PURIFICATION AND CHARACTERIZATION OF BACTERIOCINS PRODUCED BY *Lactobacillus salivarius* K4 AND K7 ISOLATED FROM CHICKEN INTESTINE

INTRODUCTION

Lactic acid bacteria (LAB) are of major economic importance to the food industry. They are the natural microflora of many fermented foods where they serve preservative or a spoilage role (Stiles and Holzapfel, 1997). In addition, their ubiquitous are found intestinal epithelium and the human gastrointestinal tract (Donohue and Salminen, 1996). They also play an important role in the digestive tract of human and animal (Stiles and Holzapfel, 1997). Members of the genus *Lactobacillus* are most commonly given safe or generally recognized as safe (GRAS) status, whilst members of the genera *Streptococcus* and *Enterococcus* contain many opportunistic pathogens (Donohue and Salminen, 1996).

LAB are regarded as a major group of probiotic bacteria (Collins *et al.*, 1998). The probiotic concept has been defined by Fuller (1989) to mean a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. Lambert and Hull (1996) reported that the predominant of LAB in the upper gastrointestinal tract are *Lactobacillus* species. Lactobacilli may colonise the mucosal surface of the duodenum as well as the stomach. For this to occur they must be posses certain properties including adhesion, competitive exclusion ability and bacterial inhibitor production.

Recently, a great deal of interest has been focused on some member of LAB with regard to their use as probiotics (Niamsup *et al.*, 2003). Several beneficial functions have been suggested for probiotic bacteria such as inhibition of intestinal pathogenic bacteria by production of organic acids and by pH reduction, prevention of pathogens adherence to the intestinal mucosa, production of bacteriocins, increase of

food assimilation and of detoxification processes, immune stimulation and decrease of heart failure and cancer incidence (Vassu *et al.*, 2001). In addition, nutritional benefits have been reported such as vitamin production, availability of minerals and trace element, production of important digestive enzymes (e.g. β-galactosidase) (Holzapfel and Schillinger, 2002).

LAB are widely used in food industry (Huot *et al*., 1996) such as food fermentation which include dairy, meat and vegetable (Noonpakdee *et al*, 2003). In addition, they are able to inhibit the growth of other microorganisms (Huot *et al*., 1996) because they produce various antibacterial compounds, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Holzapfel and Wood, 1995). This anti-microbial activity can contribute in a number of ways towards improving the quality of fermented foods, for example, through the control of pathogens, extending shelf life and improving qualities (O'Sullivan *et al.*, 2002).

Among the anti-microbial substances, bacteriocins have demonstrated great potential as food preservatives (Nes and Johnsborg, 2004). Bacteriocins are ribosomally synthesized antibacterial polypeptides that are usually inhibitory to strains closely related to the producing bacteria (Marrec *et al*., 2000; Nes and Johnsborg, 2004). Many bacteriocins of LAB are safe and effective natural inhibitors against pathogenic and food spoilage bacteria in various foods (Delves-Broughton *et al*., 1996) that could replace chemical preservatives (Marrec *et al*., 2000). Bacteriocins generally exert their antibacterial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death (O'Sullivan *et al*., 2002).

Bacteriocin producing LAB have received enormous application in food preservation. Consumers demand food products without using chemical but increased safety, quality and shelf-life. These demands have led to interest in the use of natural antibacterial to preserve foods. Even through good management, sanitation and hazard analysis critical control point (HACCP) program can be used as a basis for reducing contamination in the production chain (McMullen, 2000) but the numbers of food-

borne illnesses and intoxications are still on the increase (Soomro *et al*., 2002). According to the Council for Agricultural Science and Technology, microbial pathogens in food cause an estimated 6.5-33 million cases of human illness and up to 9000 deaths annually, with the main foods implicated including meat, poultry, eggs, sea food and dairy products. The bacterial pathogens that account for much of these cases include *Salmonella, Campyrobacter jejuni, Escherichia coli* 0157:H7, *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium botulinum* (O'Sullivan *et al.*, 2002).

LAB account for an important part of the intestinal microflora of chickens and contribute to maintain the ecological balance among the different microorganisms (Zacconi *et al.*, 1999). There are some reports that make mention of bacteriocins produced by *Lb. salivarius* which were isolated from human and animal intestine (Flynn *et al.*, 2002; Cataloluk and Gülakan, 2003). In addition, it has been reported that *Lb. salivarius* CTC 2197 strain isolated from the crop of chicken was selected as a potential probiotic strain because of its high degree of adhesiveness to chicken intestinal epithelial cell, antagonistic activity against some pathogenic bacteria, and competitiveness in vivo (Pascual *et al*., 1999). Therefore, the purpose of this study is to find bacteriocin producing-LAB strains from chicken intestine to use as biopreservative in food and animal industry.

OBJECTIVES

The objectives of this study are:

1. To isolate strains of LAB from chicken intestines and screen for their abilities to produce bacteriocins against pathogenic and food spoilage bacteria.

2. To identify the selected bacteriocin-producing LAB based on physiological, biochemical and molecular methods.

3. To purify and characterize of bacteriocins produced by selected bacteriocin-producing LAB.

LITERATURE REVIEW

1. Characteristic of lactic acid bacteria (LAB)

1.1 Taxonomy of LAB

LAB are a phylogenetically diverse group of bacteria that are gram-positive, nonsporulating coccus or rod-shaped with less than 50 mol% G+C (guanine and cytocine) in their DNA (Van Belkum and Stiles, 2000). They are catalase-negative organisms that are devoid of cytochromes and of nonaerobic habit but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative. However, exceptions from this general description do occur because some species can form catalase or cyanochromes on media containing hematin or related compounds. The production of a nonheme catalase, called pseudocatalase, by some lactobacilli can also cause some confusion in the identification LAB (Schleifer and Ludwig, 1995). They ferment carbohydrates to lactic acid as the sole (homofermentative) or lactic acid, acetic acid and CO_2 a major (heterofermentative) end product of fermentation (Van Belkum and Stiles, 2000).

Modern bacterial taxonomy based on phenotypic and genotypic characteristics has resulted in the reclassification of many LAB. The current genera included in the LAB and their common ecological sources are listed in Table 1 (Van Belkum and Stiles, 2000)

Table 1 Origins and associations of LAB

Genus	Origin and common association
Aerococcus	
Carnobacterium	Derived from nonaciduric lactobacilli that grow in meat
Enterococcus	Previously group D streptococci (enterococci). Intestinal organism; opportunistic pathogen; possible probiotic
Lactobacillus	Widespread intestinal and extraintestinal organisms; food fermentations
Lactococcus	Previously group N (lactic) streptococci; starter cultures for most cheeses
Leuconostoc	Meat preservation and spoilage; sugar spoilage
Oenococcus	Melolactic fermentation of wine
Pediococcus	Traditional genus involved in meat fermentation, beer spoilage
Streptococcus	Mainly pathogens, except *S. thermophilus* that is used in cheese and yogurt making
Tetragenococcus	Previously *Pediococcus*; used in production of soy sauce
Vagococcus	Motile " streptococci"
Weissella	New genus including species of *Lactobacillus* and *Leuconostoc*. Associated with meats and other foods.

Source: Van Belkum and Stiles (2000)

1.2 LAB from chicken intestine

Many LAB have been isolated from chicken intestine. Sarra *et al.* (1992) reported that *Lactobacillus* colonization of the chicken intestinal system takes place soon after hatching, during food ingestion. The species mainly represented were reported to be *Lb. acidophilus*, *Lb. salivarius*, *Lb. fermentum* and *Lb. reuteri*. Boris *et al.* (2001) reported that *Lb. delbrueckii* is a natural inhabitant of the intestinal tract of humans and animals.

Nitisinprasert *et al.* (2000) isolated hundred fifty-six isolates of LAB from chicken intestines. Three effective thermotolerant isolates, i.e., KUB-AC5, KUB-AC16 and KUB-AC20 against the target strains resistant to antibiotics (*Escherichia coli* and *Salmonella sp*.) exhibited the broad-spectrum inhibition against those target strains. Based on physiological, biochemical and molecular systemic method, these isolates were categorized as *Lactobacillus reuteri.*

Paco *et al.* (2003) isolated *Lactobacillus* species from chicken intestine and found that out of one hundred samples studied, 23% *Lactobacillus* species were isolated: *Lb. plantarum* (39.1%), *Lb. casei* subsp. *pseudoplantarum* (17.4%), *Lb. delbrueckeii* subsp. *delbrueckeii* (8.7%), *Lb.reuteri* (8.7%), *Lb. agalis* (4.3%), *Lb.ruminus* (4.3%), *Lb. delbrueckeii subsp. lactis* (4.3%), *Lb. salivarius subsp. salicinius* (4.3%), *Lb. viridencens* (4.3%) and *Lb. amylophilus* (4.3%). In addition, Paco *et al.* (2003) reported that several factors may interfere on *Lactobacillus* viability in the litter, such as bird age, environmental temperature *etc*. In this study, *Lb agalis*, *Lb murinus* and *Lb. viridiensis* were isolated from boiler litters. These species are commonly found neither in avian environments, nor in commercial birds' enteric tract, as some other *Lactobacillus* species, which are often isolated from avian gut. Therefore, their importances for the enteric microbiota balance, as well as their role in the development of intensively raised birds are being speculated.

Many papers have been reported that some species of *Lactobacillus* isolated from chicken faeces and intestine. Fujisawa *et al.* (1984) isolated a novel *Lactobacillus* species, *Lb. aviarius*, from the intestine of chickens, which consists of two subspecies, *Lb. aviarius* subsp. *aviarius* and *Lb. aviarius* subsp. *araffinosus*. Gusils *et al.* (1999) isolated *Lb. fermentum*, *Lb. fermentum* subsp. *cellobiosus* and *Lb. animalis*, when studying lectin-like protein fractions in *Lactobacillus* strains isolated from the gastrointestinal tracts of chickens. Moreover, Fujisawa *et al.* (1992) also isolated other novel *Lactobacillus* spp. from chicken faeces, namely *Lb. gallinarum* and *Lb. johnsonii*. Althrough several *Lactobacillus* spp. have been isolated from chicken faeces, most of the strains isolated so far have been mesophiles. However, recently Niamsup *et al.* (2003) isolated thermotolerant LAB from chicken faeces and

found novel species within the genus *Lactobacillus*, for which the name *Lactobacillus thermotolerans* was given. However, Fuller (1989) reported that stress conditions of different origin (diet, environment, diseases) could lead to an abnormal presence of microorganisms in chicken intestine.

2. Antimicrobial compounds produced by LAB

Many LAB can produce a variety of antimicrobial substances including organic acids, diacetyl, hydrogen peroxide, carbon dioxide, reuterin, fatty acid, ethanol and bacteriocin (Holzapfel and Wood., 1995; Yin *et al*., 2003; Røssland *et al*., 2005). They can consequently inhibit the natural microflora including spoilage bacteria and pathogens (Yin *et al*., 2003). In the following, the antimicrobial compounds produced by LAB will be discussed.

2.1 Organic acids

Fermentation reduces the amount of viable carbohydrates and results in a range of small molecular mass organic molecules that exhibit antimicrobial activity, the most common being lactic, acetic and propionic acid (Blom and Mortvedt, 1991). The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren and Dobrogoze, 1990). Lactic acid is the major metabolite of LAB fermentation. Upon fermentation of hexose, lactic acid is produced by homofermentation (Blom and Mortvedt, 1991; Davidson and Hoover, 1993). At low pH, lactic acid is toxic to many bacteria, fungi and yeasts (Woolford, 1975). Acetic and propionic acids are other organic acids produced by LAB through heterofermentative pathways (Caplice and Fitzgerald, 1999). They are produced in trace amounts by a wide variety of LAB and have higher pKa values than lactic acid (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87) and therefore have higher undissociated ratio than lactic acid at the same pH. This difference may be one reason for their increased antimicrobial efficacy compared to lactic acid (Earnshaw, 1992).

2.2 Hydrogen peroxide (H_2O_2)

A wide range of LAB, which does not produces catalase but possess flavoprotein oxidases, which react with dioxygen produces H_2O_2 (Earnshaw, 1992). The antimicrobial activity of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing at a number of enzymes, and form the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davison, 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide and hydroxyl radicals, which can damage DNA (Byczkowski and Gessner, 1988).

2.3 Carbon dioxide

Carbon dioxide is mainly formed during heterofermentative LAB of hexoses. Carbon dioxide has a dual antimicrobial effect. Its formation creates an anaerobic environment by replacing existing molecular oxygen. It has extra- and intracellular pH-decreasing effect and its destroying effects on the cell membrane make carbon dioxide to be an inhibitory substance towards several microorganisms (King and Nagel, 1975). Carbon dioxide can effective inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotropic bacteria (Hotchkiss *et al.*, 1999) whereas LAB, some yeasts showed high tolerance (Adams, 2001).

2.4 Diacetyl

Diacetyl (2,3 butanedione) is a product of citric metabolism and is responsible for the aroma and flavor of butter and some other fermented milk products (Caplice and Fitzgerald, 1999). It is produced by some species and strains of the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus*, and *Pediococcus*, as well as by other organisms. Serveral investigators demonstrated the in vitro effectiveness of diacetyl against *Mycobacterium tuberculosis, Corynebacterium diphtheriae, Enterobacter aerogenes, Escherichia coli, Erysipelothrix sp., Klebsiella pneumoniae,*

lactic acid bacteria, *Mycobacterium phlei, Pseudomonas sp., Salmonella sp., Staphylococcus aureus* and *Streptococcus* sp. (Jay, 1982). Gram-negative bacteria, yeasts and mould are more sensitive to diacetyl than Gram-positive bacteria and its mode of action is believed to be due to interference with the utilization of arginine by reacting with arginine-binding proteins of Gram-negative bacteria (Mishra and Lambert, 1996).

2.5 Reuterin

Reuterin (3-hydroxypropanal) produced during stationary phase by anaerobic growth of *Lb. reuteri* on a mixture of glucose and glycerol or glyceraldehydes (Tararico and Dobrogosz, 1989; Gänzle *et al.*, 2000). Reuterin exhibits a broad spectrum of antimicrobial activity against certain Gram-positive and Gram-negative bacteria, yeast and causative agent of Shaga's disease, *Trypanosoma cruzi* (Earnshaw, 1992).

2.6 Bacteriocins

Bacteriocins are family of antimicrobial peptides. These substances have increasing interest. Their proteinaceous nature implies their putative degradation in the gastro-intestinal tract of man and animals. This suggests that some bacteriocin-producing LAB or purified bacteriocin could be used as natural preservative in food industry (Atrih *et al*., 2001).

3. Proteinaceous antimicrobial metabolites or bacteriocins

3.1 Definition of bacteriocin

Bacteriocins are ribosomally synthesized anitimicrobial compounds (O' Sullivan *et al*., 2002) that are produced by many different bacteria species, including many member of the LAB (Garneau *et al*., 2002). Some bacteriocins produced by LAB inhibit not only closely related species but also effective against food-borne

pathogens such as *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus.* It is also inhibit other Gram-positive spoilage microorganisms including *Bacillus* sp. and *Enterococcus faecalis* (Delves-Broughton, 1990). Van Belkum and Stiles (2000) reported that bacteriocins of LAB are a heterogenous group of bacterial antagonist ranging in molecular size from a few thousand Daltons to complex protein structures that possibly contain carbohydrate or lipid moieties.

