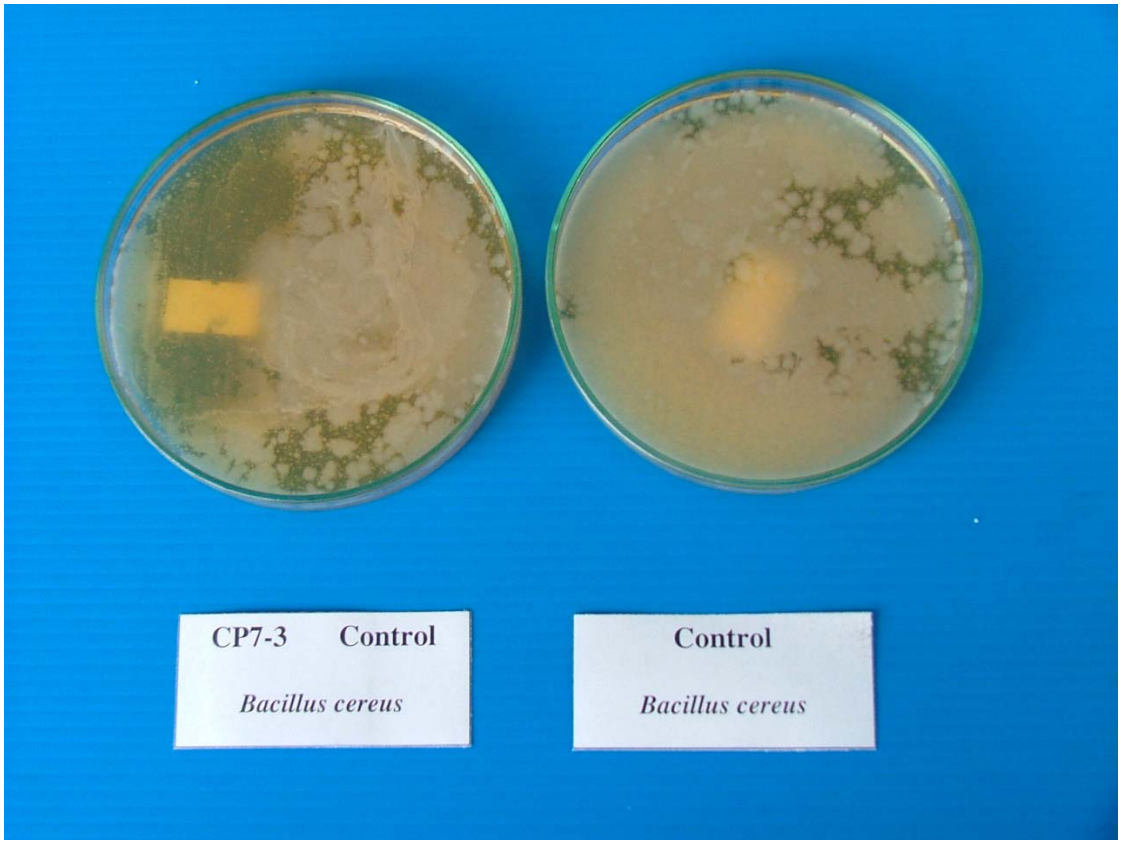

Figure 4.6. Inhibition zone against *B. cereus* by CP7-3

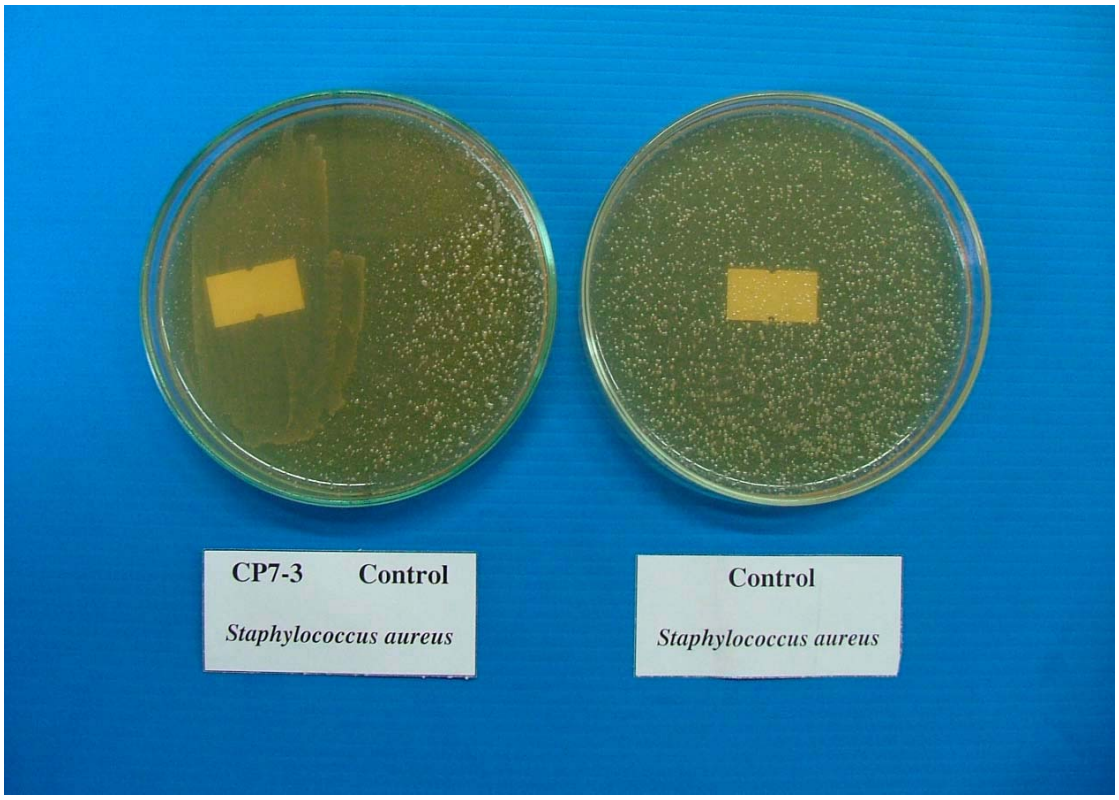

Figure 4.7. Inhibition zone against *S. aureus* by CP7-3

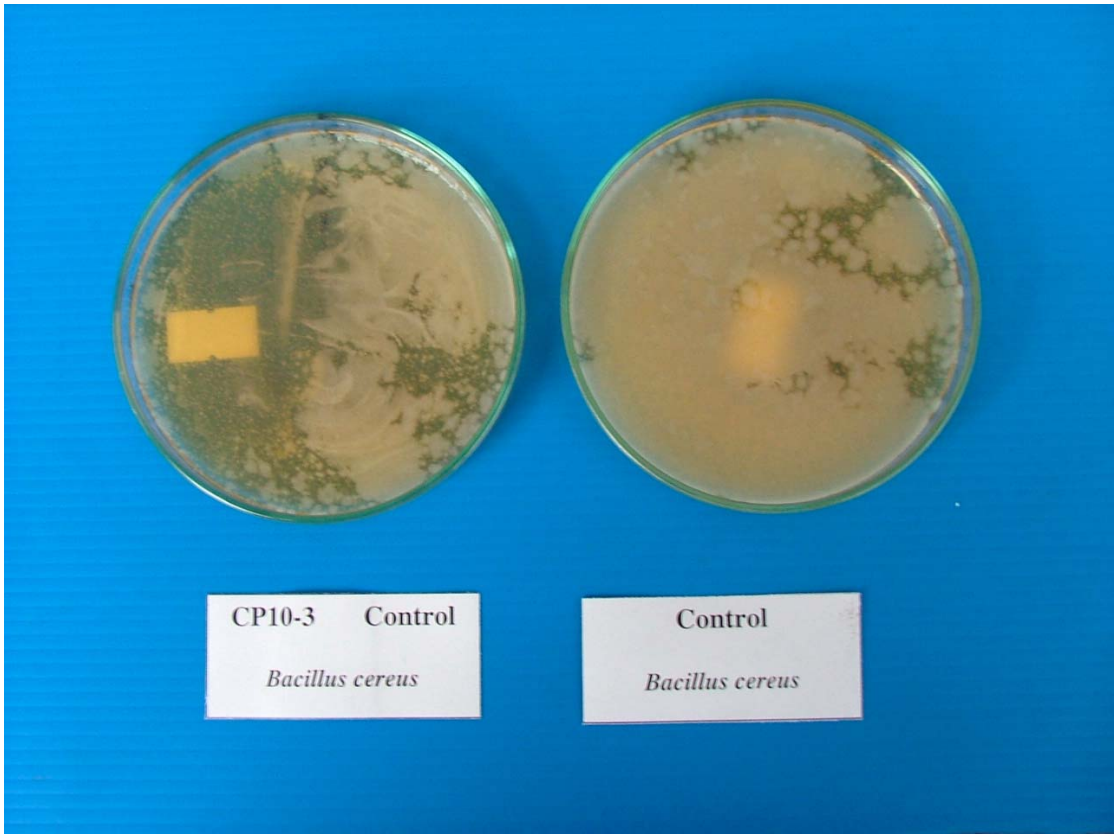

Figure 4.8. Inhibition zone against *B. cereus* by CP10-3

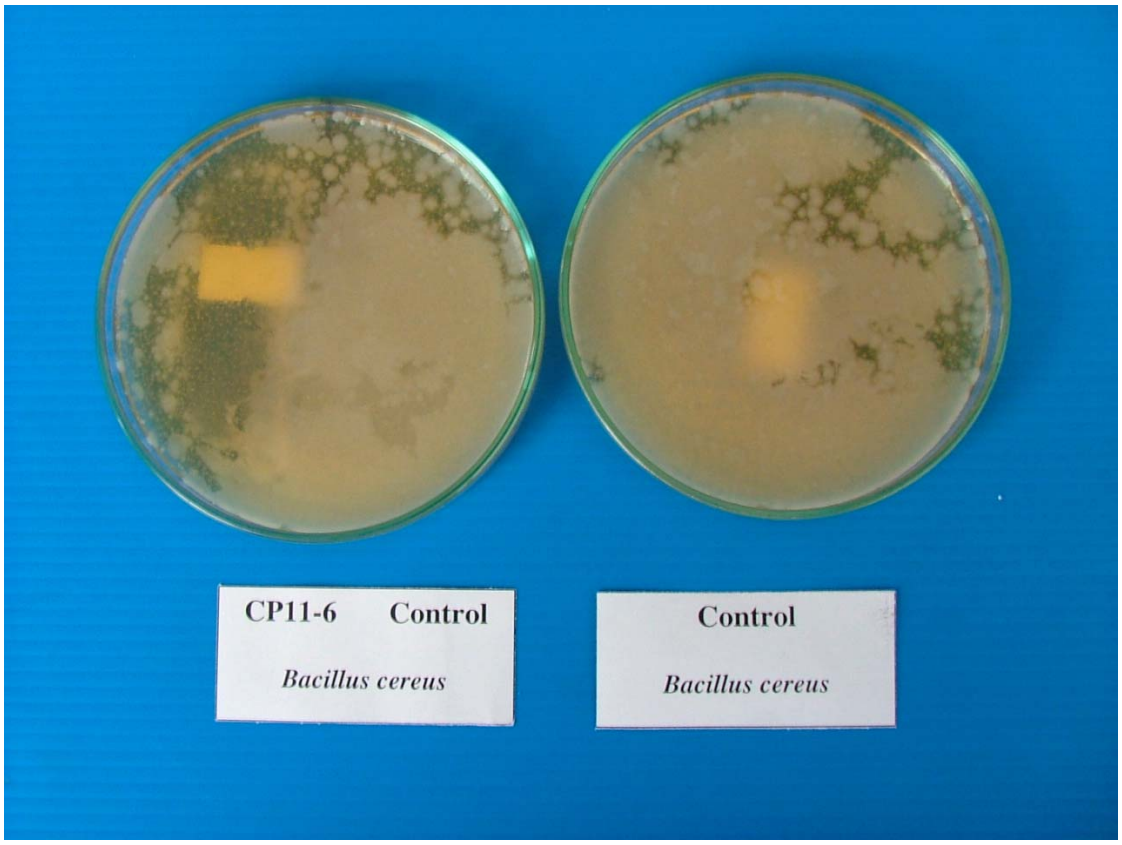

Figure 4.9. Inhibition zone against *B. cereus* by CP11-6

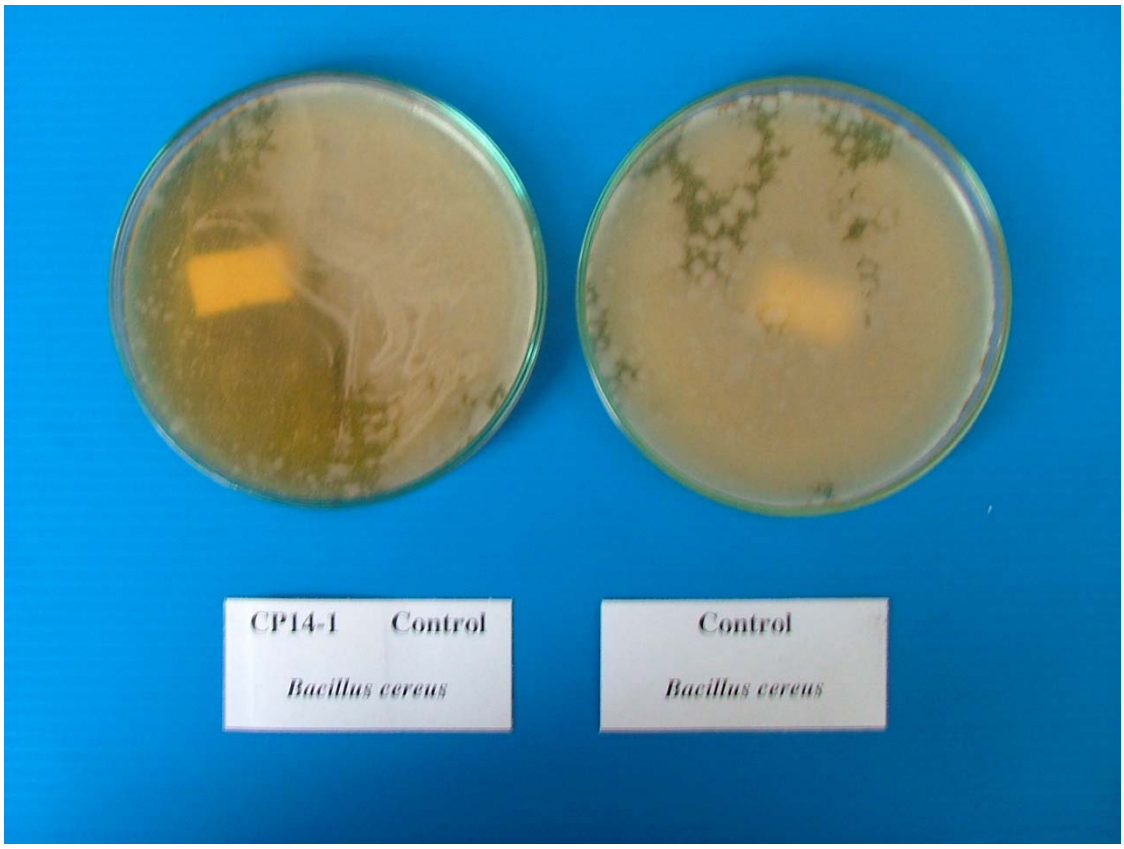

Figure 4.10. Inhibition zone against *B. cereus* by CP14-1

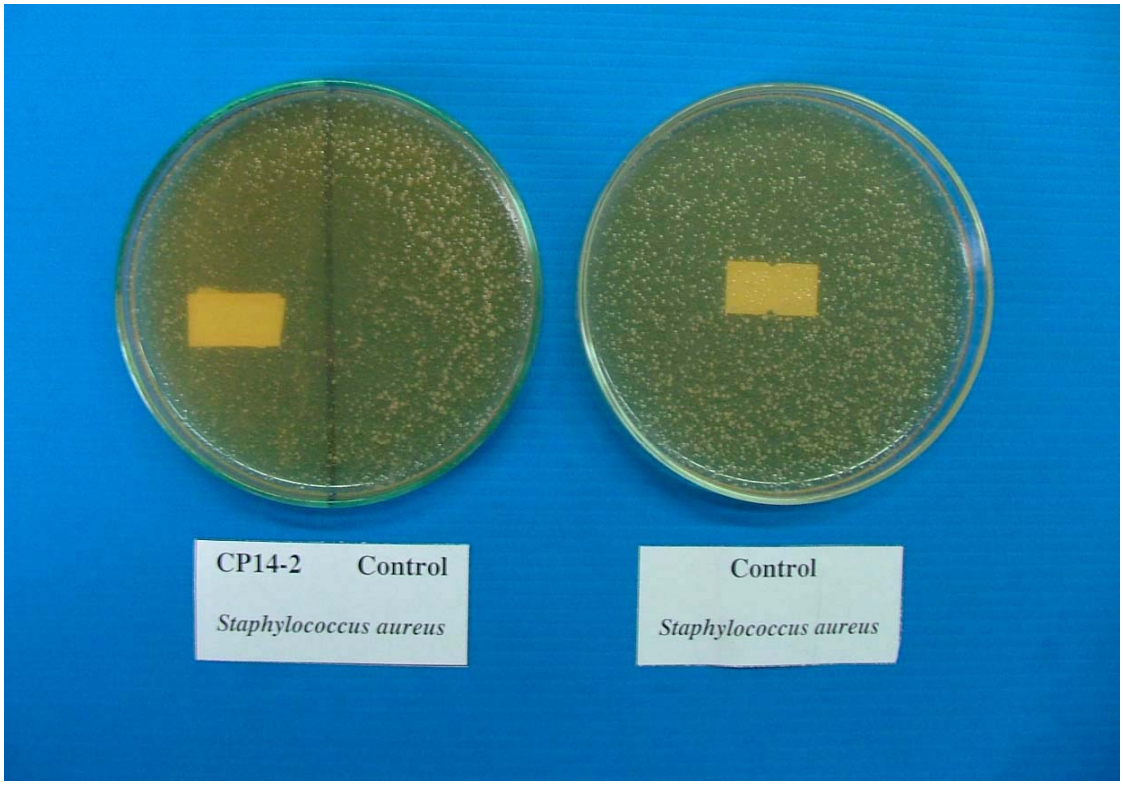

Figure 4.11. Inhibition zone against *S. aureus* by CP14-2