Eijsink *et al.* (2002) also defined bacteriocins the major peptides usually consisting of 20-60 amino acids. There is large variation among the peptides, e.g. in terms of length, amino acid sequence and composition, secretion and processing machinery, post-translational modifications, and antimicrobial activity (alone or in combination with other peptides). Almost all bacteriocins have a net positive charge at neutral or slightly acidic pH and they usually contain stretches of sequence that are hydrophobic and/or amphiphilic.

3.2 Classification of bacteriocins

Bacteriocins are commonly divided into four groups (Ennahar *et al*., 2000; Oscáriz and Pisabarro, 2001) as shown in Table 2. They are I) Lantibiotics; II) small hydrophobic heat-stable peptides (< 13,000 Da); III) large heat-labile proteins (> 30,000 Da) and IV) complex bacteriocins showing the complex molecule of protein with lipid and/or carbohydrate (Ouwehand, 1998).

Table 2 Classification of bacteriocins from Gram-positive bacteria

Class	Subclass	Description
Class I		Lantibiotics; Ribosomally produced peptides that undergo extensive post-translational modification
	Ia	Lantibiotics, small (<5kDa) peptides containing lanthionine and β-methyllanthionine
	Ib	Globular peptides with no net negative charge
Class II		Small (<10kDa), moderate (100° C) to high (121° C) heat stable non-lanthionine-containing membrane-active peptides
	IIa	Listeria-active peptides with –Y-G-N-G-V-X-C near the amino terminus
	IIb	Two-peptide bacteriocins
	IIc	Thiol-activated peptides
	a	Antibiotics with one or two cysteine residues (thiobiotics and cystibiotics, respectively)
	b	Antibiotics without cysteine
Class III		Lage (>30kDa) heat labile proteins
Class IV		Complex bacteriocins: protein with lipid and or/ carbohydrate

Source: Ouwehand (1998); Cleveland *et al.* (2001); Eijsink *et al.* (2002)

3.2.1 Class I bacteriocins

Class I bacteriocins (lantibiotics) consist of post-translationally modified peptides (Van Belkum ad Stiles, 2000; O'Sullivan *et al*., 2002) which are small (<5kDa) peptides containing unusual amino acids lanthionine (Lan), β-methyllanthine (MeLan) and a number of dehydrated amino acids (McAuliffe *et al.,* 2001) as shown in Table 3. In general, class I peptides have typically from 19 to more than 50 amino acids (Cleveland *et al*., 2001)

Table 3 Examples of lantibiotics produced by LAB

Lantibiotic	Producing strain
Nisin A	*L. lactis* NIZOR5, 6F3,NCFB894, ATCC11454
Nisin Z	*L. lactis* N8, NIZO22186
Lactocin S	*Lb. sake* 145
Lactococcin	*Lb. lactis* ADRI85L030
Lacticin 481	*Lactococcus lactis* CNRZ481, ADRIA85LO30
Cytolysin	*E. faecalis* DS16
Lacticin 3147	*Lc. lactis* DPC3147
Salvaricin A	*Streptococcus salvarius* 20P3
Streptococcin A-FF2	*St. pyrogens* FF22
Carnocin U149	*Carnobacterium pisicola*
Variacin 8	*Micrococcus varians* MCV8

Source: Ouwehand (1998); McAuliffe *et al.* (2001)

3.2.2 Class II bacteriocins

Class II bacteriocins are ribosomally synthesized as in active prepeptides that are modified by post translational cleavage of the N-terminal leader peptide generally at a double glycine (-2, -1) (Van Belkum and Stiles, 2000).

Class II bacteriocins comprise a very large group of heat-stable unmodified peptide bacteriocins (O'Sullivan *et al*., 2002) with molecular masses smaller than 10 kDa (Oscáriz and Pisabarro, 2001). None of the bacteriocin in this class displays any post-translational modification beyond the cleavage of an 18-21 amino acid leader region from the pro-bacteriocin molecule. All are small, between 36 and 57 amino acids after loss of leader peptides (Hill, 1995). Member of this class can be further subdivided into 3 groups: (1) Group IIa includes listeria-active peptides that conserved N-terminal sequence Try-Gly-Asn-Gly-Val and two cysteines forming a S-S (disulfide bridges) in their N-terminal half of the peptide. (2) Group IIb consists

of pore-forming complexs requiring two petides for their activity. (3) Group IIc includes thiol-activated peptides, which require reduced cysteine residues for activity (Carolissen-Mackay *et al*., 1997; Cleveland *et al*., 2001; Oscáriz and Pisabarro, 2001; O'Sullivan *et al*., 2002).

a). Class IIa

Characteristically, class IIa bacteriocins, like other low-molecular mass bacteriocins, are first formed as ribosomally synthesized precursors or pre-peptides, which appear not to be biologically active and contain an N-terminal extension or leader sequence. Subsequent cleavage of the prepeptide at a specific processing site removes the leader sequence from the antimicrobial molecule concomitantly with its export to the outside of the cell. The leader peptide's removal during transmembrane translocation is accomplished by the same protein that is associated with the bacteriocin transport. One important feature of the majority of these leaders is the presence of two glycine residues in the C-terminus of leader peptide, at positions -2 and -1 relative to the processing site, though this is not distinctive of the class IIa. These leaders are believed to serve as signal peptides for the processing and the secretion of class IIa bacteriocins by a dedicated transport system involving two distinct proteins: an ABC type translocator and an accessory protein. The two conserved glycine residues may serve as a recognition signal for this *sec*-independent transporter system (Ennarhar *et al*., 2000).

In addition, class IIa bacteriocins are characterized by occurrence of a YGNGVXCXXXXCXV sequence motif in their N-terminal half, including two cysteines that form disulfide bridge (Eijsink *et al*., 1998). Another shared characteristic of these bacteriocins is their strong inhibitory effect on *Listeria*. Class IIa bacteriocins have been encountered in a great variety of LAB belonging to the genera *Lactobacillus*, *Enterococcus, Pediococcus, Carnobacterium, and Leuconostoc* (Eijsink *et al*., 2002). Examples of class IIa bacteriocins are shown in Table 4.

Table 4 LAB producing class IIa bacteriocins and their origins

Bacteriocin	Producer strain
Mesentericin Y105	*Leuconostoc mesenteroides* Y105
Mundticin	*E. mundtii* AT06
Piscicocin 126	*Carnobacterium piscicola* JG126
Bavaricin A	*Lb. sake* MI401
Sakacin P	*Lb. sake* LTH 673
Pediocin AcH	*Pediococcus acidilactici* H
Bavaricin MN	*Lb. sake* MN
Divercin V41	*C. divergen* V41
Enterocin A	*E. faecium* CTC 492/T136
Enterocin P	*E. faecium* P13
Piscicocin V1b	*C. piscicola* V1
Curvacin A	*Lb. curvatus* LTH 1174
Sakacin A	*Lb. sake* LB 706
Carnobacteriocin B2	*C. piscicola* LV17A

Source: Ennahar *et al.* (2000)

b). Class IIb

Group IIb consists of pore-forming complexes requiring two peptides for their activity. These two peptides can be either individually active but synergistic when acting together (Enterocins L50A and L50B), or they may both be necessary for their antimicrobial activity (lactococcin Gα and Gβ, lactococcin M and N, plantaricin EF and plantaricin JK) (Oscáriz and Pisabarro, 2001). Abp 118 composed of abp 118 α and abp118 β, which exhibited the antimicrobial activity (Flynn *et al*., 2002).

It is important to note that one-peptide bacteriocins may display synergistic effects when applied in combination; the term two-peptide bacteriocins

(class IIb) refer only to sets of peptides whose genes are in the same operon (Eijsink *et al*., 2002; Garneau *et al.,* 2002). Examples of class IIb bacteriocins are shown in Table 5.

Table 5 Summary of known nonlantibiotic two peptide bacteriocins (Class IIb)

Bacteriocin	Producer strain
ABP118 (Abp 118 α and β)	*Lb. salivarius* UCC118
Enterocin 1071A and 1071B	*E. faecalis* BFE1071
Enterocin L50A and L50B	*E. faecium* L50
Lactacin F (LafX and LafA)	*Lb. johnsonii* VPI11088
Lactocin 705 α and β	*Lb. casei* CRL505
Lactocoocin Gα and β	*Lc. Lactis* LMG2081
Lactocoocin M and N	*Lc. Lactis subsp. cremoris* 9B4
Plantaricin E and F	*Lb. plantarum* C-11
Plantaricin J and K	*Lb. plantarum* C-11
Plantaricin Sα and β	*Lb. plantarum* PLCO10
Thermophilin 13 A and B	*S. thermophilus* SFi13
Lacticin 3147 A1 and A2	*Lc. Lactis* DPC3147
Plantaricin Wα and β	*Lb. plantarum* LMG2379

Source: Garneau *et al.* (2002).

c). Class IIc

Group IIc includes all class II bacteriocins that do not fall into groups IIa or IIb (Table 6). Two types of bacteriocins can be found within this group: (a) antibiotics with one or two cysteine residues (thiolbiotics and cystibiotics, repectively), and (b) bacteriocins without cysteine (lactococcin A and acidocin B). Some bacteriocins belonging to class IIc are exported via a *sec*-dependent pathway, whereas others are exported by a *sec*-independent mechanism (Oscáriz and Pisabarro, 2001).

Table 6 Class IIc bacteriocins

Bacteriocin	Producer strain
Cerein 7/8	*Bacillus cereus* Bc7
Enterocin B	*E. faecium* T136
	E. faecium CECT 492
Lactococcin A	*Lactococcus lactis* LMG 2130
Lactococcin B	*Lactococcus cremoris*
	Lactococcus lactis WM4
Divergicin A	*Carnobacterium divergens* LV13
Acidocin B	*Lb. acidophilus* M46

Source: Oscáriz and Pisabarro (2001)

3.2.3 Class III bacteriocins

This group consists of bacteriocins that are heat-labile proteins with a molecular mass larger than 30 kDa (Van Belkum ad Stiles 2000; Oscáriz and Pisabarro, 2001). They are usually inactivated with in 30 minutes by temperature of 100 °C or less (Dodd and Gasson, 1994). Most of them are produced by bacteria of the genus *Lactobacillus* (Ouwehand 1998; Oscáriz and Pisabarro, 2001), as shown in Table 7.

Table 7 Class III bacteriocins produced by LAB

Bacteriocin	Producer strain
Acidophilucin A	*Lb. acidophilus*
Caseicin 80	*Lb. casei* B80
Helviticin J	*Lb. heviticus*
Helviticin V-1829	*Lb. heviticus*
Lacticin A	*Lb. delbrueckii*
Lacticin B	*Lb. delbrueckii*

Source: Ouwehand (1998)

3.2.4 Class IV bacteriocins

This class contains complex bacteriocins (Ouwehand 1998) as shown in Table 8. Lipoid or carbohydrate moieties appear to be necessary for activity (Van Belkum and Stiles, 2000). The existence of class IV is not generally accepted as it may include regular peptide bacteriocins that have not been properly purified (Nes *et al.,* 1996).

Table 8 Class IV bacteriocins produced by LAB

Bacteriocin	Producer strain
Lactocin 27	*Lb. helveticus*
Leuconocin S	*Leuconostoc paramesenteroides*
Pediocin SJ-1	*Pediococcus acidilactici*

Source: Ouwehand (1998)

3.3 Detection and assay of bacteriocin activity

There are many techniques for detecting bacteriocin production. Most are based on the diffusion of bacteriocins through solid or semisolid culture media to inhibit growth of target strains (Lewus and Montville, 1991). The methods frequently used to detect bacteriocin activity involve (i) deferred antagonism or indirect methods include the flip streak and the spot-on-lawn-assays. In the flip-streak method, the putative bacteriocin-producing strain is streaked on a medium, incubated and a bacteriocin-sensitive organism is streaked perpendicular to it on the reverse side of the agar (which must be flipped) (Harris *et al*., 1989; Lewus and Montville, 1991). For the spot-on-lawn method, the putative bacteriocin producer is spotted on an agar medium and a lawn of sensitive bacteria seeded over the resultant colony (Ivanova *et al.*, 2000); (ii) direct assay is the well-diffusion assay. In this method, supernatants from putative bacteriocin-producing cultures are placed in wells cut into agar seeded with a sensitive organism (Harris *et al*., 1989).

However, Muriana and Luchansky (1993) concluded the methods frequently used to detect bacteriocin activity follow in the Table 9.

Table 9 Methods of testing bacteria for production of bacteriocins

Application	Method	Procedure
Culture supernatants		
	1.Spot-on-lawn test	Spot neutralized and filter- strerized supernatant onto an indicator lawn
	2. Agar well diffusion	Add neutralized and filter- strerized supernatant into wells bored into an indicator lawn
	3. Activity assays	Spot serial dilutions of neutralized and filter- strerized supernatant onto indicator lawn
Bacterial colonies		
	1. Flip plate method	Grow colonies on agar, ‘’flip’’ agar onto inside of Petri plate cover, and overlay with indicator
	2. Sandwich overlay	Plate bacterial dilution, cover with sterile medium, incubate until colonies are visible, and overlay with indicator lawn
	3. Lutri-plates	Special two-sided agar plates: grow colonies on one side, and overlay other side with indicator lawn

Source: Muriana and Luchansky (1993)

3.4 Purification of LAB bacteriocins

Bacteriocins are secreted into the growth medium. Most approaches for purification start with a concentration step from the culture supernatant, such as salt precipitation (e.g. ammonium sulphate), acid precipitation, or by extraction with organic solvents (Muriana and Luchansky, 1993), adsorbtion of bacteriocins onto the

producing cells at pH 5.5-6.8 (Yang *et al*., 1992, Elegado *et al*., 1997) or hydrophobic matrix such as amberlite XAD-16 (Cintas *et al*., 2000). Subsequently, several chromatographic step including size exclusion (gel filtration), adsorbtion (ion-exchange), and/or hydrophobic interaction (reverse-phase) chromatography have been used to achieve significant purification of bacteriocins (Muriana and Luchansky, 1993; Wu *et al*., 2004).

Todorov *et al*. (2004) summarized the purification method used by others researchers as follow: (a) anion-exchange chromatography (DEAE-Sephadex A-25) and reverse-phase HPLC; (b) ammonium sulphate precipitation (80%), cation-exchange chromatography (SP-sepharose fast-flow cation exchange column), hydrophobic interaction chromatography (phenyl-sepharose CL-4B column) and C_2/C_{18} reverse-phase chromatography; (c) ammonium sulphate precipitation (55%), hydrophobic interaction (C_8), cation exchange chromatography Mono S cation exchange column (phamacia, Biotech); (d) ammonium sulphate precipitation (40%), and cation exchange-SP-sepharose; (e) ammonium sulphate precipitation , and cation exchange-SP-sepharose, reversed-stationary-phase (octyl-sepharose-CL-4B), stationary-phase C_2/C_{18} chromatography.

3.5 Characterization of bacteriocins

Most of the bacteriocins from LAB are cationic, hydrophobic, or amphiphilic molecules composed of 20 to 60 amino acid residues (Chen and Hoover, 2003). In addition, Oscáriz and Pisabarro (2001) supported that most bacteriocins must fulfill two principal to be cationic and highly hydrophobic. Most small-size bacteriocins are active over a wide pH range (3.0-9.0), and while resistance to extreme pH values of 1.0 (acidocin B) and 11.0 (bavaricin A) has been observed, most of these bacteriocins are cationic at pH 7.0, lactocin S, with a net charge of -1 at neutral pH, being the exception. Their high isoelectric point allows them to interact at physiological pH values with the anionic surface of bacterial membranes. This interaction can suffice, in the case of broad-spectrum bacteriocins, or facilitate, in the

case of receptor-requiring compound, insertion of the hydrophobic moiety into the bacterial membrane.

Various bacteriocins from LAB have been identified and characterized (Eguchi *et al*., 2001). The study has demonstrated that the inhibitory factor contained an essential proteinaceous component since several proteolytic enzymes inactivated it (Jack *et al*., 1994). A sensitive, rapid physical detection method for bacteriocins could be a useful to track purification procedures, to detect bacteriocin production in experiments involving genetic manipulation, and or to detect bacteriocins in food. Detecting the bacteriocin by searching a compound with the appropriate molecular weight is one method to confirm the presence of bacteriocins in culture or food products. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) appears to have potential as one such method (Rose *et al*., 1999). MALDI-TOF MS is effective for peptides and proteins with molecular masses ranging from 0.5-30 kDa and has been used to determine the masses of purified class I and II bacteriocins (Hindre *et al*., 2003). In addition, molecular weight of SA-FF22 bacteriocin-like substance was analyzed by ESI-MS (Jack *et al*., 1994). Moreover, electrospray-ionization mass spectrometry (ESI-MS) is an extraordinarily powerful tool in the determination of molecular mass, especially of biological samples (Walk *et al*., 1999)

The last most important data required for complete characterization are their amino acid sequence determined by Edman degradation using automated protein sequencer (Jack *et al*., 1996; Meyer *et al*., 1994). However, Walk *et al*. (1999) reported that a number of problems might arise during automated protein sequencing of compounds containing nonproteinogenic amino acids. In some cases, the unusual amino acids effectively ‘block’ the further determination of sequential information. Using the coupled sequenator-ESI-MS system was able to determine the complete amino acid sequence.

To get a complete sequence of bacteriocin, the DNA sequence encoding the bacteriocin can be analyzed (Zendo *et al.*, 2003). Moreover, the analysis of the

gene cluster involved in the production and immunity of bacteriocin should be studied (Folli *et al*., 2003).

3.6 Bacteriocin nomenclature

The current basis for allocating a name to the agent responsible for bacteriocin-like activity produced by Gram-positive bacterium has been to adopt some derivation of either the genus or the species name of the producer strain together with an alphabetical and/or numerical code designation specifying that strain (Jack *et al*., 1995).

Bacteriocins nomenclature is straightforward. Just as ‘ ase’ is use in enzyme nomenclature, the sufflix ‘cin’ is used to denote bacteriocinogenic activity. The ‘cin’ suffix is appended either to the genus name or to the species name. The colicins were originally isolated from *E. coli* (Montville and Kaiser, 1993**).** In addition, Plantaricin NC8 is produced by *Lb. plantarum* NC8 (Maldonado *et al*., 2003), Enterocin 1071A and Enterocin 1071B are produced by *Enterococcus faecalis* BFE 1071 (Balla *et al*., 2000), Pentocin L and S are produced by *Pediococcus pentosaceus* L and S (Yin *et al*., 2003) and Lactococcin 972 is produced by *Lactococcus lactis* (Martínez *et al*., 2000). However, bacteriocins that have only minor conservative difference in their amino acid sequences, resulting in no significant change in their secondary structures, activity spectra, and cross-specificity of producer strain self protection, should be referred to as natural variants. For example, nisin A and nisin Z could be considered natural variants (Jack *et al*., 1995). They differ in a single amino acid at position 27 (a His in nisin A and an Asn in nisin Z), but have similar antimicrobial activity (Mulders *et al.*, 1991), membrane insertion (Demel *et al.*, 1996).

Recently, it has been reported a new natural nisin variant, nisin Q. It has been found that six amino acids were different between nisin A and nisin Q prepebtides, while only one amino acid between nisins A and Z. In major peptides after the post- translational modification, there were four amino acid substitutions

between nisin A and Q, and three between nisins Z and Q. However, the antibacterial spectrum of nisin Q was similar to that nisins A and Z (Zendo *et al*., 2003). Also, irrespective of the species of origin, bacteriocins having the same amino acid sequences should have one name that the first one published (Jack *et al*., 1995).

3.7 Comparison of bacteriocins and antibiotics

Bacteriocins are often confused in the literature with antibiotics. This would limit their use in food applications from a legal standpoint. The main differences between bacteriocins and antibiotics are summarized in Table 10 (Cleveland *et al*., 2001). The report contrasted bacteriocins to other antibiotics by the fact that bacteriocins are proteinaceous. Technically, antibiotics are made by a restricted group of organisms through the enzymatic packaging of primary metabolites into structurally related secondary metabolites that have no apparent function in the growth of producing cells and are easily secreted from the cell. (Montville and Kaiser, 1993).

All classes of bacteriocins are ribosomally synthesized, only class I is post-translationally modified to produce the active form. Different from bacteriocins, antibiotics are generally considered secondary metabolites. Antibiotics are not ribosomally synthesized. Although several antibiotics, such as Vancomycin, are composed of amino acids, they are enzymaticly synthesized (Cleveland *et al*., 2001). Moreover, bacteriocins differ from traditional antibiotics in one critical way; they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Riley and Wertz, 2002).

Table 10 Bacteriocins and antibiotics

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few case possibly cell wall biosynthesis	Cell membrane or intracellular targets
Toxicology/side effects	None known	Yes

Source: Cleveland *et al*. (2001)

3.8 Biosynthesis of bacteriocins

In general, bacteriocin production by LAB displayed primary metabolite kinetics followed by inactivation upon prolonged fermentation (Messens *et al*., 2003). The production of bacteriocin is growth-associated because production occurs during mid-exponential phase and increase to reach a maximal level at the end of the exponential phase or the beginning of the early-stationary phase (Cheigh *et al*., 2002). In addition, other reports supported that this loss in bacteriocin activity may be due to degradation by endogenous protease induced during the growth phase and/or the adsorbtion of bacteriocin on the surface of the producer (Messens *et al*., 2003; Onda *et al*., 2003).

Genes coding for active bacteriocins are usually in operon clusters (Cleveland *et al*., 2001). The genes encoding bacteriocin production and immunity are usually organized in operon clusters (McAuliffe *et al*., 2001). In general, at least four different genes are required to achieve the production of bacteriocins by LAB: (i) a structural gene encoding the prepeptide; (ii) a dedicated immunity gene; (iii) a gene encoding a dedicated ABC-transporter; and (iv) a gene encoding the secretion machinery (Garneau *et al*., 2002; Chen and Hoover, 2003). Genes encoding bacteriocin production can be located on the chromosome or encoded in a plasmid or transposon (Cleveland *et al*., 2001; McAuliffe *et al*., 2001; Chen and Hoover, 2003).

Bacteriocins are synthesized first as precursor or pre-peptides which are biologically inactive and contain an N-terminal extension or leader sequence. Subsequence cleavage of this pre-peptide at a specific processing site removes the leader sequence from the antimicrobial molecule concomitantly with its export to the outside of the cell. This translocation of the mature bacteriocin across the cytoplasmic membrane is mediated by ABC-transpoter and accessory protein. The 3-component regulatory system typically includes histidine proteinase (HPK), response regulator (RR) and induction factor (IF), which is required as a signal to induce the transcription of target genes. The immunity proteins provide total immunity against the producer's bacteriocin (Eijsink *et al*., 2002). This process described in Figure 1.

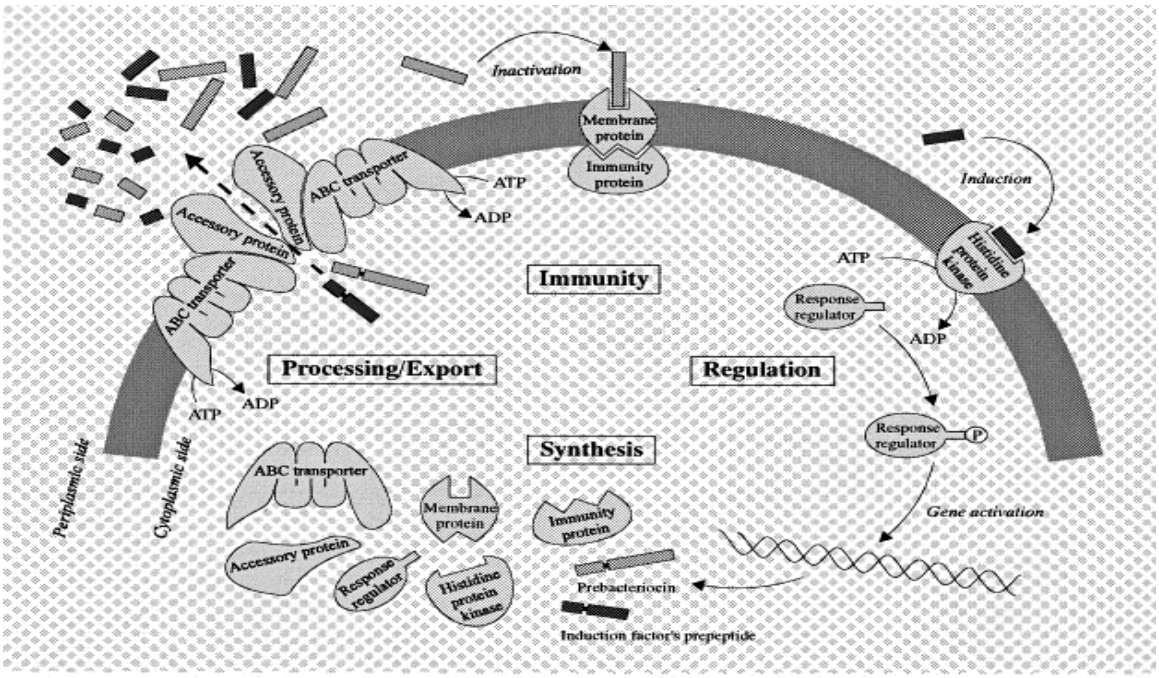


Figure 1 Biosynthesis of Class IIa bacteriocins

Source: Ennahar *et al*. (2000)

3.9 Inhibitory spectrum of bacteriocins

In general, LAB bacteriocins tend to be active against a wide range of mostly closely related Gram-positive bacteria (Messens and De Vuyst, 2002). The cytoplasmic membrane of Gram-positive bacteria is the primary target for the action of bacteriocins (Garneau *et al*., 2002). Gram-negative bacteria are generally insensitive to bacteriocins from LAB strains because of their outer membrane providing them with a permeability barrier. The sensitivity of Gram-negative bacteria can be increased by sublethal injury of the cells, using for instance high hydrostatic pressure and pulsed electric field as non-thermal methods of preservation (Caplice and Fitzgerald, 1999). Bacteriocin affects Gram-negative bacteria when their outer membrane is impaired (Abee *et al*., 1995). In addition, food grade chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA) and citrate can be used to bind magnesium ions in the lipopolysacharide outer layer of Gram-negative bacteria to render them susceptible to bacteriocins. Yeast and Fungi are not inhibited by LAB

bacteriocins. The producer strains are immune towards their own bacteriocin (Messens and De Vuyst, 2002).

Most of class I bacteriocins have a broad inhibitory spectrum. They not only inhibit closely related bacteria, such as species from the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Streptococcus*, but also inhibit many less closely related Gram-positive bacteria, such as *L. monocytogenese, Staphylococcus aureus, Bacillus cereus*, and *Clostridium botulinum*. Several bacteriocins in this class, such as nisin and thermophilin 13, prevent out-growth of spores of *B. cereus* and *C. botulinum* (Suma *et al*., 1998; Chen and Hoover, 2003).

Compared to class I bacteriocins, most class II bacteriocins have comparatively narrow activity spectra and only inhibit closely related gram-positive bacteria. In general, members of the genera *Enterococcus, Lactobacillus, Pediococcus* are sensitive to class IIa bacteriocins, and members of genus *Lactococcus* are resistant (Chen and Hoover, 2003). Some class II bacteriocins such as pediocin PA-1 have fairly broad inhibitory spectra and can inhibit some less closely related Gram-positive bacteria, such as *S. aureus* and vegetative cells of *Clostridium* spp. and *Bacillus* spp. Some class II bacteriocins, such as mundticin from *Enterococcus mundtii*, even prevent the out-growth of spores of *C. botulinum* (Eijsink *et al*., 1998).

3.10 Mechanism of action of bacteriocins

Due to the great variety of their chemical structures, bacteriocins affect different essential functions of the living cell (transcription, translation, replication, and cell wall biosynthesis), but most of them act by forming membrane chanels or pores that destroy the energy potential of sensitive cells (Oscáriz and Pisabarro, 2001). It has been established that the primary target for many of these small, cationic peptides is the cytoplasmic membrane of sensitive cells, where they act to dissipate the proton motive force (PMF) through the formation of discrete pores in the cytoplasmic membrane, and thus deprive cells of an essential energy source (Montville and Bruno., 1994; McAuliffe *et al*., 2001). The PMF, which is composed

of a chemical component (the pH gradient: ΔpH) and an electrical component (the membrane potential; Δψ), drive ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the membrane. Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy-requiring reactions (Ennahar *et al*., 2000; McAuliffe *et al.*, 2001; Chen and Hoover, 2003).This mechanism is described in Figure 2.

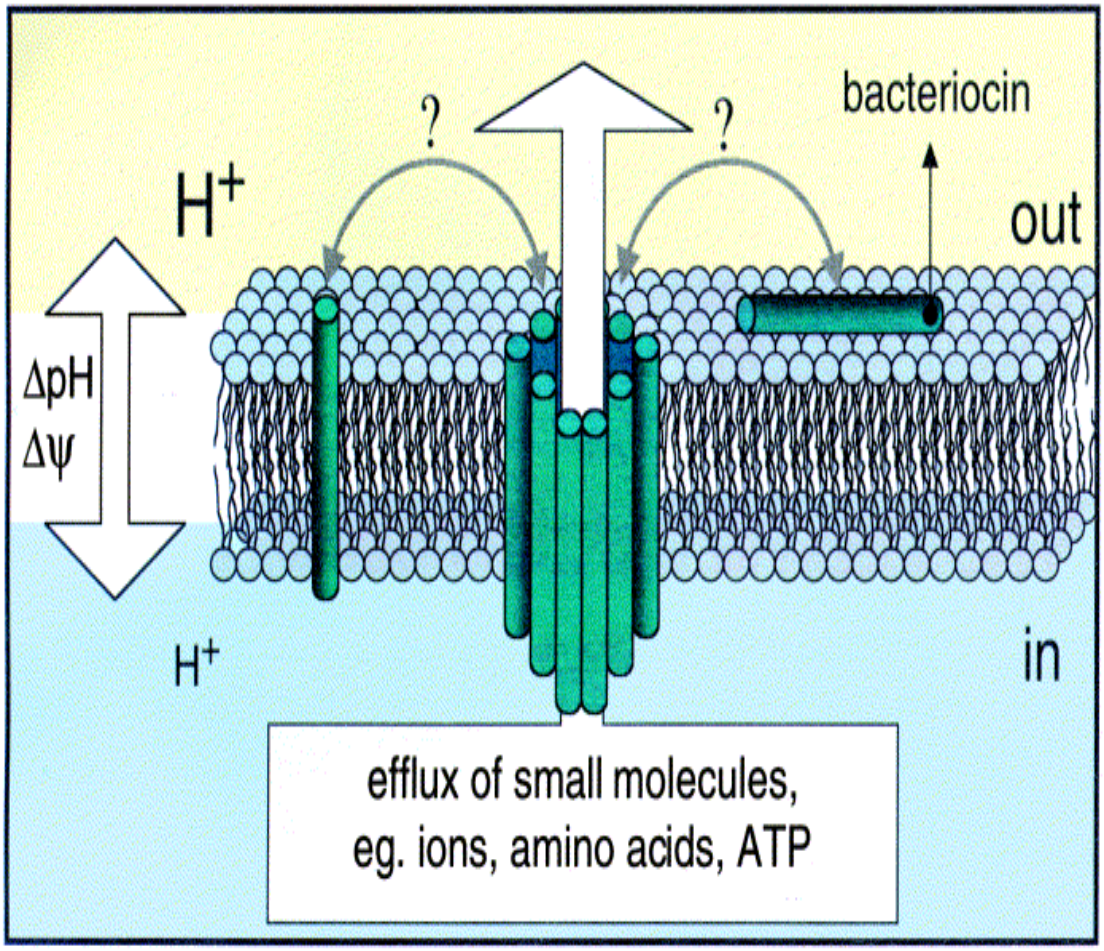


Figure 2 General models for mechanism of pore formation by bacteriocins
Source: McAullife *et al*., (2001)

The presence in class IIa bacteriocins of amphiphilic segments that are putative transmembrane helices, their water solubility and membrane-binding ability suggest that they may form poration complexes following a 'barrel-stave' model. The

initial step of class-IIa-bacteriocin interaction with the membrane surface is generally believed to be an electrostatic binding mediated by a putative membrane-bound receptor-type molecule. It has been suggested that functional binding of the positively charged and polar residues of class IIa bacteriocin occurs primary in conjugation with anionic phospholipids head groups in the membrane. As a subsequence step, hydrophobic interactions would occur between the hydrophobic/amphiphilic domains with in the C-terminal half of the bacteriocin and the lipid acyl chains, and have been shown to be crucial for the pore formation process. In fact, the C-terminal half of class IIa bacteriocins, which is more hydrophobic than the N-terminal half, contain a domain which appears to be involved in hydrophobic interactions with the membrane (Ennahar *et al*., 2000). These steps of the interaction of bacteriocin with the cell membrane of the target strain are presented in Figure 3.