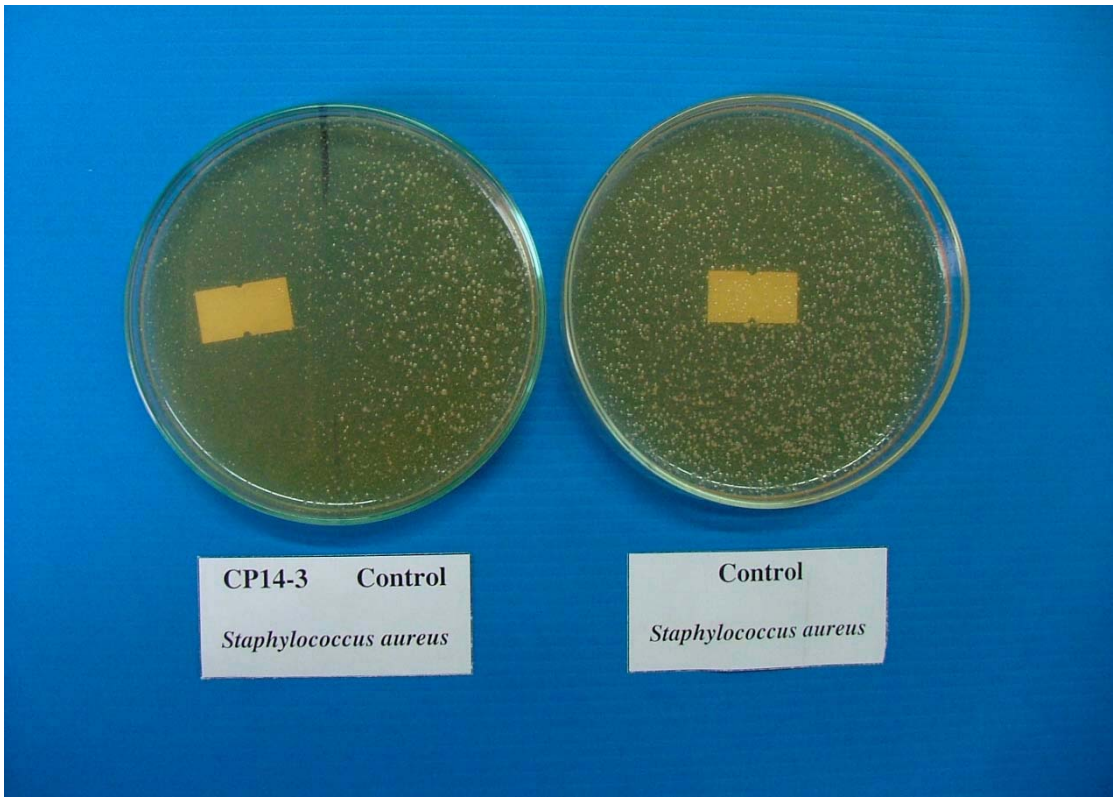

Figure 4.12. Inhibition zone against *S. aureus* by CP14-3

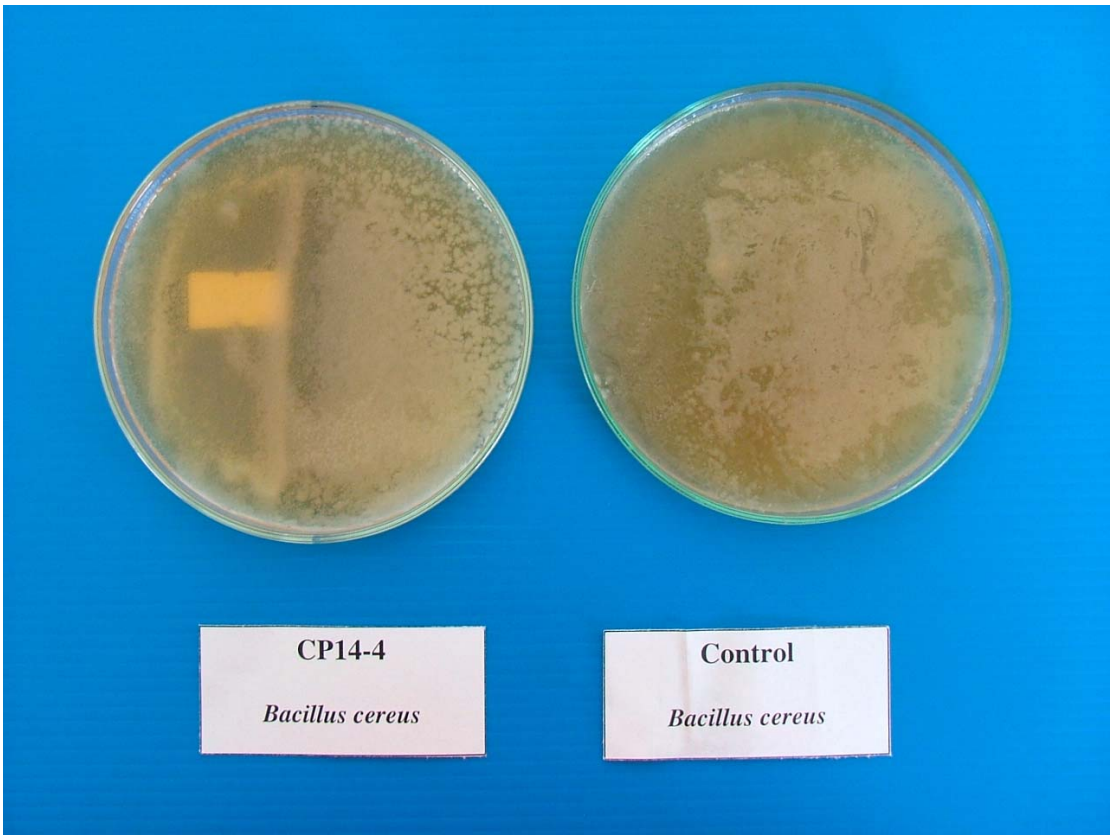

Figure 4.13. Inhibition zone against *B. cereus* by CP14-4

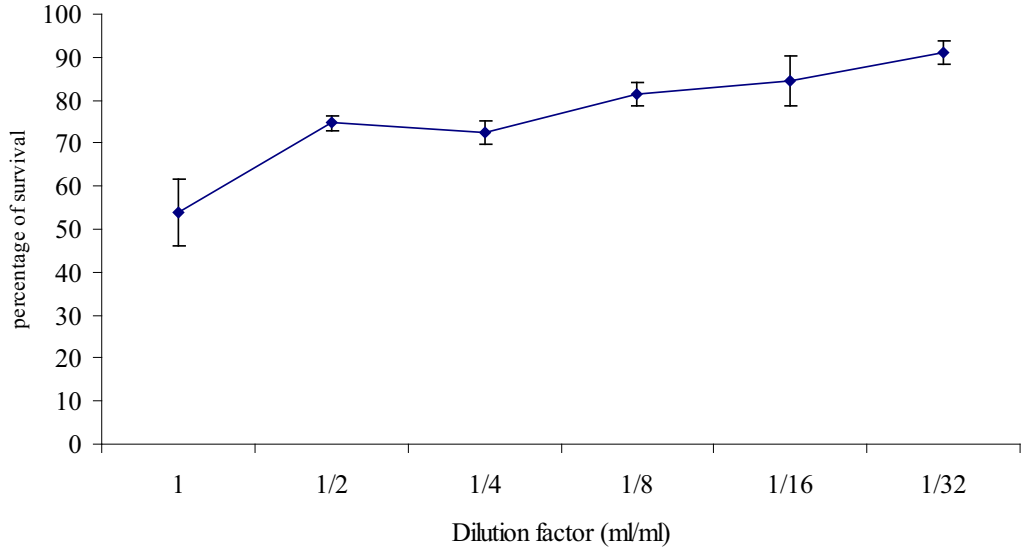

Fig. 4.14. Percentage of survival of *B. cereus* at each dilution of LAB (CP1-15) filtrate (ml filtrate/ml total volume)

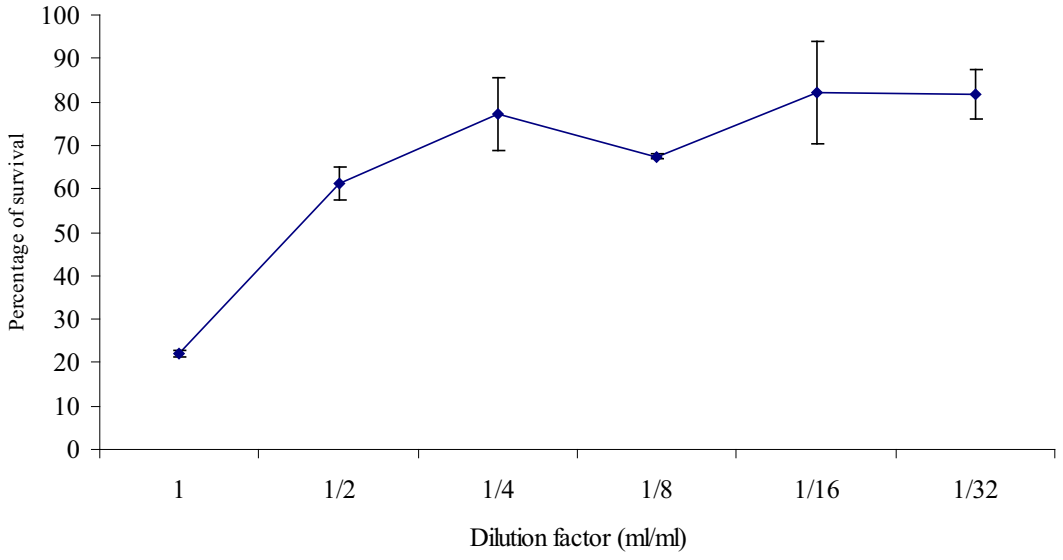

Fig. 4.15. Percentage of survival of *B. cereus* at each dilution of LAB (CP2-11) filtrate (ml filtrate/ml total volume)

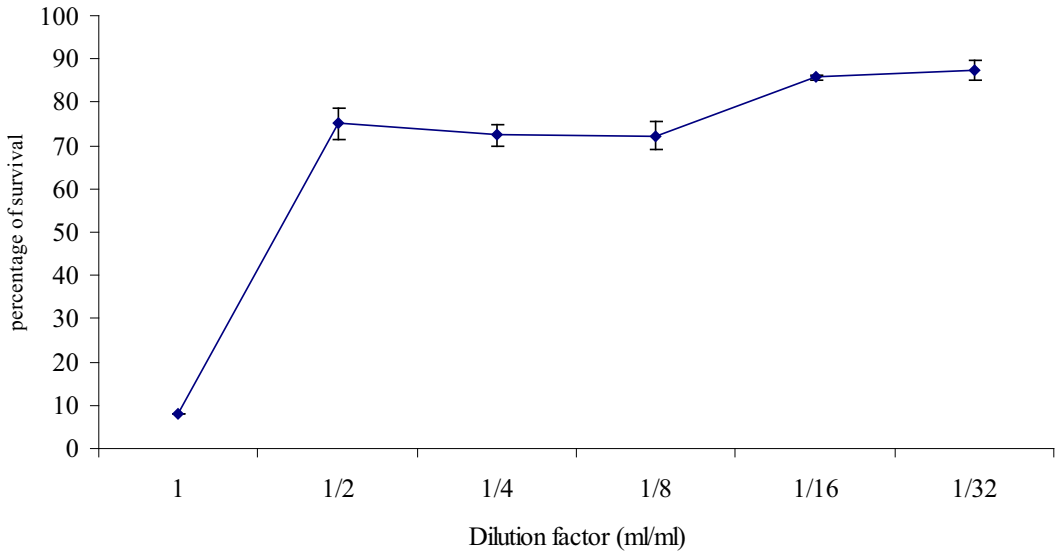