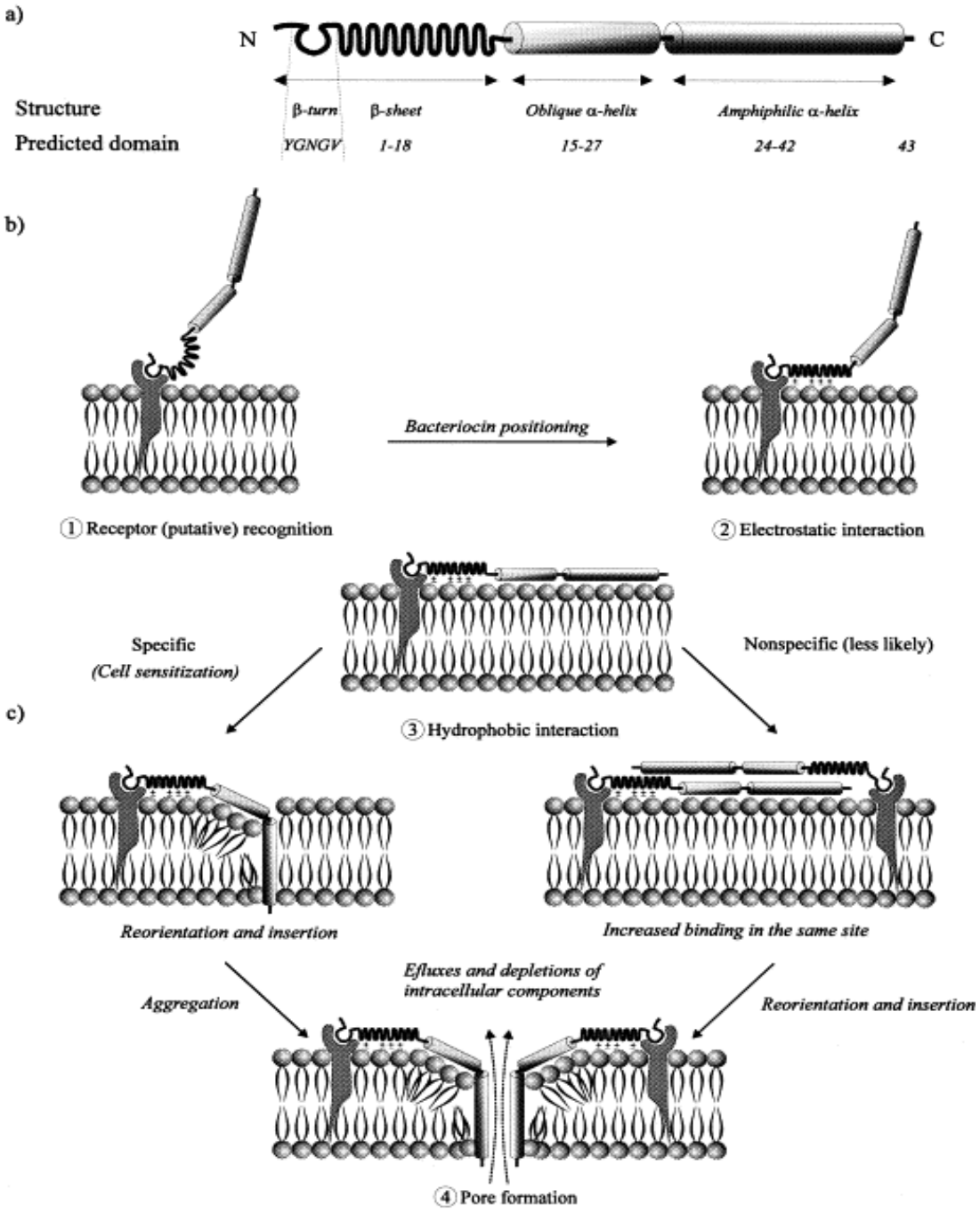


Figure 3 Schematic representation for bacteriocin and membrane interactions

a). bacteriocin predicted structural domain

b). possible interaction of each domain with the target membrane surface

c). bacteriocin insertion and formation of hydrophilic pores

Source: Ennahar *et al*. (2000)

4. Application of LAB and bactriocins

4.1 Food biopreservation

Interested in LAB bacteriocins has been sparked by growing consumer demands for natural and minimally processed foods that mandate the combination of measures or hurdles to assure microbial food safety. LAB bacteriocins have lethal activity against foodborne pathogens and spoilage microorganisms (Montville *et al*., 1995) and can play a vital role in the design and application of hurdle preservation technology (Leistner and Gorris, 1995).

One important attribute of many LAB is their ability to produce antimicrobial compounds called bacteriocins. In recent years interest in these compounds has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety. There is growing consumer awareness of the link between diet and health (Soomro *et al*., 2002).

Bacteriocins, produced by LAB, may be considered natural preservative or biopreservatives. Biopreservation refers to the use of antagonistic microorganisms or their metabolic products to inhibit or destroy undesired microorganisms in food to enhance food safety and extend shelflife (Schillinger *et al*., 1996). Three approaches are commonly used in the application of bacteriocins for biopreservation of food; (i) inoculation of food with LAB that produced bacteriocin in the products; (ii) addition of purified or semi-purified bacteriocins as food preservatives; (iii) use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing (Schillinger *et al*., 1996; Chen and Hoover, 2003).

Some of the examples that demonstrated the successful applications of bacteriocins as food preservatives are listed in Table 11.

Table 11 Bacteriocins as food preservatives: example of suggested applications

Bacteriocin	Application	Conclusion
Nisin A	Incorporation of nisin into meat binding system	Addition of nisin can reduce undesirable bacteria in restructured meat products
Pediocin AcH	Use of pediocin AcH producer *Lb. plantarum* cheese surface at the beginning of the ripening period	Spray prevents outgrowth of *L. monocytogenes* and can be used as an antilisterial treatment
Enterocin 4	Use of Enterocin 4 producer *Ent. faecalis* INIA4 as starter culture for production of Manchego cheese	Use of *Ent. faecalis* INIA4 starter inhibits *L. monocytogenes* Ohio, but not *L. monocytogenes* Scott A
Nisin A	Use of nisin to control *L. monocytogenes* in ricotta cheese	Nisin effectively inhibits *L. monocytogenes* for 8 weeks
Piscicolin 126	Use of piscicolin 126 to control *L. monocytogenes in devilled ham paste*	More effective than commercially available bacteriocin
Leucocin A	Use of leucocin-producing *Leu. gelidum* UAL 187 to control meat spoilage	Inoculation of vacuum packed beef with the bacteriocin-producer delays the spoilage by *Lb. sake* for up to 8 weeks
Lactocin 705	Use of lactocin 705 to reduce growth of *L. monocytogenes* in ground beef	Lactocin 705 inhibits growth of *L. monocytogenes* in ground beef
Pediocin AcH	Use of the pediocin producer *P. acidilactici* to inhibit *L. monocytogenes*	*P. acidilactici* (Ped+) starter culture contributes to effective reduction of *L. monocytogenes* during manufacture of chicken summer sausage
Pediocin AcH	Add pediocin preparation to raw chicken	Control growth of *L. monocytogenes* at 5 ºC for 28 days
Enterocin	Add eonterocin to inoculated ham, pork, chicken breast, pate, sausage	Control growth of *L. monocytogenes* under several conditions

Source: Cleveland *et al*., (2001)

4.2 LAB strains as probiotics

As we enter in the new millennium, people are aware that for spending a healthy life style diet play a major role in preventing disease and promoting health. (Soomro *et al*., 2002). Therefore, functional foods, designer foods, phamafoods and

nutraceuticals are synonyms for foods with ingredients that can prevent and treat disease. A probiotic may also be a functional food, but more specifically it is a live microbial feed supplement that beneficially affects the host beyond correction for traditional nutrient deficiencies by improving its intestinal balance. Hence, it may be considered a functional of the special property of containing live, beneficial microorganisms. Regulation of the intestinal balance results from the competition among the many bacterial species that survive passage through the upper gastrointestinal tract and colonize the human colon. A health benefit can also arise from the ability of an ingested microorganism to contribute an enzyme to the small intestine e.g. β-galactosidase (lactase) that many adults lack (Scheinbach, 1998).

Probiotics are commonly uses as viable microbial feed supplements that affect the host animal by improving its intestinal microbial balance Reported beneficial effects on cattle, pigs and chickens include improved general health, more efficient feed utilization, faster growth rate and increased milk and egg production (Hyronimus *et al*., 2000).

Traditional probiotic dairy strains of LAB have a long history of safe use. There is considerable interest in extending the range of foods incorporating probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice based products, cereal based products and pharmaceuticals. *Lactobacillus* spp. and *Bifidobacterium* spp. are prominent members of the commensal intestinal flora and are the commonly studied probiotic bacteria. They cause reduced lactose intolerance, alleviation of some diarrhea, lowered blood cholesterol, increased immune responses and prevention of cancer (Marteua and Rambaud., 1993; Soomro *et al*., 2002).

Microorganisms used as probiotics are *Lb. acidophilus, Lb. plantarum, Lb. casei, Lb. casei* subsp. *Rhamnosus, Lb. delbreuckii* subsp. *bulgaricus, Lb. fermentum, Lb. reuteri, Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Bifidobacterium bifidum, Streptococcus salivarius* subsp. *thermophilus, Enterococcus faecalis* and *E. faecium* (Hyronimus *et al*., 2000; Soomro *et al*., 2002).

MATERIALS AND METHODS

1. Bacterial strains

About 300 isolates of LAB were isolated from 20 chicken intestines obtained from a farm of King Mongkut's Institute of Technology, Ladkrabang, in Thailand**.** Bacteria used as indicator strains for detecting antibacterial activities of bacteriocins from LAB consisted of groups of LAB, Gram-positive bacteria, and Gram- negative bacteria, which are listed, in Table 12. All of these strains were obtained from Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Japan.

2. Media and growth conditions

LAB strains were cultivated in MRS broth (De Man, Rogosa and Sharpe broth) (Oxoid, Basingstoke, United Kingdom) under anaerobic conditions. The Indicator strains were cultivated in MRS at 30 or 37ºC for 24 hr, except *Bacillus*, *Listeria*, *Staphylococcus, Micrococcus, E. coli* and *Brochotrix* which were grown in Tryptic Soy Broth (TSB; Difco, Sparks, Md) containing 0.6% Yeast extract (YE; Nacalai Tesque, Kyoto, Japan).

For long term preservation, LAB, other Gram-positive bacteria, and Gram-negative bacteria were kept at -80ºC as stock cultures in MRS broth containing 15% glycerol and TSB containing 0.6% YE with 15% glycerol, respectively. Before use, the bacteria were propagated in proper medium (MRS or TSB-YE) by incubating overnight at optimal temperature as showed in Table 12.

E. coli JM109 was used as a host for cloning. It was grown in Luria-Bertani (LB) broth (Sambrook *et al*., 2001), supplemented with 100 μg/ml ampicillin at 37°C, and vigorously agitated overnight.

Table 12 List of indicator strains and their growth conditions

Indicator strains	Media	Temperature	Condition
Lactic acid bacteria			
Lactococcus lactis subsp. *lactis* ATCC 19435^T	MRS	30ºC	Anaerobic
Enterococcus faecium TUA 1344L	MRS	30ºC	Anaerobic
Lactobacillus plantarum ATCC 14917^T	MRS	30ºC	Anaerobic
Pediococcus pentosaceus JCM 5885	MRS	30ºC	Anaerobic
Lactobacillus sakei subsp. *sakei* JCM 1157^T	MRS	30ºC	Anaerobic
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS	30ºC	Anaerobic
Enterococcus faecalis JCM 5803^T	MRS	37ºC	Anaerobic
Other gram positive bacteria			
Bacillus circulans JCM 2504^T	TSB-YE	30ºC*	Aerobic
Bacillus subtilis JCM 1465^T	TSB-YE	30ºC*	Aerobic
Bacillus coagulans JCM 2257^T	TSB-YE	37ºC*	Aerobic
Bacillus cereus JCM 2152^T	TSB-YE	30ºC*	Aerobic
Staphylococcus epidermis JCM 2414^T	TSB-YE	37ºC	Aerobic
Listeria innocua ATCC 33090^T	TSB-YE	37ºC	Aerobic
Micrococcus luteus IFO 12708	TSB-YE	30ºC*	Aerobic
Brochotrix campestris NBRC 11547 ^{T}	TSB-YE	26ºC	Aerobic
Gram negative bacteria			
Escherichia coli JM109	TSB-YE	37ºC*	Aerobic

ATCC = American Type Culture Collection, Rockville, Md

JCM = Japanese culture of Microorganisms, Wako, Japan

JM = Commercial strain from Toyobo, Osaka, Japan

TUA = Tokyu University of Agriculture, Tokyo Japan

IFO = Institute for Fermentation, Osaka, Japan

NBRC = National Institute of Technology and Evaluation (NITE) Biological Resource Center

* = shaking

3. Isolation of LAB from chicken intestine

The primary screening LAB were isolated from 20 chicken intestines. The intestinal content was taken from each part such as duodenum, jejunum, ileum and ceacum. Ten grams of each part of intestinal content were homogenized in 90 ml diluent (0.85% NaCl) and subsequently 10-fold serial dilution was carried out. The dilutions were, therefore, spread-plate on MRS (Oxoid, Basingstoke, UK) agar containing 0.5% $CaCO_3$ in duplicate. The plates were incubation at 37°C for 24-48 hr under anaerobic condition (candle jar). Only clear zone producing-colonies were randomly selected due to dissolution of $CaCO_3$ by acids production. Numbers of colonies on each plate were recorded, and LAB populations were expressed in log cfu/g. The selected colonies then were purified by transferring from the master plate to new MRS agar plate for two passages. The colonies were maintained in MRS broth under anaerobic conditions at 37°C for overnight, and then stored at -80°C with 15% glycerol for bacteriocin screening in future study.

4. Screening of LAB strains for antibacterial activities

The secondary screening of three-hundred isolates for bacteriocin was carried out by spot-on-lawn method (Ennahar *et al*., 1999). One percent inoculums of LAB culture (v/v) were mixed in MRS broth at 37°C for 16-20 hr. Cell Free Supernatant (CFS) was obtained by centrifugation at 5369 x g for 10 min at 4°C. CFS was adjusted to pH 5.5 by 0.5 M NaOH (to exclude the effect of organic acids) and then sterilized by filter (0.2 µm, polysulfone, Kanto chemical, Japan). Antibacterial activities of bacteriocins were tested by spotting 10 µl of CFS onto the surface of agar plate which was overlaid with 5 ml of soft agar (1% w/v) seeded with 10 µl of freshly-grown indicator strain (about 10^7 cfu/ml), *Lactobacillus sakei* subsp. *sakei* JCM 1157^T. After overnight incubation at proper temperature as shown in Table 12, inhibition zone was observed. Only positive bacteriocin-producing LAB were further investigated with other indicator strains (Table 12). The antibacterial activities were measured with a critical dilution method and expressed as arbitrary unit (AU). The CFS was two-fold serially diluted in 96-wells microtiter plate (Greiner bio-one,

German). An arbitrary unit is defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain. AU was calculated as (1000/10) D where D was the dilution factor (Parente *et al.,* 1995).

5. Physicochemical stability of the antibacterial activity of CFS

5.1 Effect of enzymatic treatments

The CFS was treated with proteolytic enzymes at a final concentration of 1 mg/ml as the followings: trypsin (Sigma, St. Louis, Mo, U.S.A), alpha-chymotrypsin (sigma), ficin (sigma), protease type XIII (sigma), pepsin (sigma), papain (Merck, Darmstadt, Germany), proteinase K (Merck) and actinase E (Kaken Pharmaceutical, Tokyo, Japan). All samples were adjusted to pH 7, except that treated with pepsin, which was adjusted to pH 3, and then were sterilized by filtering through filter membrane (0.22 μm, Acrylic copolymer, Kanto Chemical, Japan). The filtrates were incubated at 37°C for 3 hr. Residual enzyme activities were finally stopped by boiling for 5 min. Antibacterial activities of CFS were determined by spot-on-lawn against *Lb. sakei* subsp. *sakei* JCM 1157^T (Ennahar *et al*., 1999).

5.2 Effect of pH and heat treatments

To determine the effect of pH on antibacterial activities, the CFS was adjusted to various pH levels of 3-10 and incubated at 30°C for 2 hr. All samples were then adjusted to pH 6 and sterilized by filtering through 0.22 μm filter membrane. Thermal stability was studied by heating the neutralized CFS at 100°C for 5, 30 min and at 121°C for 15 min. Antibacterial activities of CFS were determined by spot-on-lawn against *Lb. sakei* subsp. *sakei* JCM 1157^T (Ennahar *et al*., 1999).

6. Growth determination and bacteriocin production

6.1 Effect of temperature and incubation time on bacteriocin production

The effect of incubation time on growth and bacteriocin production of bacteriocin-producing LAB (two isolates, K4 and K7) were studied at several growth temperatures at 30, 37 and 42°C. The bacteriocin production was determined by inoculating an overnight culture of K4 and K7 at 1% (v/v) into 300 ml of MRS broth and incubating at 30, 37 and 42°C for 24 hr. Ten milliliters of samples were collected from the cultures and examined for bacterial count (cfu/ml), measuring the optical density (OD) at 600 nm. Then, antibacterial activity was evaluated by spot-on-lawn method against *Lb. sakei* subsp. *sakei* JCM 1157^T.

6.2 Effect of NaCl and pH on production of bacteriocins

NaCl was added to MRS broths at the final concentration 1, 2, 3 and 4%. Similarly, effect of pH on production of bacteriocins was studied by adjusting pH values of MRS broth to pH 3, 4, 5, 6, 7, 8, 9 and 10. On the previous studied, the optimum temperature for bacteriocins production of K4 and K7 was 30°C. Therefore, K4 and K7 were grown at 30°C for 16 and 12 hr, respectively. The optical density at 600 nm was measured to determine the growth of K4 and K7. Bacteriocin production was evaluated by spot-on-lawn method against *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans* JCM 2257^T as indicator strains.

6.3 Determination of mode of action

6.3.1 Determination of mode of action of partially purified bacteriocin

Mode of action study was carried out by using partially purified bacteriocin prepared by Amberlite XAD-16 (Sigma, St. Louis, Mo, U.S.A) to adsorb proteins from CFS as described by Cintas *et al*., (2000). One hundred milliliters of

MRS broth were inoculated with indicator strain culture at 1% level, and then incubated at 30°C for 1 hr. Subsequently, the partially purified bacteriocin at a final concentration of 100 AU/ml was added to indicator strain culture, *Lb. sakei* subsp. *sakei* JCM 1157^T. The samples were taken at 2 hr time intervals for 8 hr and examined for viable cell count (cfu/ml), cell density (600 nm).

6.3.2 Determination of mode of action of purified bacteriocin

After growing for 1 hr, *Lb. sakei* subsp. *sakei* JCM 1157^T was divided into 3 protions. The first portion was added by the purified bacteriocin with a final concentration of 100 AU/ml. The second was added by 45% acetonitrile used for eluting in HPLC to serve as a control. In addition, the third portion, without bacteriocin and 45% acetonitrile was used as a control. Subsequently, the cultures were incubated at 30°C for 24 hr. Then, samples from each culture were taken at 2 hr time intervals for 8 hr. The optical density of the samples at 600 nm and viable cell count per ml (cfu/ml) were examined.

7. Identification of bacteriocin-producing strains

7.1 Determination of morphological and other physiological characteristics

LAB isolates were subjected to Gram-staining reaction and examined by phase contrast microscope to determine the cell morphology. The test of catalase activity and gas production from glucose were determined according to the methods described by Forbes *et al*., (1998).

The growth of LAB isolates at temperatures 5, 15, 30, 37, 42, 45 and 50°C and pH 4.5 and 9.6 were determined by inoculating one colony from an overnight culture into MRS broth. Salt tolerance was observed in MRS containing NaCl concentrations at 6.5 and 18%. All samples were examined for cell turbidity to determine the cell growth after 24 and 48 hr incubation times (Axelsson, 1998).

7.2 Determination of carbohydrate fermentation pattern

Carbohydrate fermentation pattern was carried out by using API 50 CH Rapid fermentation strips (API, BioMerieux, France) in CHL medium as specified by the manufacturer at 37°C for 24 and 48 hr.

7.3 Nucleotide sequence analysis of the 16S rDNA

7.3.1 PCR amplification of 16S rRNA gene of K4 and K7

PCR amplification of 16S rDNA was carried out to confirm the identification result. Genomic DNA of K4 and K7 were isolated by using Genomic DNA Mag extractor Genome kit (Toyobo, Osaka, Japan). Polymerase Chain Reaction (PCR) amplification was conducted with pair of bacterial universal primer 8f 5'-AGA GTT TGA TCA TGG CTC AG-3' and 1510r 5'-GTG AAG CTT ACG GCT ACC TTG TTA CGA CTT-3' (Martínez-Murcia *et al*., 1995; Christine *et al*., 2002). PCR mixture composed of 1 X reaction buffer with 25mM $MgCl_2$, 2.5 mM deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Promega, U.S.A), 20 pM of each primer and genomic DNA about 50-200 ng were used as a template for amplification. The PCR condition as following: 94°C for 5 min, 30 cycles including denaturation 94°C (30s), annealing at 52°C (30 s), and extension at 72°C (45 s) and the final step72°C for 7 min.

7.3.2 Cloning and sequencing of 16S rRNA gene of K4 and K7

The PCR product was purified by QIAquick PCR purification kit (Qiagen, U.S.A) and cloned into pGem-T vector system (Promega, U.S.A). The ligation product was transformed into *E. coli* JM109. Screening of *E. coli* JM109 positive clone was determined by colony PCR. Each clone was suspended in 20 µl of 1 X reaction buffer with 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate, 1 U of *Taq* polymerase (Promega, U.S.A), and 20 pM of each primer (M13 universal and 519B). PCR condition was as following: denaturation 94°C for 5 min, 30 cycles

including denaturation 94°C for 30 s, annealing at 52°C for 20 s, and extension at 72°C for 1 min and the final extension 72°C for 7 min.

7.3.3 Nucleotide sequence analysis of the 16S rDNA of K4 and K7

Plasmid DNA from *E. coli* JM109 transformant was extracted by using Mag extractor plasmid kit (Toyobo, Osaka, Japan). The DNA sequences of positive clones were determined by using the ABI PRISM 3730 XL sequencer with bigdye terminator version 3.1. A database searched from GenBank was done by BLAST program.

8. Bacteriocin purification

8.1 Concentration of bacteriocins using Amberlite XAD-16

Lb. salivarius K4 was grown in 1 liter MRS broth at 30°C for 14-15 hr until OD at 600 nm reached about 5. CFS was obtained by centrifugation at 5369 x g for 10 min at 4°C and the purification processes were carried out as described by Cintas *et al.,* (2000). Bacteriocin adsorption was achieved by adding 20 g of Amberlite XAD-16 (Sigma, St.Luis, Mo) into 1 liter of CFS and shaking at room temperature for 2 hr. The amberlite adsorbing hydrophobic substances was collected and washed with 100 ml of distilled water followed by 100 ml of 40 % (v/v) ethanol in distilled water. Finally the bacteriocin was eluted with 100 ml of 70% isopropanol solution containing 0.1% Trifluroacetic acid (TFA) in distilled water. The bacteriocin antibacterial activities of fractions were determined against *Lb. sakei* subsp. *sakei* JCM 1157^T. Therefore, the eluates that showed high bacteriocin activities were separately subjected to cation exchange chromatography as follows.

8.2 Cation exchange chromatography of the eluates

The eluates that showed the highest antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^T were then evaporated using rotary evaporator to get rid

of isopropanol and then diluted with 20 mM phosphate buffer pH 5.7. Subsequently, pH of the solution was adjusted to 5.7. The sample was then applied to SP-Sepharose fast flow cation-exchange column (Amersham Phamacia Biotech, Tokyo). The column was first washed with 20 mM phosphate buffer pH 5.7 and elution was performed with a stepwise gradient from 0.25, 0.5, 0.75 and 1 M NaCl in 20 mM phosphate buffer pH 5.7. Four fractions, 20 ml each, were collected using a bench top peristaltic pump. These fractions were evaluated for their antibacterial activities against indicator strain, *Lb. sakei* subsp. *sakei* JCM 1157^T. The fraction that showed the highest bacteriocin activity was selected and subjected to reverse phase High Performance Liquid Chromatography (RP-HPLC) (Shimadzu, Japan).

8.3 Purification of bioactive fractions by Reversed-Phase HPLC-1

Among the fractions of cation exchange chromatography, the fraction which showed the highest activity against *Lb. sakei* subsp. *sakei* JCM 1157^T was selected and further purified by reversed phase-HPLC using Resource RPC 3 ml column (Amersham Bioscience). Elution was carried out with linear gradient of 100% A to 20%A/80%B, which solvent A was 0.1% TFA in water; and solvent B was acetonitrile containing 0.1% TFA. Elution was carried out at flow rate 1 ml/min for 30 min and fractions were detected by the absorbance at 220 nm. These fractions were evaluated for their antibacterial acivities against indicator strain, *Lb. sakei* subsp. *sakei* JCM 1157^T and acetonitrile containing 0.1% TFA was used as negative control.

8.4 Rechromatography of bioactive fractions by Reversed-Phase HPLC-2

Among the fractions of HPLC-1, the fractions that showed highest bacteriocin activities were separately re-chromatographed on the same column. Elution was carried out at the flow rate of 1 ml/min for 30 min gradient of 75%A/B25% to 35%A/65%B. Solvent A was 0.1%TFA in water and solvent B was 0.1%TFA in Acetonitrile. was carried out at flow rate 1 ml/min for 30 min and fractions were detected by the absorbance at 220 nm. These fractions were evaluated

for their antibacterial acivities against indicator strain, *Lb. sakei* subsp. *sakei* JCM 1157^T and acetonitrile containing 0.1% TFA was used as negative control.

9. Determination of protein concentration

The protein concentration of CFS, partially purified and fully purified bacteriocins were determined using the Bio-Rad protein Assay kit, which was based on the Bradford method (Kruger, 2002).

10. Determination of N-terminal amino acid sequence

N-terminal amino acid sequence of bacteriocins produced by *Lb. salivarius* K4 were performed by Edman degradation on gas-phase automatic sequence analyzer (PSQ-1, Shimazu, Japan).

11. Determination of Molecular weight of purified bacteriocins

11.1 MALDI-TOF-MS measurement

The molecular weight of partially purified bacteriocins was measured by a Voyager-RP matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Perceptive Biosystem, Framingham, MA, USA). In this analysis, a saturated α-cyano-4-hydroxycinnamic acid solution was used as matrix (Ennahar *et al*., 2001).

11.2 ESI-MS

Molecular weight of fully purified bacteriocin was determined by electrospray-ionization mass spectrometry (ESI-MS; JMS-T100LC, JEOL, Japan) (Walk *et al*., 1999).

12. Nucleotide sequence analysis of bacteriocin genes

12.1 Design of specific primers

The primer sets were designed from data base of bacteriocin-like prepeptide (ORF 3) and bacteriocin abp 118 β gene cluster produced by *Lb. salivarius* UCC 118 as shown in Figure 4 and Table 13 (Flynn *et al*., 2002).

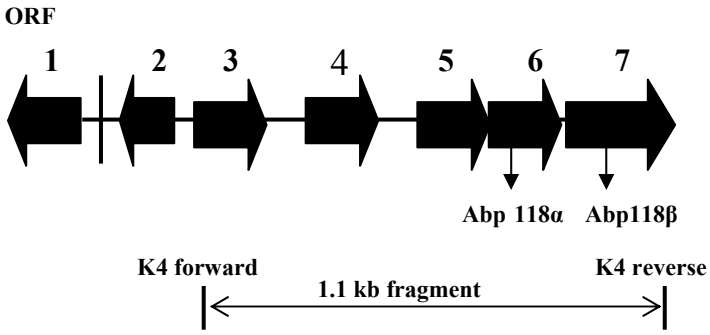


Figure 4 Illustration of primer set designed from database of ORF 3 and abp 118 β

Table 13 Primers used for PCR amplification of bacteriocin gene

Primer set	Nucleotide sequence
K4 forward	5’ AGAGCAGATGAAGTGAATAATTA3’
K4 reverse	5’ CTTACACTTGACACTACTTGA3’
M13 universal	5’ CAGGAAACAGCTATGACC 3’
M13 reversal	5’ AACAGCTATCAGCATG 3’

12.2 PCR amplification of bacteriocin genes

Genomic DNA of K4 was isolated by using Genomic DNA purification kit (Mag extractor Genome; Toyobo, Japan). To study structural genes of bacteriocins, genomic DNA of 50-200 ng was used as a template for amplification in

25 µl of 1x reaction buffer with 1.25 units of Premix *Taq* DNA polymerase (Ex *Taq* version, Takara Bio Inc, Japan) and 20 pM of each primer. The PCR program consisted of denaturation 94°C for 3min, followed by 30 cycles including denaturation 94°C (30 s), annealing at 52°C (30 s), and extension at 72°C (45 s) and the final extension 72°C for 2 min.