Fig. 4.16. Percentage of survival of *B. cereus* at each dilution of LAB (CP3-1) filtrate (ml filtrate/ml total volume)

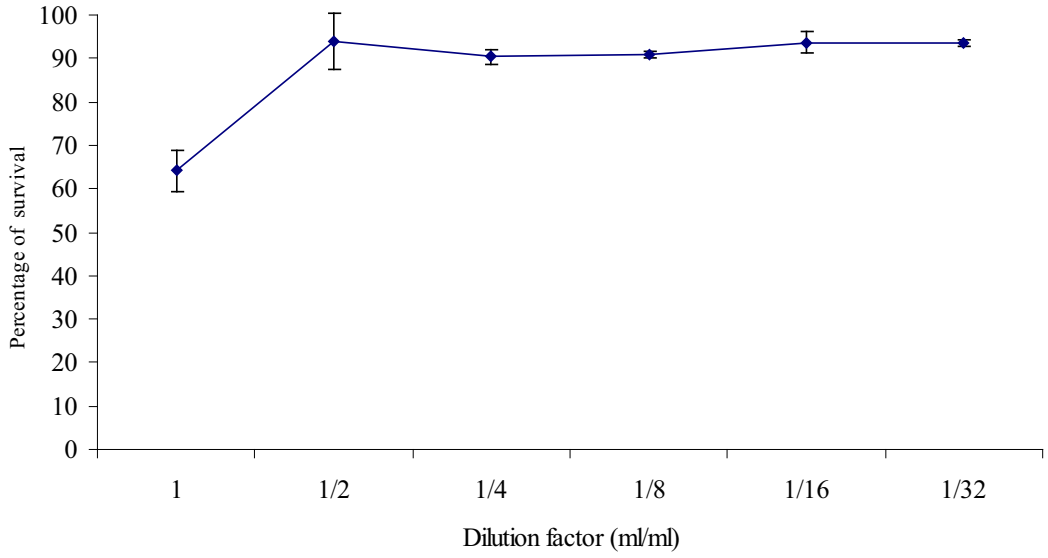

Fig. 4.17. Percentage of survival of *B. cereus* at each dilution of LAB (CP7-3) filtrate (ml filtrate/ml total volume)

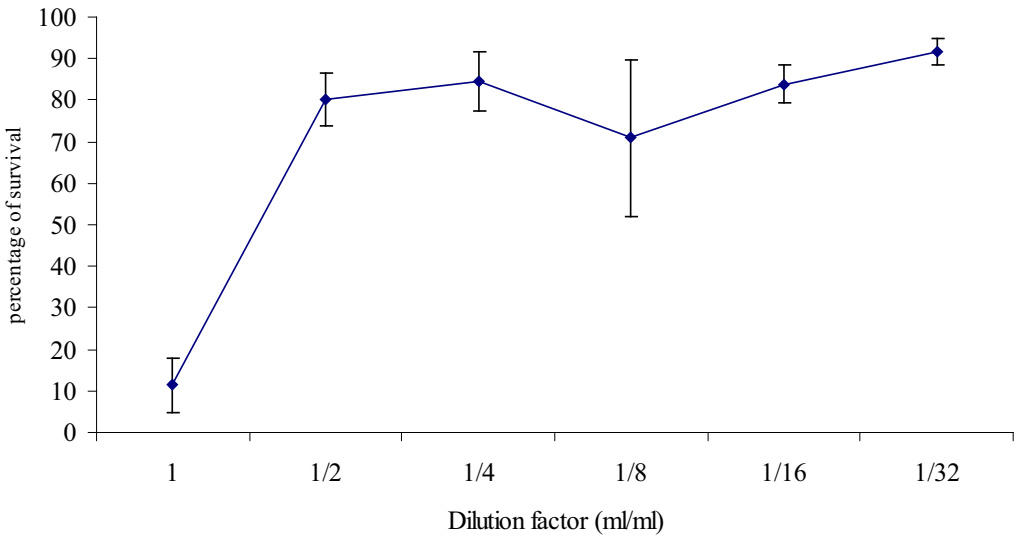

Fig. 4.18. Percentage of survival of *B. cereus* at each dilution of LAB (CP10-3) filtrate (ml filtrate/ml total volume)

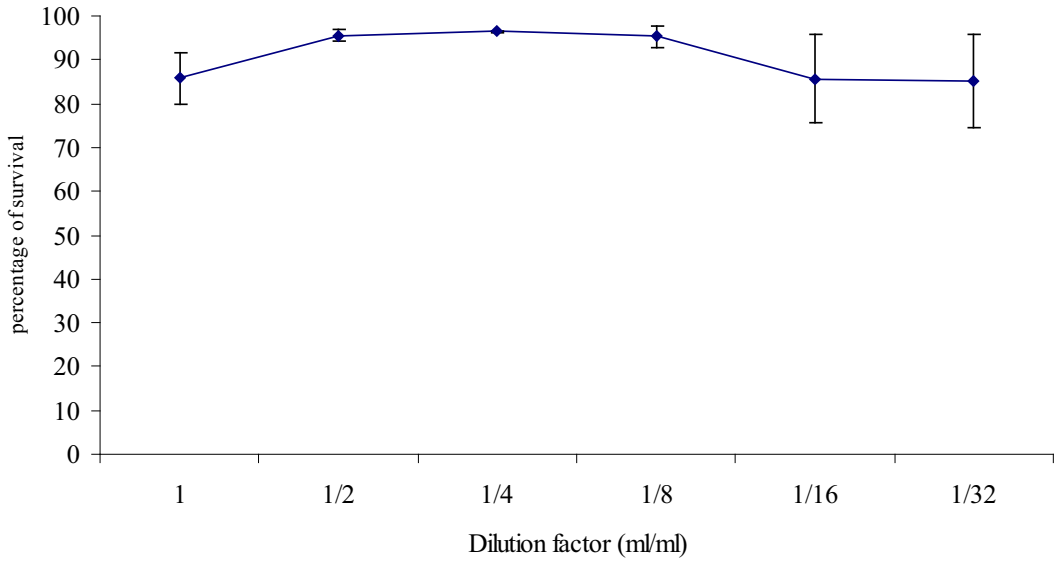

Fig. 4.19. Percentage of survival of *B. cereus* at each dilution of LAB (CP11-6) filtrate (ml filtrate/ml total volume)