12.3 Cloning and sequencing of bacteriocin genes

The PCR product was purified by QIAquick PCR purification kit (Qiagen, USA) and cloned into pGem-T vector system (Promega, USA). The ligation product was transformed into *E.coli* JM109. Positive clone was screened by colony PCR. Each clone was suspended in 20 µl of 1X reaction buffer with 25 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphate, 1 U of *Taq* polymerase (Promega, USA), 20 pM of each primer (M13 universal and M13 reversal). PCR condition was: denaturation 94°C for 5 min, 30 cycles including denaturation 94°C for 30s, annealing at 52°C for 20 s, and extension at 72°C for 1 min and the final extension 72°C for 7 min. Plasmid DNA from *E. coli* JM109 transformant was extracted by using Mag extractor plasmid kit (Toyobo, Japan). The DNA sequences of positive clones were determined by using the ABI PRISM 3730 XL sequencer with bigdye terminator version 3.1.

12.4 Computer analysis of DNA and protein sequences

Analysis of DNA and protein sequences were performed by SDC-GENETYX program (software Development, Tokyo, Japan) and the database was searched by BLAST program (Genbank).

13. Places

1. Isolation of LAB from chicken intestines was conducted at Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, 10520, Thailand.

2. Screening of LAB for antibacterial activities was carried out at Department of Pathology and Department of Veterinary Public Health, Faculty of Veterinary Medicine, Kasetsart University, Kamphangsean Campus, 73140, Thailand.

3. Purification and characterization of bacteriocins were carried out at

3.1 Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.

3.2 Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate school, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.

14. Duration

The experiments were carried out from May, 2002-January 2005.

15. Funding source

The Center for Agricultural Biotechnology through the fund from Subproject Graduate Study and Research in Agricultural Biotechnology under High Education Development Project, Commission on Higher Education, the Ministry of Education, Thailand. Kasetsart University, Kamphangsean Campus, 73140, Thailand.

RESULTS

1. Isolation of LAB strains from chicken intestine (Primary screening of bacteriocin-producing LAB)

LAB were isolated from different sections of chicken intestinal tract. The total number of LAB from each part were counted and calculated in cfu/g as shown in Table 14. The highest mean of 8.72 log cfu/g occurs in ceacum.

Table 14 Average number of LAB isolated from different part of chicken intestine

Intestine	The number of LAB (log cfu/g)
Duodenum	7.00
Jejunum	7.37
Ileum	8.34
Ceacum	8.72

2. Screening and characterization of LAB strains producing antibacterial substance (Secondary screening of bacteriocin-producing LAB)

2.1 Inhibitory activity of CFS of K4 and K7

Three hundred of LAB isolates were evaluated for their antibacterial activities against indicator strain, *Lb. sakei* subsp. *sakei* JCM 1157^T. Their CFS were adjusted to pH 5.5 to eliminate acid condition. Out of them, six isolates (2% of primary screening of bacteriocin-producing LAB) were shown antibacterial activity at pH 5.5 and were designated as K4, K7, K8, K9, K10, and K11. The K4 and K7 had 3200 and 1600 AU/ml antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^T, respectively, which were higher than those of the others (100 AU/ml). Since LAB with broader inhibition spectra are preferred, only two isolates, K4 and K7, were selected for further studies.

The CFS of K4 and K7 displayed antibacterial activities against only Gram-positive bacteria as shown in Table 15. CFS of K4 exhibited antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^{T}, *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^{T} , *B. coagulans* JCM 2257^{T}, *E. faecalis* JCM 5803^{T} , *L. innocua* ATCC 33090^{T} and *Br. campestris* NBRC 11547 ^{T}. K7 displayed antibacterial activities against only *Lb. sakei* subsp. *sakei* JCM 1157^{T}, *Leu. mesenteroides* subsp. *mesenteroides* JCM 6124^{T} and *B. coagulans* JCM 2257^{T}. However, CFS of K4 and K7 did not inhibit Gram-negative bacteria at pH 5.5. For K4, the highest antibacterial activity of 3200 AU/ml was found on *Lb. sakei* subsp. *sakei* JCM 1157^{T}. The highest antibacterial activity of 1600 AU/ml of K7 was also found on *Lb. sakei* subsp. *sakei* JCM 1157^{T}. Therefore, this indicator strain was chosen for the further study of other properties of the bacteriocins.

Table 15 Antibacterial activities of K4 and K7 against indicator strains

Indicator strains	Antibacterial activity (AU/ml)	
	K4	K7
Lactic acid bacteria		
Lactococcus lactis subsp. *lactis* ATCC 19435^T	-	-
Enterococcus faecium TUA 1344L	-	-
Lactobacillus plantarum ATCC 14917^T	-	-
Pediococcus pentosaceus JCM 5885	-	-
Lactobacillus sakei subsp. *sakei* JCM 1157^T	3200	1600
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	100	200
Enterococcus faecalis JCM 5803^T	200	-
Other gram positive bacteria		
Bacillus subtilis JCM 1465^T	-	-
Bacillus circulans JCM 2504^T	-	-
Bacillus coagulans JCM 2257^T	1600	400
Bacillus cereus JCM 2152^T	-	-
Staphylococcus epidermis JCM 2414^T	-	-
Listeria innocua ATCC 33090^T	800	-
Micrococcus luteus IFO 12708	-	-
Brochotrix campestris NBRC 11547 ^{T}	800	-
Gram negative bacteria		
Escherichia coli JM109	-	-

2.2 Physicochemical stability of the antibacterial activities of CFS

2.2.1 Effect of proteolytic enzyme

K4 and K7 were grown at 37°C for overnight. Bacterial cultures were centrifuged and treated with proteolytic enzymes. The CFS antibacterial activities of K4 and K7 were completely destroyed by various proteolytic enzymes such as trypsin, alpha-chymotrypsin, papain, ficin, actinase E, proteinase K, pepsin and protease type XIII as shown in Table 16. This complete inactivation of antibacterial activities indicated that there was a proteinaceous structure classified as a bacteriocin (Vaughan *et al*., 2001).

Table 16 Effect of enzymes on the antibacterial activities produced by K4 and K7 against *Lb. sakei* subsp. *sakei* JCM 1157^T

Treatment	Antibacterial activity (AU/ml)	
	K4	K7
Enzyme stability		
Control pH 3	1600	800
Control pH 5.5	1600	800
Control pH 7	1600	800
Trypsin	0	0
alpha-chymotrypsin	0	0
Papain	0	0
Ficin	0	0
Actinase E	0	0
Proteinase K	0	0
Pepsin	0	0
Protease XIII	0	0

2.2.2 Effect of pH and heat treatment

The antibacterial activity of K4 was stable at a wide range pH of 3-10 while the antibacterial activity of K7 was stable at pH range of 4-7. But, the higher activity was obtained at high pH range of 8-10 and at low pH to 3. The antibacterial substances of K4 and K7 were heat tolerants since the antibacterial activities were retained under heat treatment at 100°C for 30 min. The antibacterial activities from CFS of both K4 and K7 were slightly reduced, when heated up to 121°C (autoclave) for 15 min (Table 17).

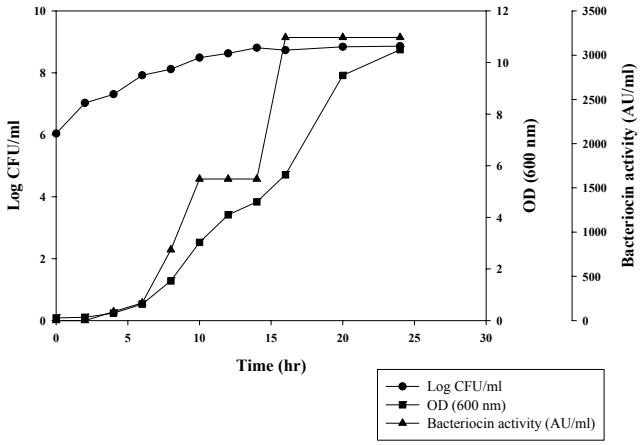
Table 17 Effect of pH and heat on the antibacterial activities produced by K4 and K7 against *Lb. sakei* subsp. *sakei* JCM 1157^T

Treatment	Antibacterial activity (AU/ml)	
	K4	K7
pH stability		
Control (not incubated)	3200	1600
pH3	3200	1600
pH4	3200	800
pH5	3200	800
pH5.5	3200	800
pH6	3200	800
pH7	3200	800
pH8	3200	1600
pH9	3200	1600
pH10	3200	1600
Heat stability		
Control	1600	800
100°C at 5 min	1600	800
100°C at 30 min	1600	800
121°C at 15 min	800	400

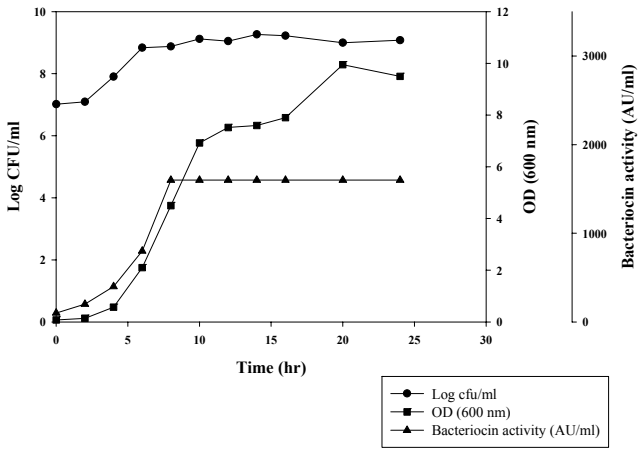
2.3 Profile of growth and bacteriocin production of K4 and K7 at various temperatures

The growth of K4 and K7 and production of bacteriocins were studied at various temperatures: 30, 37 and 42°C as shown in Figure 5 (A, B and C) and Figure 6 (A, B and C). The profile of both isolates showed typical growth phase of bacteria. The cell number and OD (600 nm) revealed the same trend of growth. At temperature 37 and 42°C, the bacterial grew to stationary phase in less time than the growth at 30°C. The highest bacteriocin activity was observed in late exponential phase at all temperatures. The maximum bacteriocins production was found at about 12-16 hr of 30°C incubation period and 6 hr of incubation time at 37°C or 42°C. The bacteriocins activity of K4 increased up to 3200 AU/ml at 30°C, while at temperature 37°C or 42°C the bacteriocins activity was 1600 AU/ml. The maximum bacteriocins activity of K7 was found at 30°C which equivalent to 1600 AU/ml and decreased to 800AU/ml at temperature 37 and 42°C after 6 and 8 hr of incubation period, respectively.

A) Profile of growth of K4 and bacteriocin production at 30°C



B) Profile of growth of K4 and bacteriocin production at 37°C



C) Profile of growth of K4 and bacteriocin production at 42°C

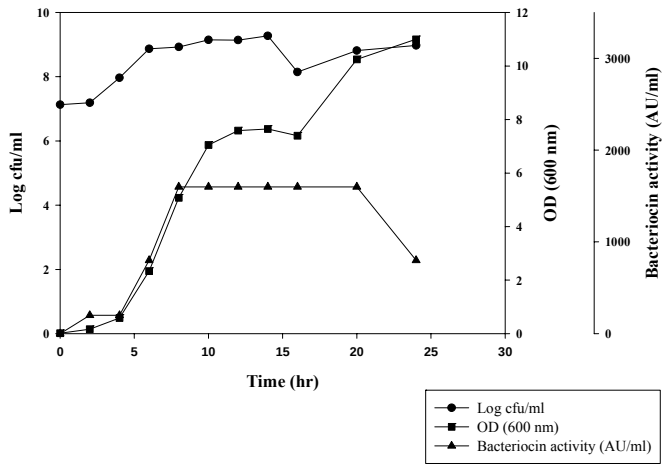
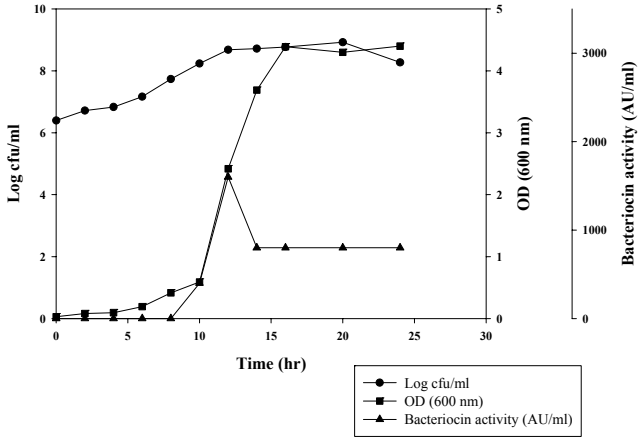
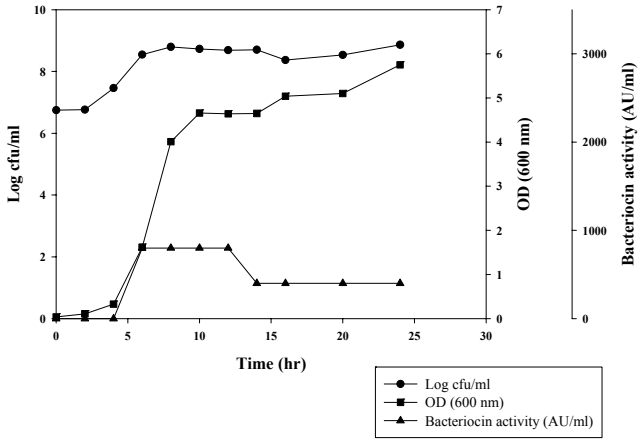


Figure 5 Profile of growth of K4 and bacteriocin production at various temperatures

A) Profile of growth of K7 and bacteriocin production at 30°C



B) Profile of growth of K7 and bacteriocin production at 37°C



C) Profile of growth of K7 and bacteriocin production at 42°C

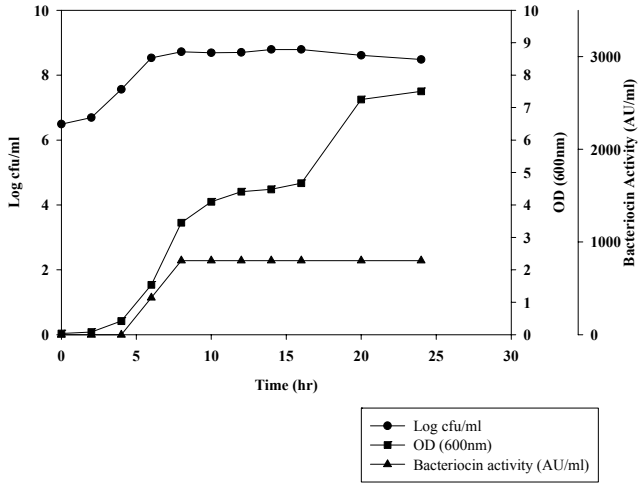


Figure 6 Profile of growth of K7 and bacteriocin production at various temperatures

2.4 Effect of NaCl and pH on growth and bacteriocin production of K4 and K7

On the previous studies, the optimum temperature for bacteriocin production of K4 and K7 was 30°C. Therefore, K4 and K7 were grown at 30°C for 16 and 12 hr, respectively. The results were shown that K4 and K7 produced bacteriocins in wide range pH of 3-10 and up to 4 % NaCl in MRS broth. The highest growth rate of K4 was observed at pH7 and in 1% concentration of NaCl. In addition, the high production of bacteriocin was found at pH 5.5-7 against the indicator strains, *Lb. sakei* subsp. *sakei* JCM 1157^T and *B. coagulans*. On the other hand, at the concentration of 1-2% NaCl in MRS, K4 was also showed high production of bacteriocins (Table 18 and 19).