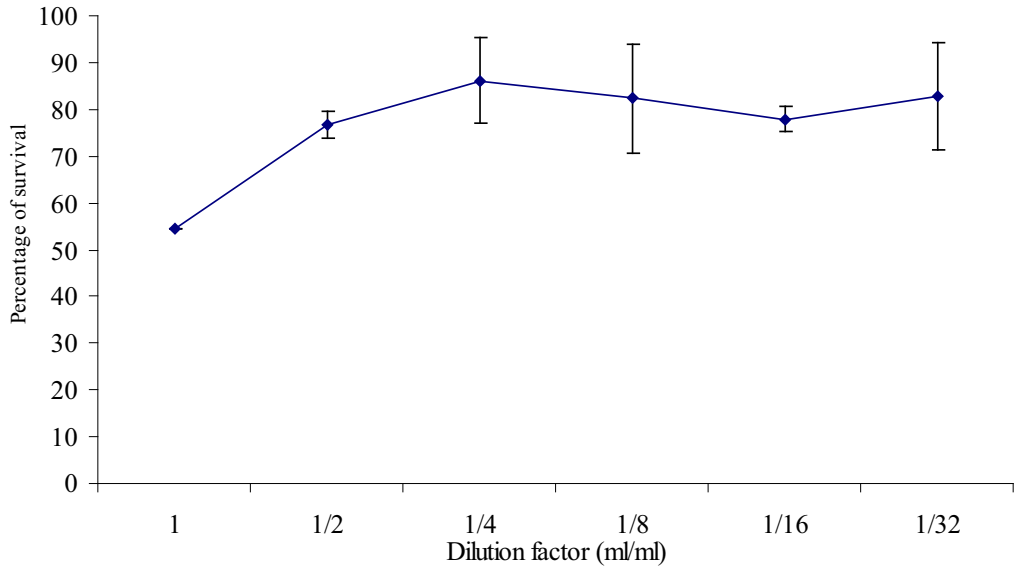

Fig. 4.20. Percentage of survival of *B. cereus* at each dilution of LAB (CP14-1) filtrate (ml filtrate/ml total volume)

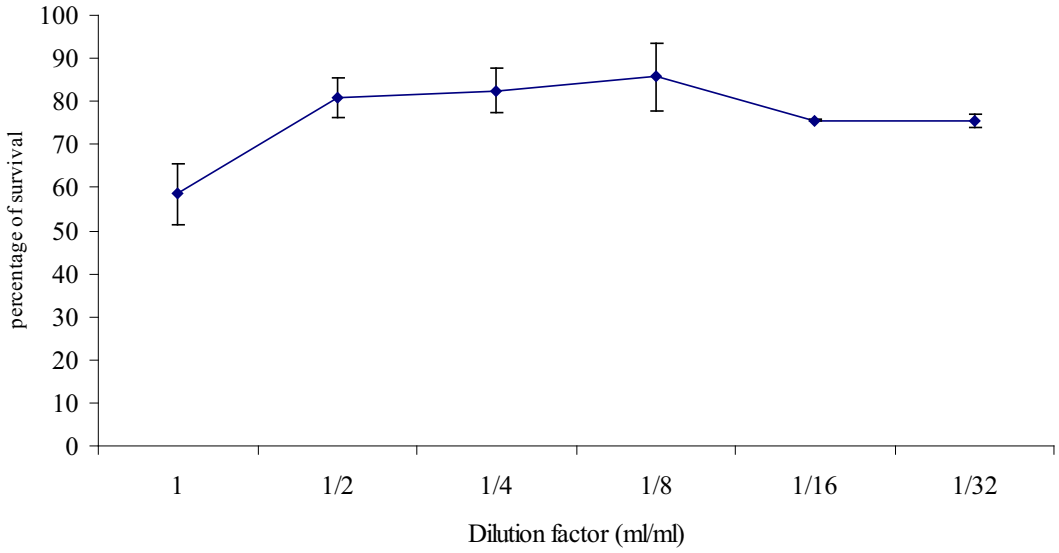

Fig. 4.21. Percentage of survival of *B. cereus* at each dilution of LAB (CP14-4) filtrate (ml filtrate/ml total volume)

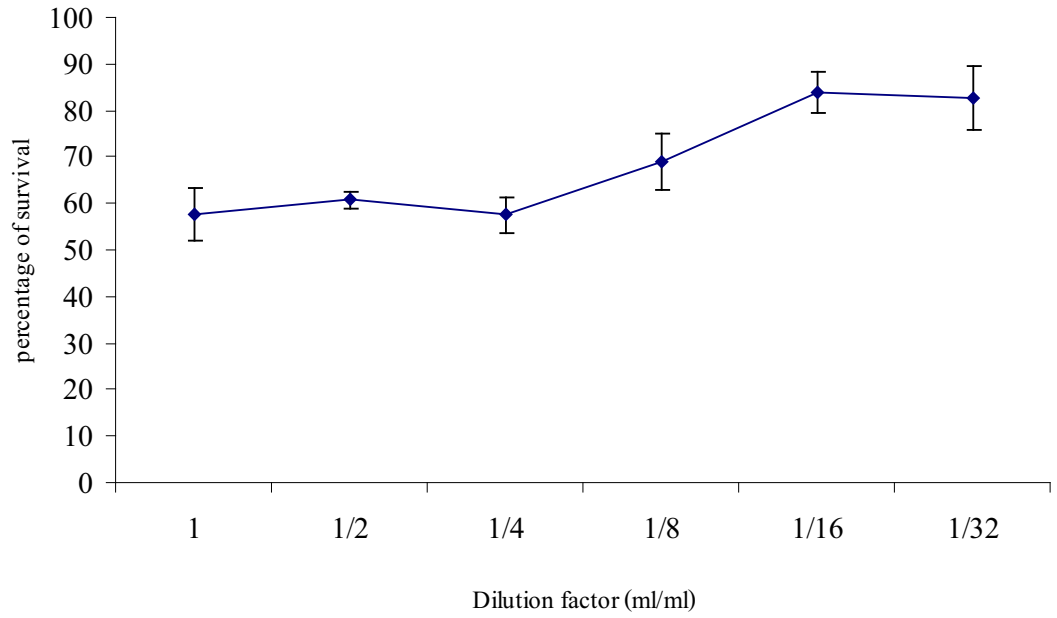

Fig. 4.22. Percentage of survival of *S. aureus* at each dilution of LAB (CP14-3) filtrate (ml filtrate/ml total volume)

Table 4-8 Bacteriocin unit (BU) of isolated strains that inhibited tested pathogens

Isolated No.	Tested pathogens	
	B. cereus	*S. aureus*
CP1-15	1.08	To be determined
CP2-11	0.44	-
CP3-1	0.16	-
CP7-3	1.28	To be determined
CP10-3	0.23	-
CP11-6	1.72	-
CP14-1	1.09	-
CP14-2	-	To be determined
CP14-3	-	1.16
CP14-4	1.17	-

CHAPTER 5
DISCUSSION

Certain isolates were selected for taxonomic determination. The isolates were included in the member of the genus *Lactobacillus* (35), *Pediococcus* (22), and *Weissella* (8) on the basis of morphological, cultural, biochemical, and physiological characteristics, fermentation type, isomer of lactic acid and 16S rRNA gene sequences. They were grouped at the species level on the basis of key phenotypic characteristics and 16S rRNA gene sequences.

Group A1 contained 8 isolates (CP1-8, CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP4-6, and CP4-11). The strains in this group were heterofermentative sphere-shaped, produced slime on sucrose, did not grow at 45°C, had no DAP in peptidoglycan, produced DL-lactic acid and were identified as of genus *Weissella*. Similarly to the type strain *W. confusa* ATCC10881^T, the isolates produced NH_3 from arginine and hydrolyzed esculin, produced acids from D-cellobiose, D-fructose, D-glucose, maltose, D-mannose, salicin, sucrose and xylose, but not from D-melezitose, D-melibiose, raffinose, D-sorbitol, and D-trehalose (Collins et al., 1993; Hammes et al., 1992; Kandler and Weiss, 1986; Schillinger et al., 1989; and Tanauspawat et al., 1993a and 2000). Based on 16S rRNA gene sequences of selected isolates, CP3-8 represented 99% similarity to *W. confusa* ATCC 10881^T and CP1-8, CP3-9, CP4-6, and CP4-11 represented 98%, 98%, 99%, and 99% similarities respectively to *W. kimchii* JCM12495^T.

Group A21 contained 20 strains (CP1-5, CP1-18, CP7-4, CP7-8, CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15, CP12-4, CP13-2, CP13-3, CP13-4, CP13-7, and CP13-11). The strains in this group were homofermentative sphere-shaped, grew at 45°C but not at 50°C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production, reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose and D-ribose, but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics (Tanasupawat et al., 1993b), they were identified as *P. pentosaceus*. CP1-18, CP7-4, and CP11-11 were selected for 16S rRNA gene sequence analysis,

and represented 99%, 98%, and 98% similarities respectively to *P. pentosaceus* ATCC33316^T.

Group A22 contained 2 strains (CP6-2 and CP7-3). The strains in this group were homofermentative sphere-shaped, and grew at 50^{o}C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production and reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose, and D-ribose but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics (Tanasupawat et al., 1993b), they were identified as *P. acidilactici*. Based on 16S rRNA gene sequence analysis, CP6-2 and CP7-3 represented 98% similarities to *P. acidilactici* DSM20284^T.

Group B11 contained 3 strains (CP7-2, CP7-9, and CP13-1). The strains in this group were heterofermentative rod-shaped, grew at 45^{o}C, produced DL-lactic acid and had no DAP in peptidoglycan. Similarly to the type strain *L. fermentum* ATCC14931^T, these strains produced acid from D-glucose, D-fructose, D-ribose and sucrose, but failed to produce acid from gluconate, glycerol, and α-methyl-D-glucoside. Based on 16S rRNA gene sequence analysis, CP7-2, CP7-9 and CP13-1 represented 100%, 100% and 99% similarities respectively to *L. fermentum* ATCC14931^T.

Group B12 contained 4 strains (CP1-13, CP1-14, CP1-19, and CP6-8). The strains in this group were heterofermentative rod-shaped, and did not grow at 45^{o}C, produced DL-lactic acid and had no DAP in peptidoglycan. All strains had the following common characteristics with *L. brevis* ATCC14869^T and the other 3 strains identified on the basis of DNA relatedness as *L. brevis* by Tanasupawat et al. (1993a): negative for acid production and reduction in litmus milk; negative for growth at pH 8.5 and at 6% NaCl; positive for acid production from L-arabinose, fructose, D-glucose and D-xylose; negative for acid production from D-amygdalin, D-cellobiose, gluconate, glycerol, lactose, D-mannose, D-mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, and D-trehalose. The two strains selected for 16S rRNA gene sequence analysis, CP1-19 and CP6-8 represented 98% similarities with *L. brevis* ATCC14869^T.

Group B21 contained 4 strains (CP2-3B, CP5-2, CP5-6, and CP5-9). The strains in this group were homofermentative rod-shaped, did not grow at 45°C, had no DAP in peptidoglycan, and produced L-lactic acid from glucose. All strains had the following key characteristics of *L. farciminis* (Hammes and Vogel, 1995): positive for NH_3 production from arginine; positive for acid production from D-cellobiose, D-galactose, D-glucose, lactose, D-mannose, salicin, sucrose and D-trehalose; negative for acid production from D-mannitol, D-melibiose, and raffinose; positive for growth at 10% NaCl. The two strains selected for 16S rRNA gene sequence analysis, CP5-2 and CP5-9 represented 98% and 99% similarities respectively to *L. farciminis* ATCC29644^T.

Group B22 contained 23 strains (CP1-15, CP1-17, CP1-20, CP2-3A, CP2-10, CP2-11, CP2-16, CP3-16, CP4-5, CP4-12, CP4-16, CP4-17, CP4-18, CP6-3, CP6-10, CP7-5, CP7-7, CP7-10, CP7-12, CP7-13, CP8-4, CP10-2, and CP10-3). The strains in this group were homofermentative rod-shaped, did not grow at 45°C, had DAP in peptidoglycan, and produced DL-lactic acid from glucose. All isolates hydrolyzed esculin, but not arginine. They all produced acid from D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and D-trehalose, but not from glycerol. These are the common characteristics with *L. plantarum* ATCC14917^T and the other 16 strains identified on the basis of DNA relatedness as *L. plantarum* by Tanasupawat et al. (1992). The eight strains selected for 16S rRNA gene sequence analysis, CP1-15, CP1-20, CP2-3A, CP3-16, CP6-10, CP7-7, CP8-4, and CP10-3 represented 98% similarities to *L. plantarum* ATCC14917^T.

Group B223 contained on strain, CP8-1. This strain was homofermentative rod-shaped, did not grow at 45°C, had no DAP in peptidoglycan and produced DL-lactic acid from glucose. This strain was separated from group B22 because it hydrolyzed arginine. Similarly to *L.sakei* ATCC15521^T, CP8-1 produced acid from L-arabinose, D-cellebiose, D-fructose, D-glucose, gluconate, D-melibiose, D-ribose and sucrose, but failed to produce acid from D-mannitol, D-melezitose, raffinose and D-sorbitol. The 16S rDNA sequence analysis of this strain showed 99% similarity to *L.sakei* ATCC15521^T. Fig. 5.1 showed a phylogenetic tree based on 16S DNA sequences of representative strains of each group and type strains. The numbers on the branches indicate the number of times the partition of the species into the two sets which are

separated by that branch occurred among the trees, out of 1.00 trees (bootstrap values derived from 1,000 replications).

Most lactic acid bacteria isolated in this study were assigned to *P. pentosaceus* (31%) and *L. plantarum* (35%). *L. pentosus* and *L. plantarum* that contain *meso*-diaminopimelic acid in the cell wall were previously reported to be the predominant rod-shaped LAB, and *P. pentosaceus* strains were the major coccal bacteria in other fermented Thai foods e.g. fish cake, sweetened rice, fermented rice noodle and pickle (Tanasupawat and Komagata, 1995). Table 5-1 summarized the identification of isolates and their distribution in Thai traditional fermented sausages. It was noted that *L. plantarum* was distributed in at least 8 (of 12) samples and *P. pentosaceus* in 5 (of 12) samples used in this study. On the other hand, *Weissella* sp., *P. acidilactici*, *L. fermentum*, *L. brevis*, *L. farciminis* and *L. sakei* were isolated from much fewer samples.

Previous studies reported the isolation of *L. pentosus*, *L. plantarum*, *L. fermentum*, *P. pentosaceus*, and *Enterococcus hirae* from Sai-krog-prieo, and of *L. plantarum* and *Enterococcus* sp. from mum (Tanasupawat and Komagata, 1995 and 2001). This study first reported the isolation of *W. cibaria/kimchii*, *W. confusa*, *L. brevis*, and *L. farciminis*, from Sai-krog-prieo, and *P. pentosaceus*, *P. acidilactici*, *L. fermentum*, *L. brevis*, and *L. sakei* from mum.

Table 5-1. Identification of isolates and their distribution in Thai traditional fermented sausages

Sausage names and province where collected	Days of fermentation	Isolated No.	Group or subgroup	Identification
Sai-krork-prieo Bangkok	4	CP1-8	A1	*W. cibaria/kimchii*
		CP1-5, CP1-18	A21	*P. pentosaceus*
		CP1-13, CP1-14, CP1-19	B12	*L. brevis*
		CP1-15, CP1-17, CP1-20	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	5	CP2-3B	B21	*L. farciminis*
		CP2-3A, CP2-10, CP2-11, CP2-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11	A1	*W. confusa*
		CP3-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP4-6, CP4-11	A1	*W. cibaria/kimchii*
		CP4-5, CP4-12, CP4-16, CP4-17, CP4-18	B22	*L. plantarum*
Sai-krork-prieo Bangkok	2	CP5-2, CP5-6, CP5-9	B21	*L. farciminis*
Mum (beef) Chaiyaphoom	3	CP6-3, CP6-10	B22	*L. plantarum*
		CP6-2	A22	*P. acidilactici*
		CP6-8	B12	*L. brevis*
Mum (beef) Chaiyaphoom	3	CP7-4, CP7-8	A21	*P. pentosaceus*
		CP7-3	A22	*P. acidilactici*
		CP7-2, CP7-9	B11	*L. fermentum*
		CP7-5, CP7-7, CP7-10, CP7-12, CP7-13	B22	*L. plantarum*
Mum (beef) Konkean	4	CP8-1	B23	*L. sakei*
		CP8-4	B22	*L. plantarum*
Mum (pork) Chaiyaphoom	4	CP10-2, CP10-3	B22	*L. plantarum*
Sai-krork-prieo Chaiyaphoom	4	CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15	A21	*P. pentosaceus*
Mum (pork) Chaiyaphoom	4	CP12-4	A21	*P. pentosaceus*
Sai-krork-prieo Chaiyaphoom	4	CP13-1	B11	*L. fermentum*
		CP13-2, CP13-3, CP13-4, CP13-7, CP13-11	A21	*P. pentosaceus*

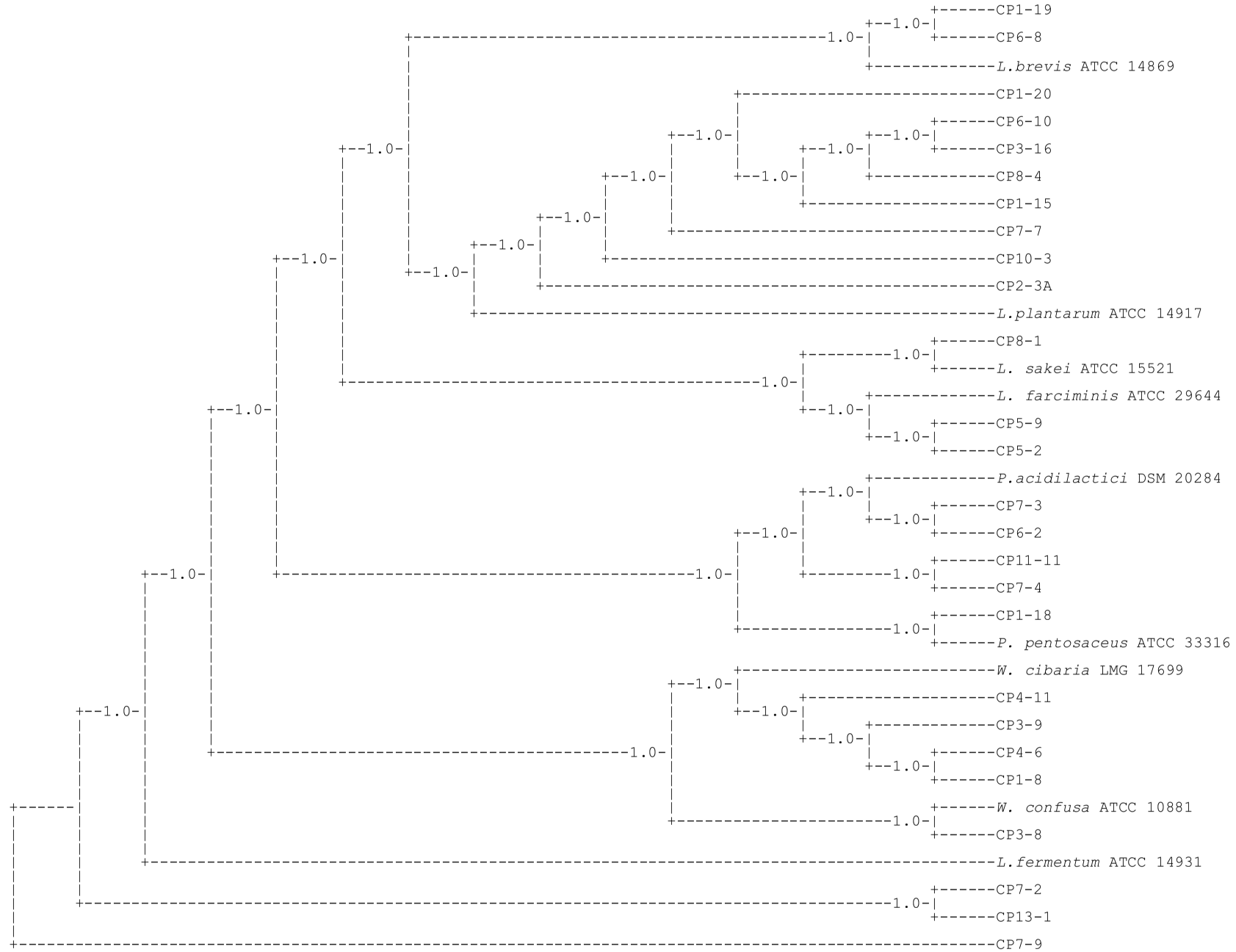

Figure 5.1. Phylogenetic relationships based on 16S rDNA sequences of representative strains of each group and type strains.

Appendix A

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Full Paper

LACTIC ACID BACTERIA FOUND IN TRADITIONAL FERMETED SAUSAGES IN THAILAND

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Key Words- fermented sausage; lactic acid bacteria; *Weissella*; *Lactobacillus*; *Pediococcus; 16S DNA; diacetyl.*

Text: 18 pages

Table: 6: 6 pages

Figure Caption: 1 page

Figure: 1: 1 page.

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Abbreviations: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms.

Abstract

Lactic acid bacteria from traditional Thai fermented sausages were characterized. The fermented sausages were mainly produced from minced pork/beef and sliced pork/beef skin. Sixty-five strains were isolated from 12 samples collected from the central and northeastern parts of Thailand. The strains were identified by conventional morphological, cultural, physiological and biochemical tests as well as 16S rDNA sequence analysis. Some of these species have not been previously isolated from Thai fermented sausages. The isolates were identified as *Weissella cibaria/kimchii* (5), *W. confusa* (3), *Pediococcus pentosaceus* (20), *P. acidilactici* (2), *Lactobacillus fermentum* (3), *L. brevis* (4), *L. farciminis* (4), *L. plantarum* (23), and *L. sakei* (1). Tested strains of each group produced DL-lactic acid from D-glucose, except those identified as *L. farciminis* which produced L-lactic acid. The distribution of these bacteria in fermented sausages in Thailand was discussed.

Key Words- fermented sausage; lactic acid bacteria; *Weissella*; *Lactobacillus*; *Pediococcus;* 16S DNA; diacetyl.