The highest growth rate and bacteriocin production of K7 were observed at pH 5.5-10 and 1% NaCl when *Lb. sakei* subsp. *sakei* JCM 1157^T was used as indictor strain. However, bacteriocin activity produced by K7 decreased when 2% NaCl was added in MRS broth and was destroyed at concentration of NaCl 3-4 % (Table 20 and 21). In addition, it was found that the growth and bacteriocins activities of both isolates were diminished when it was cultivated in MRS containing more than 3% NaCl.

Table 18 Effect of pH on growth and bacteriocin production of K4

Treatment	OD (600nm)	pH of culture	Antibacterial activity (AU/ml)	
			Lb. sakei	*B. coagulans*
pH 3	0.27	3.1	400	0
pH 4	1.45	4	800	100
pH 5	2.68	4.5	3200	800
pH 5.5	3.90	4.5	6400	800
pH 6	3.80	4.5	6400	800
pH 7	4.87	4.5	6400	800
pH 8	4.65	4.5	3200	400
pH 9	4.41	4.8	3200	400
pH 10	4.32	4.9	3200	400

Table 19 Effect of NaCl on growth and bacteriocin production of K4

Treatment	OD (600nm)	pH of culture	Antibacterial activity (AU/ml)	
			Lb. sakei	*B. coagulans*
NaCl 1%	4.38	4.3	1600	400
NaCl 2%	2.96	4.5	1600	400
NaCl 3%	2.20	4.5	800	400
NaCl 4%	0.66	5.3	400	200

Table 20 Effect of pH on growth and bacteriocin production of K7

Treatment	OD (600nm)	Initial pH of culture	Antibacterial activity (AU/ml)	
			Lb. sakei	*B. coagulans*
pH 3	0.64	3.0	400	0
pH 4	1.65	3.8	400	100
pH 5	5.27	4.3	800	400
pH 5.5	5.66	4.4	1600	400
pH 6	5.42	4.5	1600	400
pH 7	5.62	4.5	1600	400
pH 8	5.95	4.5	1600	400
pH 9	5.65	4.5	1600	400
pH 10	5.45	4.5	1600	400

Table 21 Effect of NaCl on growth and bacteriocin production of K7

Treatment	OD (600nm)	Initial pH of culture	Antibacterial activity (AU/ml)	
			Lb. sakei	*B. coagulans*
NaCl 1%	5.39	4.3	1600	400
NaCl 2%	3.67	4.5	800	100
NaCl 3%	0.86	5	400	0
NaCl 4%	0.50	5.3	400	0

3. Identification of bacteriocin-producing K4 and K7

As shown in Table 22, K4 and K7 were determined to be short rod, Gram-positive, catalase negative and did not produce gas from glucose. They grew at temperature ranging from 30°C to 45°C and at pH 4.5 and 9.6. However, both isolates did not grow in MRS broth supplemented with 6.5% NaCl. Based on comparison of their characteristics with Bergey's manual (Kandler and Weiss, 1986), K4 and K7 could be identified as genus *Lactobacillus*. For species determination, biochemical

characterization was used. Carbohydrate fermentation patterns indicated that K4 was able to ferment Galactose, D-glucose, D-Fructose, D-mannose, Mannitol, Sorbitol, N-Acetyl-glucosamine, Arbutine, Esculine, Salicine, Maltose, Lactose, Melibiose, Saccharose, Trehalose, D-Raffinose and D-Arabitol while K7 was able to ferment Galactose, D-Glucose, D-Fructose, D-Mannose, Mannitol, Sorbitol, N-Acetyl-glucosamine, Esculine, Lactose, Melibiose, Saccharose and D-Raffinose (Table 23). By using API database correlation version 5.0, both isolates were identified as *Lb. salivarius* with 99% identities.

Table 22 Characteristics of K4 and K7

Test		Results	
		K4	K7
Gram's stain		Positive	Positive
Morphology		Short rod	Short rod
Catalase		Negative	Negative
Gas from glucose		-	-
Growt at 5°C-15°C		-	-
	30°C	+	+
	45°C	+	+
	50°C	-	-
NaCl	1 %-4 %	+	+
	6.5%	-	-
	18%	-	-
pH 4.5		+	+
pH 9.6		+	+

Table 23 Carbohydrate fermentation patterns of K4 and K7

Test	Isolates			
	K4		K7	
	24 hr	48hr	24hr	48hr
1. Glycerol	-	-	-	-
2. Erythriol	-	-	-	-
3. D-Arabinose	-	-	-	-
4. L-Arabinose	-	-	-	-
5. Ribose	-	-	-	-
6. D-Xylose	-	-	-	-
7. L-Xylose	-	-	-	-
8. Adonitol	-	-	-	-
9. β-Methyl-xyloside	-	-	-	-
10. Galactose	+	+	+	+
11. D-Glucose	+	+	+	+
12. D-Fructose	+	+	+	+
13. D-Mannose	+	+	+	+
14. L-Sorbose	-	-	-	-
15. Rhamnose	-	-	-	-
16. Dulcitol	-	-	-	-
17. Inositol	-	-	-	-
18. Mannitol	+	+	+	+
19. Sorbitol	+	+	+	+
20. α-Methyl-D-mannoside	-	-	-	-
21. α-Methyl-D-glucoside	-	-	-	-
22. N-Acetyl-glucosamine	+	+	+	+
23. Amygdaline	-	-	-	-
24. Arbutine	+	+	-	-
25. Esculine	+	+	+	+
26. Salicine	+	+	-	-

Table 23 (Continued)

Test	Isolates			
	K4		K7	
	24 hr	48 hr	24 hr	48 hr
27. Cellobiose	-	-	-	-
28. Maltose	+	+	-	-
29. Lactose	+	+	+	+
30. Melibiose	+	+	+	+
31. Saccharose	+	+	+	+
32. Trehalose	+	+	-	-
33. Inuline	-	-	-	-
34. Melezitose	-	-	-	-
35. D-Raffinose	+	+	+	+
36. Amidon	-	-	-	-
37. Glycogene	-	-	-	-
38. Xylitol	-	-	-	-
39. β-Gentiobiose	-	-	-	-
40. D-Turanose	-	-	-	-
41. D-Lyxose	-	-	-	-
42. D-Tagatose	-	-	-	-
43. D- Fucose	-	-	-	-
44. L- Fucose	-	-	-	-
45. D-Arabitol	+	?	-	-
46. L-Arabitol	-	-	-	-
47. Gluconate	-	-	-	-
48. 2 ceto-gluconate	-	-	-	-
49. 5 ceto-gluconate	-	-	-	-

+, acid production (positive); -, no acid produced, ?, weakly positive

To confirm the conventional identification results, 16S rRNA gene investigation was used in this study. The PCR product of the 16S rRNA gene (about 1500 bp) was amplified and was cloned to pGem-T vector and sequenced on both strands. Comparison of the sequence with the database in GenBank (http://www.ncbi.nlm.nih.gov) by BLAST program, the alignment of 16S rRNA gene sequence of K4 (Figure 7) and K7 (Figure 8) indicated that K4 and K7 were identical to *Lb. salivarius* (accession No. AF 420311.1) for 99% and (accession No.AY 389803.1) for 98%, respectively. As a result, K4 and K7 were designated to be *Lb. salivarius* K4 and *Lb. salivarius* K7, respectively.

```
Query  3     AGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGA  62
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Query  63    AACTTTCTTACACCGAATGCTTGCATTCACCGTAAGAAGTTGAGTGGCGGACGGGTGAGT  122
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Query  123   AACACGTGGGCAACCTGCCTAAAAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCG  182
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Sbjct  121   AACACGTGGGTAACCTGCCTAAAAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCG  180

Query  183   TATATCTCTAAGGATCGCATGATCCTTGGATGAAAGATGGTTCTGCTATCGCTTTTAGAT  242
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Query  243   GGACCCGCGGCGTATTAACTAGTTGGTGGGGTAACGGCCTACCAAGGTGATGATACGTAG  302
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Sbjct  601   CTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGTAGTGCATTGGAAACTGGAAG  660

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Query  782   GCGTGGGTAGCCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCT  841
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Sbjct  899   GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA  958
```

Figure 7 Alignment of the 16S rRNA gene sequence of K4 (99% similarity with *Lb. salivarius*, accession No. AF 420311)

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Query  962   GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACC  1021
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Query  1022  ACCTGAGAGATTAGGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGCTGTCGTC  1081
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Query  1082  AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTCAGTT  1141
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Sbjct  1079  AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTCAGTT  1138

Query  1142  GCCAGCATTAAGTTGGGCACTCTGGCGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGG  1201
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Query  1262  ACAACGAGTCGCGAGACCGCGAGGTTTAGCTAATCTCTTAAAGCCGTTCTCAGTTCGGAT  1321
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Query  1322  TGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGAATCAGCATGTC  1381
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Sbjct  1379  GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAC  1438

Query  1442  ACCCAAAGCCGGTGGGGTAACCGTAAGGAGCCAGCCGTCTAAGGTGGGACAGATGATTGG  1501
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Query  1502  GGTGAAGTCGTAACAAG  1518
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Sbjct  1499  GGTGAAGTCGTAACAAG  1515
```

Figure 7 (Continued)

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Query  1     AGAGTTTGATTATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATACAAGTCGAAC  60
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Sbjct  1     AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAAC  60

Query  61    GAAACTTTCTTACACCGAATGCTTGCATTCACCGTAAGAAGTTGAGTGGCGGACGGGTGA  120
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Query  121   GTAACACGTGGGTAACCTGCCTAAAAGAAGGGGATAACACTTGGAAACAGGTGCTAATAC  180
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Query  181   CGTATATCTCTAAGGATCGCATGATCCTTAGATGAAAGATGGTTCTGCTATCGCTTTTAG  240
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Query  241   ATGGACCCGCGGCGTATTAACTAGTTGGTGGGGTAACGGCCTACCAAGGTGATGATACGT  300
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Query  361   GAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGA  420
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Sbjct  481   CTGTTCATTCGATGACGGTATCTAACCAGCAAGTCACGGCTAACTACGTGCCAGCAGCCG  540

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Query  899   GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA  958
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Sbjct  901   GGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA  960
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Figure 8 Alignment of the 16S rRNA gene sequence of K7 (98% similarity with *Lb. salivarius*, accession No. AY 389803)

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Query  959   GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGATA  1018
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Query  1019  ACCTAAGAGATTAGGCTTTCCCTTCGGGGACAAAGTGACAGGTGGTGCATGGCTGTCGTC  1078
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Query  1079  AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTGTCGGTT  1138
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Query  1499  GGTGAAGTCGTAACAAGGTA  1518
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Sbjct  1501  GGTGAAGTCGTAACAAGGTA  1520
```

Figure 8 (Continued)

4. Purification of bacteriocins produced by *Lb. salivarius* K4

Bacteriocin production of K4 and K7 indicated that K4 produced higher yield of bacteriocins than K7 did. In addition, K4 showed broader spectrum of antibacterial activities than K7 did. Therefore, for the further purification of bacteriocins, only *Lb. salivarius* K4 was selected.

Concentration of bacteriocins of *Lb. salivarius* K4: The results of bioassay (concluded in Table 24) found that all fractions showed antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^{T}. In addition, bacteriocin activity is contained mainly in the 70% isopropanol eluate. Similarly, in this study the 70% isopropanol

eluate showed the highest antibacterial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T. Therefore, this fraction was subjected to next step, cation-exchange chromatography. The antibacterial activities of the eluates are presented in Table 24.

Table 24 Antibacterial activities of the amberlite eluated of *Lb. salivarius* K4

Description of the sample	Antibacterial activity (AU/ml)*
CFS	3200
Filtrate	800
CFS after washed by distilled water	100
40% EtOH	200
70% isopropanol	12800

* *Lb. sakei* subsp. *sakei* JCM 1157^T used as an indicator strain.

Cation exchange chromatography of the bioactive eluates: As seen in Table 24, the isopropanol eluate that showed highest antibacterial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T was selected for cation exchange chromatography on SP Sepharose. The results found that four fractions were obtained and all four fractions were evaluated for their antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^T and the results are shown in Table 25. The highest bacteriocin activity was eluted by 0.25 M NaCl (Figure 9) and hence further purified by reverse-phase HPLC.

Table 25 Antibacterial activities of SP-Sepharose fractions of *Lb. salivarius* K4

Fraction no.	NaCl concentration	Antibacterial activity (AU/ml)*
1	0.25 M NaCl	3200
2	0.50 M NaCl	800
3	0.75 M NaCl	400
4	1 M NaCl	400

* *Lb. sakei* subsp. *sakei* JCM 1157^T used as an indicator strain.

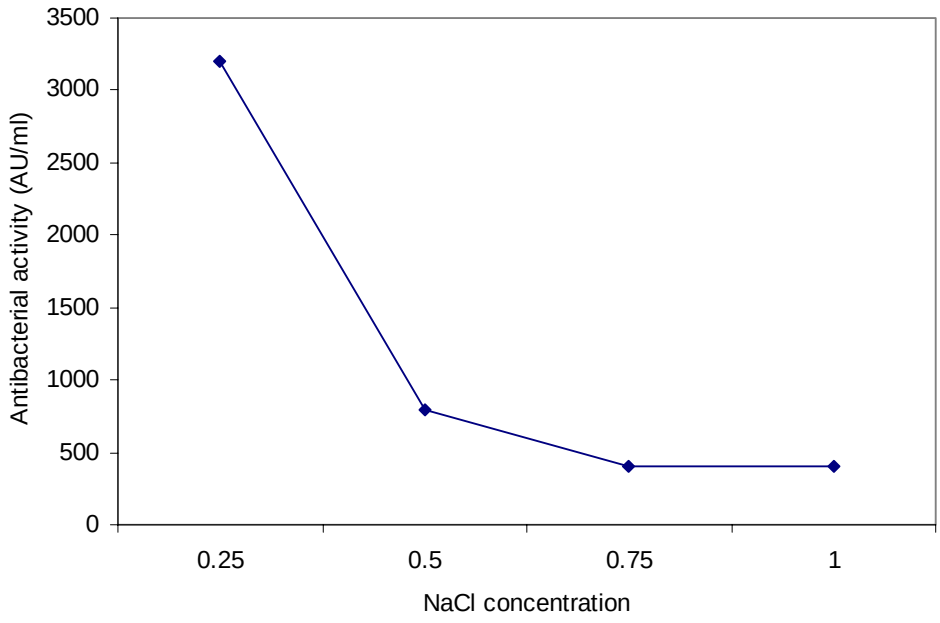

Figure 9 Antibacterial activities of SP-Sepharose in stepwise of NaCl; AU/ml

Reverse-phase HPLC-1: As seen in Table 25, fraction no. 1 was further purified by reverse-phase HPLC. Thirty fractions were obtained from HPLC-1. Bioassay of these fractions against *Lb. sakei* subsp. *sakei* JCM 1157^T indicated that fractions at 18-27 min displayed inhibition zone. These fractions were futher to evaluated molecular weight (MW) by MALDI-TOF MS. HPLC-1 chromatogram is shown in Figure 10.