Introduction

Sai-krork-prieo is a traditional Thai fermented sausage, consumed all around the country and produced from minced pork, sliced pork skin, garlic, pepper, spices, salt and sugar. Mum is a product similar to Sai-krork-prieo, but can be made from beef as well as pork, and is popular only in the Northeastern of Thailand. Lactic acid bacteria (LAB) play an important role in the ripening process of raw fermented sausages. However, the production of traditional fermented sausages in Thailand has utilized the naturally occurring LAB, resulting in various and inconstant products. Natural fermentations could vary from the simple to the complex. In general, each fermentation takes place under conditions that the producers have found to be favorable for the appropriate growth and action of microorganisms. Alternative to the natural fermentation, the use of well-studied starter cultures would result in more constant and food-safe products. The aim of this study was the isolation and identification of lactic acid bacteria from traditional fermented sausages in Thailand. In addition, their capability of producing metabolic compounds with antimicrobial property, i.e., diacetyl, was reported. The strains found in this study have a potential use for the establishing of the so-called “functional foods” (Leroy et al., 2002; Reid, 1999).

Materials and methods

Sample collection, bacterial cell counts, and isolation method. Twelve fermented sausage samples of various brands were collected from factories and local markets. The samples pHs were represented by the pH of the suspension of a 5-gram portion in 10 ml deionized water. The cell numbers were counted by a plating method on De Man, Rogasa and Sharpe agar (MRS agar) (De Man et al., 1960) at 30^{o}C after a 3- to 5-day incubation. Pure cultures were obtained by streaking cultured cells on MRS agar plates with 0.2% $CaCO_3$.

Morphological and cultural characteristics. Cell form, cell size, cell arrangement, and colonial appearance were examined on the cells grown on a half strength of MRS (MRSH) agar incubated for 3 days. Hucker-Conn modification (Hucker and Conn, 1923) was used for gram stain. Spore formation was examined in gram-stained specimens. Motility was detected by the appearances of stab cultures in soft agar (Whittenbury, 1963).

Biochemical and physiological characteristics. Catalase, nitrate reduction, hydrolysis of esculin, arginine, slime formation and reactions in litmus milk were investigated as described by Tanasupawat et al. 1992 and 1998. Catalase activity was detected on cells grown MRSH agar. Nitrate reduction was tested in medium composed of 1.0 g KNO_3, 3.0 g yeast extract, 5.0 g peptone, 0.2 g beef extract, 5.0 g NaCl, 0.25 g Tween 80, 1.0 g agar, and 1000 ml deionized water adjusted to pH 6.8, after incubation for 7 days. Growth at different temperature (30, 45, and 50^{o}C), at different starting pHs (3.5, 4.0, 4.5, 8.0, 8.5 and 9.6), and at different concentrations of NaCl (4, 6, 8, and

10%) were tested by using MRSH broth. The production of gas from D-glucose was examined by using MRS broth with a Durham tube. Acid production from carbohydrates was determined as described in Tanasupawat et al., 1998 by the use of a basal medium of GYPB broth with the omission of glucose. The GYPB medium contained 10.0 g glucose, 5.0 g yeast extract, 5.0 g peptone, 2.0 g beef extract, 2.0 g sodium acetate, 0.25 g Tween 80, 200 mg $MgSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot 4H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5.0 g NaCl, and 1,000 ml deionized water adjusted to pH 6.8. The acid produced in 3 ml broth was titrated with 0.1 N NaOH.

Peptidoglycan type of cell wall. Diaminopimelic acid in the cell wall was detected by hydrolysis of 3 mg dried cells grown in GYPB broth with 1 ml 6 N HCl at 100°C for 18 h, and the hydrolysate was applied on cellulose TLC plate (Merck no. 5577). The TLC plate was developed with the solvent system of methanol-pyridine-12 N HCl-water (32: 4: 1: 7) (v/v) (Komagata and Suzuki, 1987). Spots were visualized by spraying with 0.5% ninhydrin solution in n-butanol followed by heating at 100°C for a few minutes.

Isomers of lactic acid. The strains tested were cultivated in GYPB broth for 3 to 5 days. Lactic acid was analyzed enzymatically according to Okada et al., 1978 using D- and L- lactate dehydrogenase (Boehringer, Germany).

Screening for diacetyl formation. Diacetyl formation was screened by the method modified from Mattessich and Cooper (1989) and Phalip et al. (1994), which yielded qualitative results. Cells were grown in MMRS broth (100 ml consisted of 0.18 gram glucose, 0.5 gram yeast extract, 1.0 gram peptone, 1.0 gram beef extract, 0.5 gram

sodium acetate, 1.29 gram trisodium citrate·$2H_2O$, 0.15 gram tween 80, 0.02g $MgSO_4$·$7H_2O$, 0.005 gram $MnSO_4$·$4H_2O$, and 0.2 gram K_2HPO_4) at 35°C for 3-5 days. One ml of each test solution (0.5% creatine solution and 7.5% α- naphthol in 2.5 N NaOH) was added into each 3 ml of culture medium. The degree of change in culture medium color was recorded.

Sequencing and comparison of 16S rRNA gene. Template DNA for 16S rRNA gene amplification was prepared by the method modified from Nilsson et al. (2003). Two loops from an overnight MRS plate culture were transferred into 100-500 μL of TE buffer (10 mM Tris-Cl, pH 7.5, and 1 mM EDTA). Samples were boiled for 10-15 minutes. Then, the debris was pelleted by centrifugation 12000 rpm for 10 minutes, and 50-200 μL of supernatant was collected. 16S rRNA gene was amplified using the PCR method with a 1U *Taq* DNA polymerase (BioLab Ltd., Auckland, New Zealand) and primers UFUL (GCCTAACACATGCAAGTCGA) and URUL (CGTATTACCGCGGCTGCTGG) (Great American Gene Co., California, USA). These primers target two highly conserved regions known to be variable among bacterial species (De Rijk et al., 1992; Van de Peer et al., 1996). 16S rRNA gene was sequenced by using a BigDye v. 3.1 cycle sequencing kit (Applied Biosystems, California, USA), according to the manufacturer's protocol, with UFUL as the primer. The 16S rRNA gene sequences determined (ca. 450-500 bases) were aligned along with the sequences of type strains obtained from the GenBank by using the program CLUSTAL X (version 1.82) (Thompson et al., 1997). Distance matrices for aligned sequences were calculated by the two-parameter method of Kimura (1980). A phylogenic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) with the program PHYLIP (version 3.64) available at

http://evolution.genetics.washington.edu/phylip.html. Confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1,000 samplings.

Reference strains. *Weissella confusa* ATCC10881^T, *W. cibaria/kimchii* LMG17699^T, *Pediococcus pentosaceus* ATCC33316^T, *P. acidilactici* DSM20284^T, *Lactobacillus fermentum* ATCC14931^T, *L. brevis* ATCC14869^T, *L. farciminis* ATCC29644^T, *L. plantarum* ATCC14917^T and *L. sakei* ATCC15521^T were used as reference strains.

Results

Bacterial cell counts and sausage characteristics

Sausage samples tested contained $1.4x10^{11}$-$5.5x10^{13}$ bacterial cells/g, and showed a pH between 4.2-5.0 (Table 1).

Morphological and cultural characteristics

All isolates were Gram-positive, non-motile, and non-sporing. The cells of 30 sphere-shaped strains measured 0.8 to 1.0 μm in size and appeared in pairs or in tetrads (Table 2). Their colonies on MRS agar plates were circular, low convex with entire margin, and non-pigmented. The cells of 35 rod-shaped strains measured 0.8-1.0x1.5-5.0 μm in cell size, and appeared singly, in pairs, or in chains (Table 3). Their colonies on MRS agar plates were circular, low convex with entire margin, and non-pigmented.

Physiological and Biochemical characteristics

Isolates were divided into two major groups, Groups A and B by cell shape. Group A consisted of 30 sphere-shaped isolates, which were further divided into 2 subgroups according to gas production from glucose. Subgroup A1 produced gas from glucose (8 strains), while subgroup A2 did not (22 strains). All strains in subgroup A1 did not grow at 45^{o}C. Subgroup A2 was further divided into 2 subgroups according to their growth at different temperatures. Subgroup A21 grew at 45^{o}C (20 strains); and subgroup A22 grew at 50^{o}C (2 strains).

Group B consisted of 35 rod-shaped isolates, which were further divided into 2 subgroups according to gas production from glucose. Subgroup B1 produced gas from glucose (7 strains), while subgroup B2 did not (28 strains). Subgroup B1 was further divided into 2 subgroups according to their growth at different temperatures. Subgroup B11 grew at 45^{o}C (3 strains); and subgroup B12 did not grow at 45^{o}C (4 strains). All strains in subgroup B2 did not grow at 45^{o}C. Subgroup B2 was further divided into 2 groups according to isomers of lactic acid. Subgroup B21 produced L-lactic acid from glucose (4 strains), while subgroup B22 produced DL-lactic acid (23 strains). All strains in subgroup B22 did not produce NH_3 from arginine. One strain (CP8-1) was separated from subgroup B22 and located in subgroup B23 because it hydrolyzed arginine. The division of isolated strains into groups and their other general characteristics are shown in Tables 2 and 3. Tables 4 and 5 present the characteristics in producing acid from carbohydrates of group A and B, respectively.

Peptidoglycan type of cell walls. Only subgroup B22 strains contained meso-diaminopimelic acid in the whole cell hydrolyzate (Table 3), while the others (subgroup A1, A22, A23, B11, B12, B21, B23 strains) had no meso-diaminopimelic acid.

Isomers of lactic acid and diacetyl production. Subgroup A1, A21, A22, B11, B12, B22, and B23 strains produced DL-lactic acid, while subgroup B21 strains produced L- lactic acid from D-glucose. Three strains from subgroup B22 (CP2-3A, CP2-11 and CP3-16) and one from A1 (CP3-11) produced diacetyl, while the other isolates did not.

Discussion

The isolates were included in the member of the genus *Lactobacillus* (35), *Pediococcus* (22), and *Weissella* (8) on the basis of morphological, cultural, biochemical, and physiological characteristics, fermentation type, isomer of lactic acid and 16S rRNA gene sequences. They were grouped at the species level on the basis of key phenotypic characteristics and 16S rRNA gene sequences.

Group A1 contained 8 isolates (CP1-8, CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP4-6, and CP4-11). The strains in this group were heterofermentative sphere-shaped, produced slime on sucrose, did not grow at 45^{o}C, had no DAP in peptidoglycan, produced DL-lactic acid and were identified as of genus *Weissella*. Similarly to the type strain *W. confusa* ATCC10881^{T}, the isolates produced NH_3 from arginine and hydrolyzed esculin, produced acids from D-cellobiose, D-fructose, D-glucose, maltose, D-mannose, salicin, sucrose and xylose, but not from D-melezitose, D-melibiose, raffinose, D-sorbitol, and D-trehalose (Collins et al., 1993; Hammes et al., 1992; Kandler and Weiss, 1986; Schillinger et al., 1989; and Tanauspawat et al., 1993a and 2000). Based on 16S rRNA gene sequences of selected isolates, CP3-8 represented 99% similarity to *W. confusa* ATCC 10881^{T} and CP1-8, CP3-9, CP4-6,

and CP4-11 represented 98%, 98%, 99%, and 99% similarities respectively to *W. kimchii* JCM12495^T.

Group A21 contained 20 strains (CP1-5, CP1-18, CP7-4, CP7-8, CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15, CP12-4, CP13-2, CP13-3, CP13-4, CP13-7, and CP13-11). The strains in this group were homofermentative sphere-shaped, grew at 45^{o}C but not at 50^{o}C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production, reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose and D-ribose, but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics (Tanasupawat et al., 1993b), they were identified as *P. pentosaceus*. CP1-18, CP7-4, and CP11-11 were selected for 16S rRNA gene sequence analysis, and represented 99%, 98%, and 98% similarities respectively to *P. pentosaceus* ATCC33316^T.

Group A22 contained 2 strains (CP6-2 and CP7-3). The strains in this group were homofermentative sphere-shaped, and grew at 50^{o}C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production and reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose, and D-ribose but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics (Tanasupawat et al., 1993b), they were identified as *P. acidilactici*. Based on 16S rRNA gene sequence analysis, CP6-2 and CP7-3 represented 98% similarities to *P. acidilactici* DSM20284^T.

Group B11 contained 3 strains (CP7-2, CP7-9, and CP13-1). The strains in this group were heterofermentative rod-shaped, grew at 45°C, produced DL-lactic acid and had no DAP in peptidoglycan. Similarly to the type strain *L. fermentum* ATCC14931^T, these strains produced acid from D-glucose, D-fructose, D-ribose and sucrose, but failed to produce acid from gluconate, glycerol, and α-methyl-D-glucoside. Based on 16S rRNA gene sequence analysis, CP7-2, CP7-9 and CP13-1 represented 100%, 100% and 99% similarities respectively to *L. fermentum* ATCC14931^T.

Group B12 contained 4 strains (CP1-13, CP1-14, CP1-19, and CP6-8). The strains in this group were heterofermentative rod-shaped, and did not grow at 45°C, produced DL-lactic acid and had no DAP in peptidoglycan. All strains had the following common characteristics with *L. brevis* ATCC14869^T and the other 3 strains identified on the basis of DNA relatedness as *L. brevis* by Tanasupawat et al. (1993a): negative for acid production and reduction in litmus milk; negative for growth at pH 8.5 and at 6% NaCl; positive for acid production from L-arabinose, fructose, D-glucose and D-xylose; negative for acid production from D-amygdalin, D-cellobiose, gluconate, glycerol, lactose, D-mannose, D-mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, and D-trehalose. The two strains selected for 16S rRNA gene sequence analysis, CP1-19 and CP6-8 represented 98% similarities with *L. brevis* ATCC14869^T.

Group B21 contained 4 strains (CP2-3B, CP5-2, CP5-6, and CP5-9). The strains in this group were homofermentative rod-shaped, did not grow at 45°C, had no DAP in peptidoglycan, and produced L-lactic acid from glucose. All strains had the following key characteristics of *L. farciminis* (Hammes and Vogel, 1995): positive for NH_3 production from arginine; positive for acid production from D-cellobiose, D-

galactose, D-glucose, lactose, D-mannose, salicin, sucrose and D-trehalose; negative for acid production from D-mannitol, D-melibiose, and raffinose; positive for growth at 10% NaCl. The two strains selected for 16S rRNA gene sequence analysis, CP5-2 and CP5-9 represented 98% and 99% similarities respectively to *L. farciminis* ATCC29644^T.

Group B22 contained 23 strains (CP1-15, CP1-17, CP1-20, CP2-3A, CP2-10, CP2-11, CP2-16, CP3-16, CP4-5, CP4-12, CP4-16, CP4-17, CP4-18, CP6-3, CP6-10, CP7-5, CP7-7, CP7-10, CP7-12, CP7-13, CP8-4, CP10-2, and CP10-3). The strains in this group were homofermentative rod-shaped, did not grow at 45^{o}C, had DAP in peptidoglycan, and produced DL-lactic acid from glucose. All isolates hydrolyzed esculin, but not arginine. They all produced acid from D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and D-trehalose, but not from glycerol. These are the common characteristics with *L. plantarum* ATCC14917^T and the other 16 strains identified on the basis of DNA relatedness as *L. plantarum* by Tanasupawat et al. (1992). The eight strains selected for 16S rRNA gene sequence analysis, CP1-15, CP1-20, CP2-3A, CP3-16, CP6-10, CP7-7, CP8-4, and CP10-3 represented 98% similarities to *L. plantarum* ATCC14917^T.

Group B223 contained on strain, CP8-1. This strain was homofermentative rod-shaped, did not grow at 45^{o}C, had no DAP in peptidoglycan and produced DL-lactic acid from glucose. This strain was separated from group B22 because it hydrolyzed arginine. Similarly to *L.sakei* ATCC15521^T, CP8-1 produced acid from L-arabinose, D-cellebiose, D-fructose, D-glucose, gluconate, D-melibiose, D-ribose and sucrose, but failed to produce acid from D-mannitol, D-melezitose, raffinose and D-sorbitol. The 16S rDNA sequence analysis of this strain showed 99% similarity to

L.sakei ATCC15521^{T}. Fig. 1 showed a phylogenetic tree based on 16S DNA sequences of representative strains of each group and type strains.

Most lactic acid bacteria isolated in this study were assigned to *P. pentosaceus* (31%) and *L. plantarum* (35%). *L. pentosus* and *L. plantarum* that contain *meso*-diaminopimelic acid in the cell wall were previously reported to be the predominant rod-shaped LAB, and *P. pentosaceus* strains were the major coccal bacteria in other fermented Thai foods e.g. fish cake, sweetened rice, fermented rice noodle and pickle (Tanasupawat and Komagata, 1995). Table 6 summarized the identification of isolates and their distribution in Thai traditional fermented sausages. It was noted that *L. plantarum* was distributed in at least 8 (of 12) samples and *P. pentosaceus* in 5 (of 12) samples used in this study. On the other hand, *Weissella* sp., *P. acidilactici*, *L. fermentum*, *L. brevis*, *L. farciminis* and *L. sakei* were isolated from much fewer samples.

Previous studies reported the isolation of *L. pentosus*, *L. plantarum*, *L. fermentum*, *P. pentosaceus*, and *Enterococcus hirae* from Sai-krog-prieo, and of *L. plantarum* and *Enterococcus* sp. from mum (Tanasupawat and Komagata, 1995 and 2001). This study first reported the isolation of *W. cibaria/kimchii*, *W. confusa*, *L. brevis*, and *L. farciminis*, from Sai-krog-prieo, and *P. pentosaceus*, *P. acidilactici*, *L. fermentum*, *L. brevis*, and *L. sakei* from mum.

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Table 1. Bacterial cell counts and sausage characteristics

Sample No.	Sausage names and province where collected	Days of fermen-tation	pH	Bacterial counts (cells/g)	Isolated No. (65 isolates)
CP1	Sai-krork-prieo Bangkok	4	4.2	$3.7x10^{11}$	CP1-5, CP1-8, CP1-13, CP1-14, CP1-15, CP1-17, CP1-18, CP1-19, CP1-20
CP2	Sai-krork-prieo Pathumthani	5	4.2	$2.5x10^{12}$	CP2-3A, CP2-3B, CP2-10, CP2-11, CP2-16
CP3	Sai-krork-prieo Pathumthani	4	4.9	$1.4x10^{11}$	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP3-16
CP4	Sai-krork-prieo Pathumthani	4	4.8	$1.9x10^{12}$	CP4-5, CP4-6, CP4-11, CP4-12, CP4-16, CP4-17, CP4-18
CP5	Sai-krork-prieo Bangkok	2	4.3	$4.0x10^{13}$	CP5-2, CP5-6, CP5-9
CP6	Mum (beef) Chaiyaphoom	3	4.5	$3.0x10^{13}$	CP6-2, CP6-3, CP6-8, CP6-10,
CP7	Mum (beef) Chaiyaphoom	3	4.4	$5.5x10^{13}$	CP7-2, CP7-3, CP7-4, CP7-5, CP7-7, CP7-8, CP7-9, CP7-10, CP7-12, CP7-13
CP8	Mum (beef) Konkean	4	4.6	$1.5x10^{12}$	CP8-1, CP8-4
CP10	Mum (pork) Chaiyaphoom	4	5.0	$8.0x10^{11}$	CP10-2, CP10-3
CP11	Sai-krork-prieo Chaiyaphoom	4	4.6	$1.0x10^{13}$	CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15
CP12	Mum (pork) Chaiyaphoom	4	5.0	$2.0x10^{13}$	CP12-4
CP13	Sai-krork-prieo Chaiyaphoom	4	4.8	$8.5x10^{12}$	CP13-1, CP13-2, CP13-3, CP13-4, CP13-7, CP13-11

Table 2. General characteristics of isolated strains in group A.

	Group A						
	A1	[a]*W. confusa* ATCC10881^T	[b]*W. kimchii* JCM12495^T	A21	[c]*P. pentosaceus* ATCC33316^T	A22	[c]*P. acidilactici* DSM20284^T
Number of strains	8	-	-	20	-	2	-
Cell form	Cocci						
Cell arrangement	In pairs or tetrads						
Gas from glucose	+	+	+	-	-	-	-
Growth at 45°C	-	-	-	+	-	+	+
Growth at 50°C	-	-	-	-	-	+	+
Isomers of lactic acid	DL	DL	D	DL	DL	DL	DL
Arginine hydrolysis	+	+	+	+	-	+	+
Esculin hydrolysis	+	+	+	+	+	+	+
Nitrate Reduction	-	ND	ND	-	-	-	-
Reaction in litmus milk							
Acidification	- (+2)	ND	ND	-	-	-	-
Coagulation	- (+2)	ND	ND	-	-	-	-
Reduction	-	ND	ND	-	-	-	-
Growth at pH							
3.5	- (+1)	-	-	+(-2)	ND	+(-1)	ND
4.0	+	+	W	+	+	+	+
4.5	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+
8.5	- (+3)	-	-	+(-2)	-	+	-
9.6	-	-	-	-	-	-	-
Salt tolerance							
4% NaCl	+	+	+	+	+	+	+
6% NaCl	+	+	+	+(-1)	+	+	-
8% NaCl	+	-	-	+(-2)	-	+	-
10% NaCl	-	-	-	-	-	-	-
Slime from sucrose	+	+	+	-	-	-	-
Peptidoglycan type: DAP	-	-	-	-	-	-	-

+, positive; W, weakly positive; -, negative reaction; ND, not determined. Numbers in parenthesis indicate the number of strains showing a positive or negative reaction.
DAP, diaminopimelic acid.
ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms.
[a] Data from Choi et al. (2002), Collins et al. (1993), Hammes et al. (1992), Kandler and Weiss (1986), Schillinger et al. (1989) and Tanasupawat et al. (1993a, 2000).
[b] Data from Choi et al. (2002)
[c] Data from Tanasupawat et al. (1993b).

Table 3. General characteristics of isolated strains in group B.

	Group B									
	B11	[a]*L. fermentum* ATCC 14931^T	B12	[a]*L. brevis* ATCC 14869^T	B21	[b]*L. farciminis* ATCC 29644^T	B22	[c]*L. plantarum* ATCC 14917^T	B23	[d]*L. sakei* ATCC 15521^T
Number of strains	3	-	4	-	4	-	23	-	1	-
Cell form	Rods									
Cell arrangement	Singly, in pairs or in chains									
Gas from glucose	+	+	+	+	-	-	-	-	-	+
Growth at 45°C	+	+	-	-	-	-	-	-	-	-
Growth at 50°C	-	ND	-	-	-	-	-	-	-	-
Isomers of lactic acid	DL	DL+ L(+)	DL	DL	L	L	DL	DL	DL	DL+ D(-)
Arginine hydrolysis	+	-	+(-1)	-	+	+	-	-	+	+
Esculin hydrolysis	-(+1)	-	+	-	+	+	+	+	-	+
Nitrate Reduction	-(+1)	ND	-	ND	-	ND	-	ND	-	ND
Reaction in litmus milk										
Acidification	-(+1)	-	-	-	+	ND	+(-5)	-	-	ND
Coagulation	-	-	-	-	-	ND	-(+2)	-	-	ND
Reduction	-	-	-	-	-	ND	-(+1)	-	-	ND
Growth at pH										
3.5	+	-	+(-1)	-	+(-2)	ND	+(-2)	+	-	ND
4.0	+	+	+	+	+	+	+	+	-	+
4.5	+	+	+	+	+	+	+	+	+	+
8.0	+	+	-(+1)	+	+	+	+	+	+	+
8.5	-(+1)	+	-	-	+(-1)	+	-(+9)	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-
Salt tolerance										
4% NaCl	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	-	-	-	+	+	+(-3)	+	+	-
8% NaCl	-(+1)	-	-	-	+	+	-(+11)	-	-	-
10% NaCl	-	-	-	-	+	+	-	-	-	-
Slime from sucrose	-	-	-	-	-	-	-	-	-	-
Peptidoglycan type: DAP	-	-	-	-	-	-	+	+	-	-

+, positive; W, weakly positive; -, negative reaction; ND, not determined. Numbers in parenthesis indicate the number of strains showing a positive or negative reaction.
DAP, diaminopimelic acid.
[a] Data from Tanasupawat et al. (1993a).
[b] Data from Tanasupawat et al. (1998, 2002).
[c] Data from Tanasupawat et al. (1992, 1993a, 2000).
[d] Data from Tanasupawat et al. (1993a).

Table 4. Acid production from carbohydrates by the isolated strains in group A.

	Group A						
	A1	[a]*W. confusa* ATCC10881^T	[b]*W. kimchii* JCM12495^T	A21	[c]*P. pentosaceus* ATCC33316^T	A22	[c]*P. acidilactici* DSM20284^T
Number of strains	8	-	-	20	-	2	-
D-Amygdalin	+(-1)	+	+	+	ND	+	ND
L-Arabinose	+	-	-	+(-1)	+	+(-1)	+
D-Cellebiose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galactose	+(-3)	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Gluconate	-(+3)	+	+	-	ND	-	ND
Glycerol	-	ND	-	-	-	-	-
Inulin	-	ND	-	-(+4)	-	-	-
Lactose	-(+1)	-	-	-(+7)	-	-	-
Maltose	+	+	+	-(+2)	+	-	-
D-Mannitol	-(+1)	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+
D - Melibiose	-	-	-	-(+1)	+	-	-
D - Melezitose	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	ND	-	-	-	-	-
Raffinose	-	-	-	-	W	-	-
L - Rhamnose	-(+1)	-	-	+(-3)	-	+(-1)	-
D-Ribose	-(+1)	+	-	+	+	+	+
Salicin	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	+	+	+(-5)	+	+	-
D - Trehalose	-	-	-	-(+8)	+	+	+
D-Xylose	+	+	+	+(-1)	W	+	+

+, positive; W, weakly positive; -, negative reaction; ND, not determined. Numbers in parenthesis indicate the number of strains showing a positive or negative reaction.

[a] Data from Choi et al. (2002), Collins et al. (1993), Hammes et al. (1992), Kandler and Weiss (1986), Schillinger et al. (1989) and Tanasupawat et al. (1993a, 2000).

[b] Data from Choi et al. (2002)

[c] Data from Tanasupawat et al. (1993b).

Table 5. Acid production from carbohydrates by the isolated strains in group B.

	Group B									
	B11	[a]*L. fermentum* ATCC 14931^T	B12	[a]*L. brevis* ATCC 14869^T	B21	[b]*L. farciminis* ATCC 29644^T	B22	[c]*L. plantarum* ATCC 14917^T	B23	[d]*L. sakei* ATCC 15521^T
Number of strains	3	-	4	-	4	-	23	-	1	-
D-Amygdalin	-(+1)	-	-	-	+(-2)	+	+	ND	-	+
L-Arabinose	+(-1)	-	+	+	-	-	+(-2)	+	+	+
D-Cellebiose	-(+1)	-	-	-	+	+	+	+	W	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	-	-(+2)	-	+	+	+	+	+	ND
D-Glucose	+	+	+	+	+	+	+	+	+	+
Gluconate	-	-	-	-	-	-	+ (-11)	+	W	+
Glycerol	-	-	-	-	-	-	-	-	W	ND
Inulin	-(+1)	ND	-	ND	-(+1)	ND	+ (-11)	ND	-	ND
Lactose	+(-1)	+	-	-	+	+	+(-1)	+	+	ND
Maltose	+	-	+	-	+(-2)	+	+	+	-	ND
D-Mannitol	-(+1)	-	-	-	-	-	+	+	-	-
D-Mannose	+(-1)	-	-	-	+	+	+	+	+	ND
D - Melibiose	+	-	-	-	-	-	+(-1)	+	+	+
D - Melezitose	-(+1)	-	-	-	-	ND	+(-2)	+	-	-
α-Methyl-D-glucoside	-	-	+	-	-	-	-(+7)	ND	-	ND
Raffinose	+	-	-	-	-	-	+(-1)	+	-	-
L - Rhamnose	-(+1)	-	-	-	-	-	- (+11)	-	-	ND
D-Ribose	+	+	+	-	-	-	+	+	+	+
Salicin	-(+1)	ND	-	ND	+	+	+	+	W	
D-Sorbitol	-(+1)	-	-	-	-	-	+(-4)	+	-	-
Sucrose	+	+	-	-	+	+	+	+	+	+
D - Trehalose	+	-	-	-	+	+	+	+	+	ND
D-Xylose	+	-	+	+	-	-	-(+2)	-	W	-

+, positive; W, weakly positive; -, negative reaction; ND, not determined. Numbers in parenthesis indicate the number of strains showing a positive or negative reaction.

[a] Data from Tanasupawat et al. (1993a).

[b] Data from Tanasupawat et al. (1998, 2002).

[c] Data from Tanasupawat et al. (1992, 1993a, 2000).

[d] Data from Tanasupawat et al. (1993a).

Table 6. Identification of isolates and their distribution in Thai traditional fermented sausages.

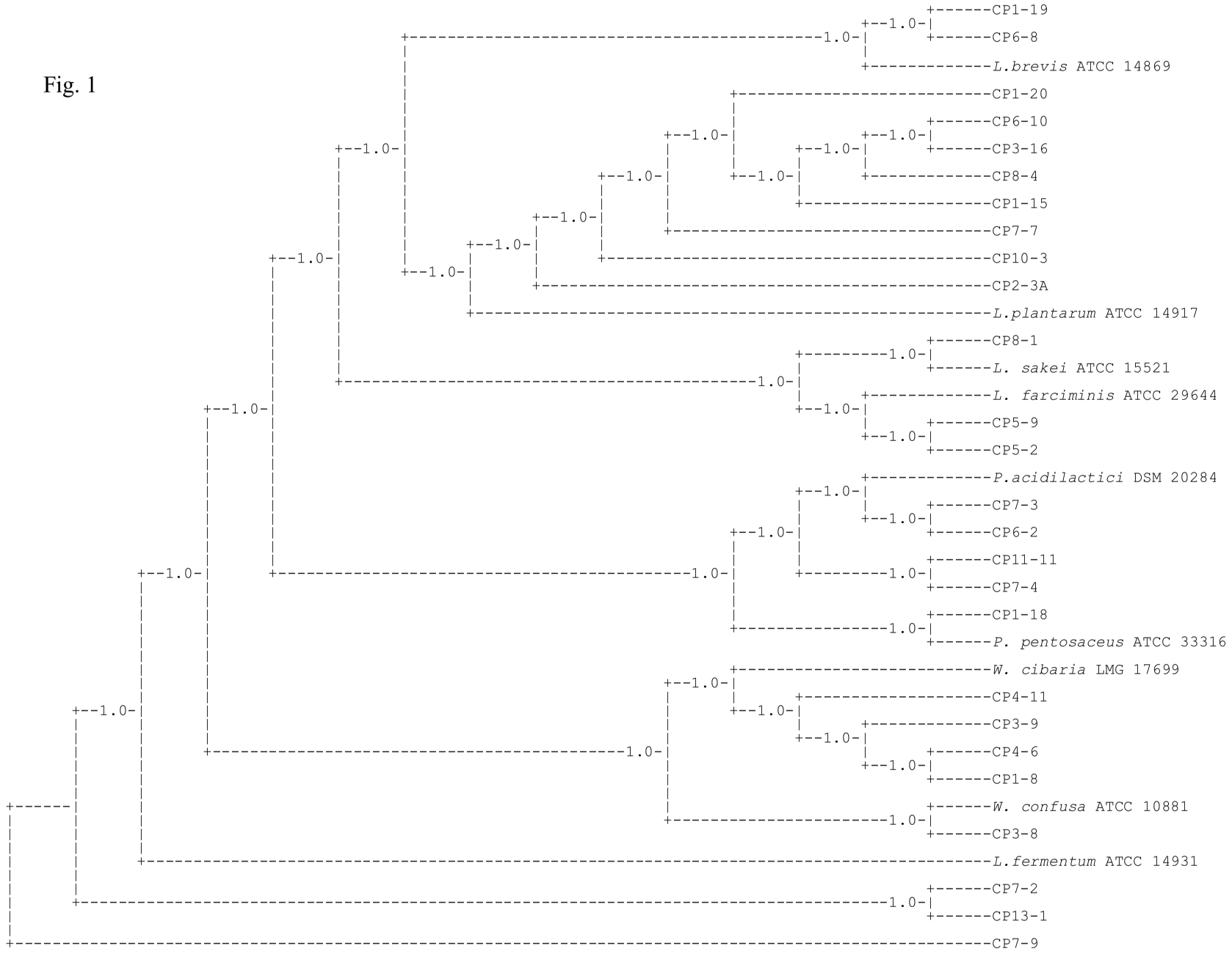
Sausage names and province where collected	Days of fermentation	Isolated No.	Group or subgroup	Identification
Sai-krork-prieo Bangkok	4	CP1-8	A1	*W. cibaria/kimchii*
		CP1-5, CP1-18	A21	*P. pentosaceus*
		CP1-13, CP1-14, CP1-19	B12	*L. brevis*
		CP1-15, CP1-17, CP1-20	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	5	CP2-3B	B21	*L. farciminis*
		CP2-3A, CP2-10, CP2-11, CP2-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11	A1	*W. confusa*
		CP3-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP4-6, CP4-11	A1	*W. cibaria/kimchii*
		CP4-5, CP4-12, CP4-16, CP4-17, CP4-18	B22	*L. plantarum*
Sai-krork-prieo Bangkok	2	CP5-2, CP5-6, CP5-9	B21	*L. farciminis*
Mum (beef) Chaiyaphoom	3	CP6-3, CP6-10	B22	*L. plantarum*
		CP6-2	A22	*P. acidilactici*
		CP6-8	B12	*L. brevis*
Mum (beef) Chaiyaphoom	3	CP7-4, CP7-8	A21	*P. pentosaceus*
		CP7-3	A22	*P. acidilactici*
		CP7-2, CP7-9	B11	*L. fermentum*
		CP7-5, CP7-7, CP7-10, CP7-12, CP7-13	B22	*L. plantarum*
Mum (beef) Konkean	4	CP8-1	B23	*L. sakei*
		CP8-4	B22	*L. plantarum*
Mum (pork) Chaiyaphoom	4	CP10-2, CP10-3	B22	*L. plantarum*
Sai-krork-prieo Chaiyaphoom	4	CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15	A21	*P. pentosaceus*
Mum (pork) Chaiyaphoom	4	CP12-4	A21	*P. pentosaceus*
Sai-krork-prieo Chaiyaphoom	4	CP13-1	B11	*L. fermentum*
		CP13-2, CP13-3, CP13-4, CP13-7, CP13-11	A21	*P. pentosaceus*

Figure Caption

Fig. 1. Phylogenetic relationships based on 16S rDNA sequences of representative strains of each group and type strains.

The phylogenetic tree was constructed by the neighbor-joining method. The numbers on the branches indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among the trees, out of 1.00 trees (bootstrap values derived from 1,000 replications).

Fig. 1


CP1-19
CP6-8
L.brevis ATCC 14869
CP1-20
CP6-10
CP3-16
CP8-4
CP1-15
CP7-7
CP10-3
CP2-3A
L.plantarum ATCC 14917
CP8-1
L. sakei ATCC 15521
L. farciminis ATCC 29644
CP5-9
CP5-2
P.acidilactici DSM 20284
CP7-3
CP6-2
CP11-11
CP7-4
CP1-18
P. pentosaceus ATCC 33316
W. cibaria LMG 17699
CP4-11
CP3-9
CP4-6
CP1-8
W. confusa ATCC 10881
CP3-8
L.fermentum ATCC 14931
CP7-2
CP13-1
CP7-9

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