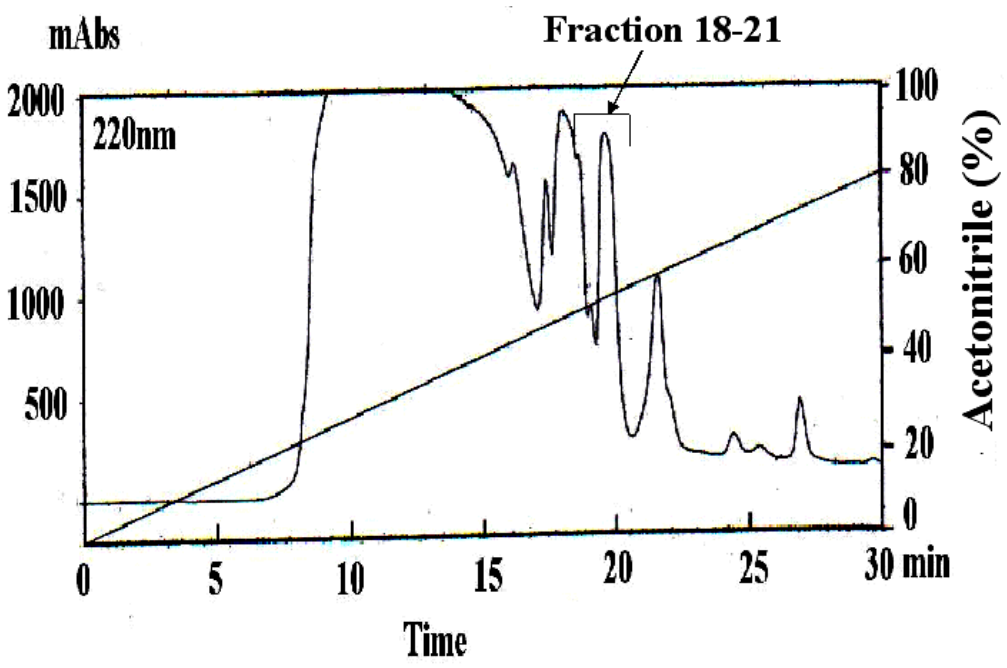

Figure 10 HPLC-1 chromatogram of the purification of fraction from SP-Sepharose

The molecular weight (MW) of bioactive fractions from Figure 10 was determined by MALDI-TOF MS. It was found that fractions at 18, 19, 20 and 21 min showed two peaks, one major and a minor peak with less than 10%. The major peaks of fraction at 18, 19, 20 and 21 min were 4432.63, 4426.9, 4337.78 and 4339.03, respectively (Figure 11, 12, 15 and 16). Therefore, fractions at 18 and 19 min were combined to get higher concentration. Similarly, fractions at 20 and 21 min were combined and then subjected to further rechromatography study.

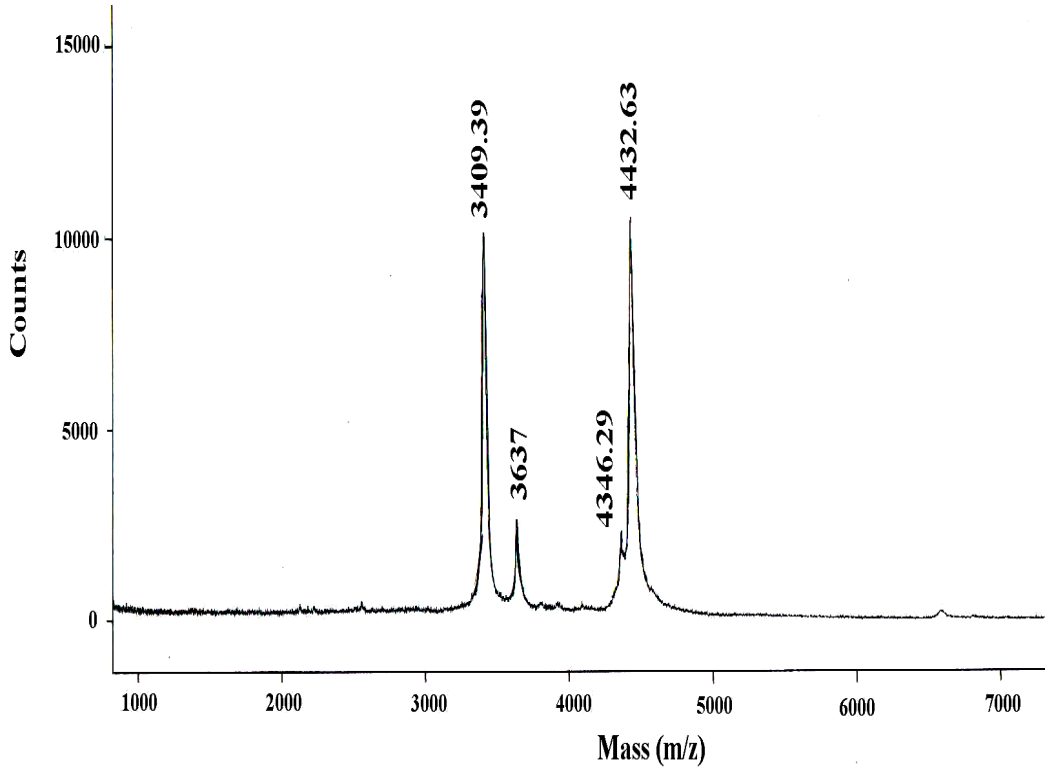

Figure 11 Mass spectrums (MALDI-TOF MS) of HPLC-1-fraction at 18 min

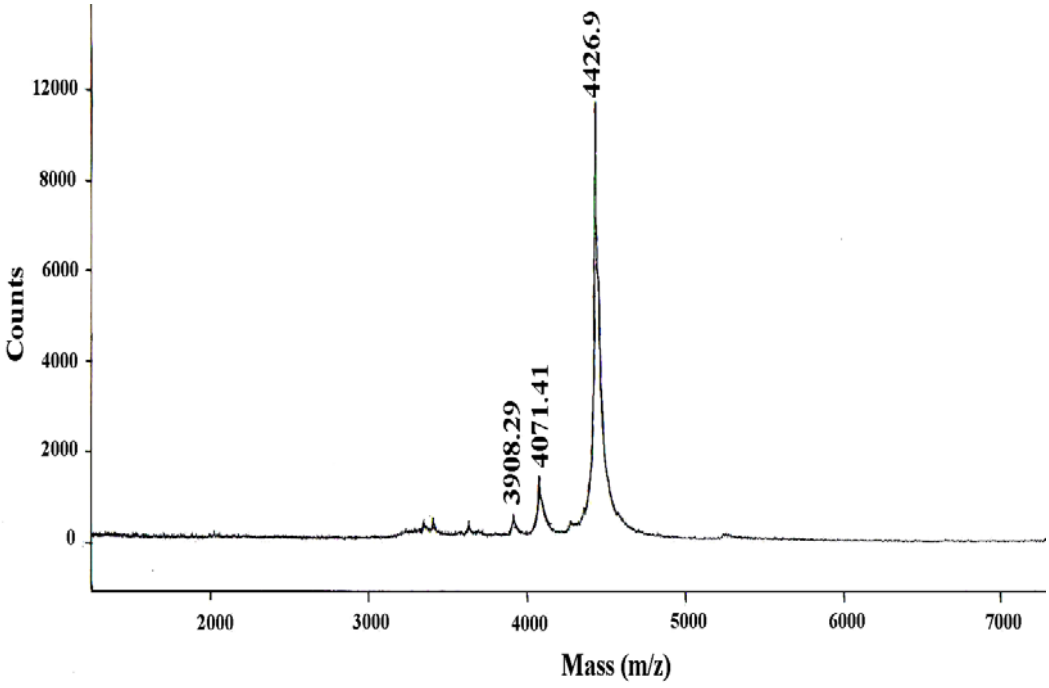

Figure 12 Mass spectrums (MALDI-TOF MS) of HPLC-1-fraction at 19 min

Reverse-phase HPLC-2 of fractions at 18 and 19 min: The bioactive fractions at 18 and 19 min from HPLC-1 were rechromatographed on the same column using gradient 75%A/25%B to 35%A/65%B. The bioactive fractions were determined by spot-on-lawn method and confirmed the single peak of MW by ESI-MS. The result found that three bioactive fractions, at 11, 12, and 13 min, showed inhibition zone againt *Lb. sakei* subsp. *sakei* JCM 1157^{T}. However, fraction at 12 min gave single peak of MW. Therefore, this purified fraction of HPLC-2 was designated FK 12 as shown in Figure 13.

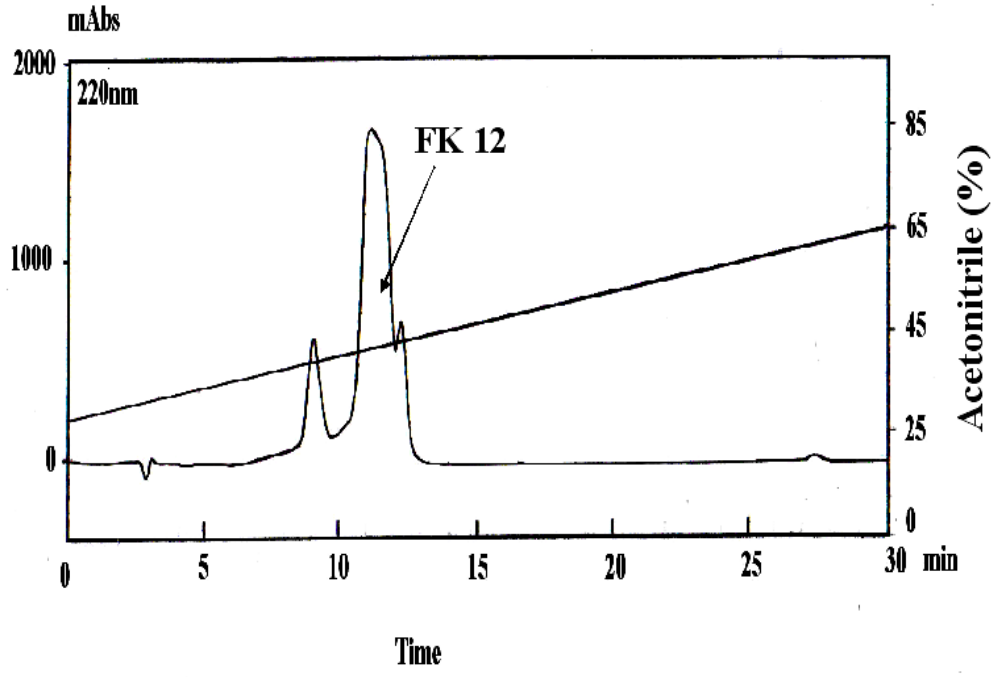

Figure 13 Chromatogram at 220 from RP-HPLC-2 of bacteriocin FK12

Molcular weight of bacteriocins FK12: The MW of purified bacteriocin FK12 was analyzed by ESI-MS and corresponded to 4436.46 Da (Figure 14).

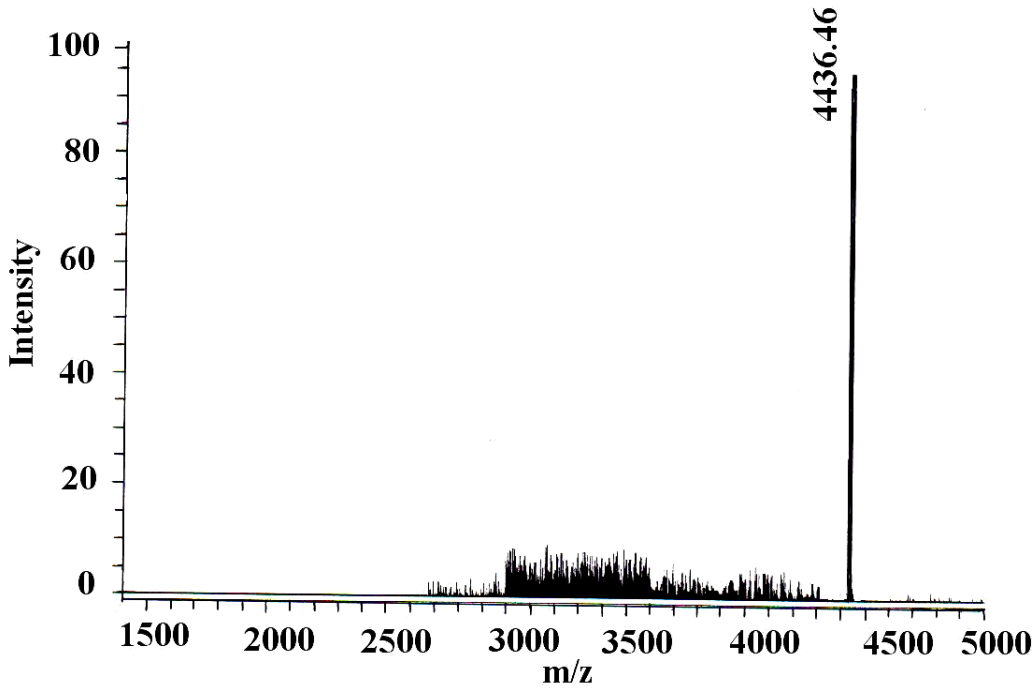

Figure 14 Molecular weight of purified bacteriocins fraction FK 12

Similarly, MW of the major peak of fractions at 20 and 21 min were 4337.78 and 4339.03 Da. Fractions at 20 and 21 min were combined to get higher concentration, and then subjected to further rechromatography experiment. MW of HPLC-1 fractions at 20 and 21 min are shown in Figure 15 and 16.

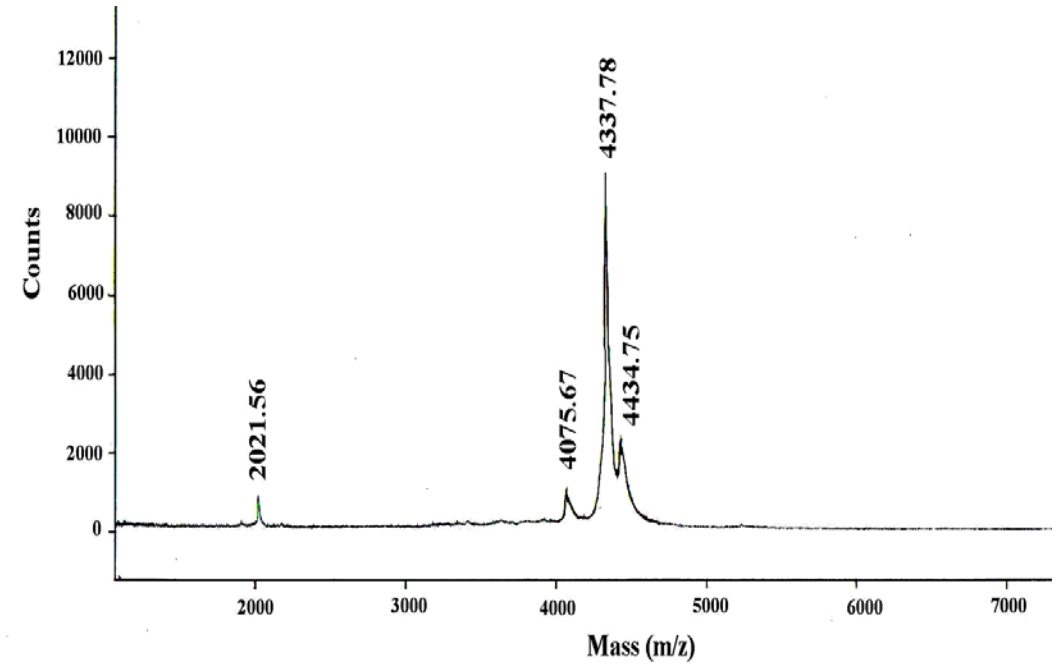

Figure 15 Mass spectrums (MALDI-TOF MS) of HPLC-1-fraction at 20 min

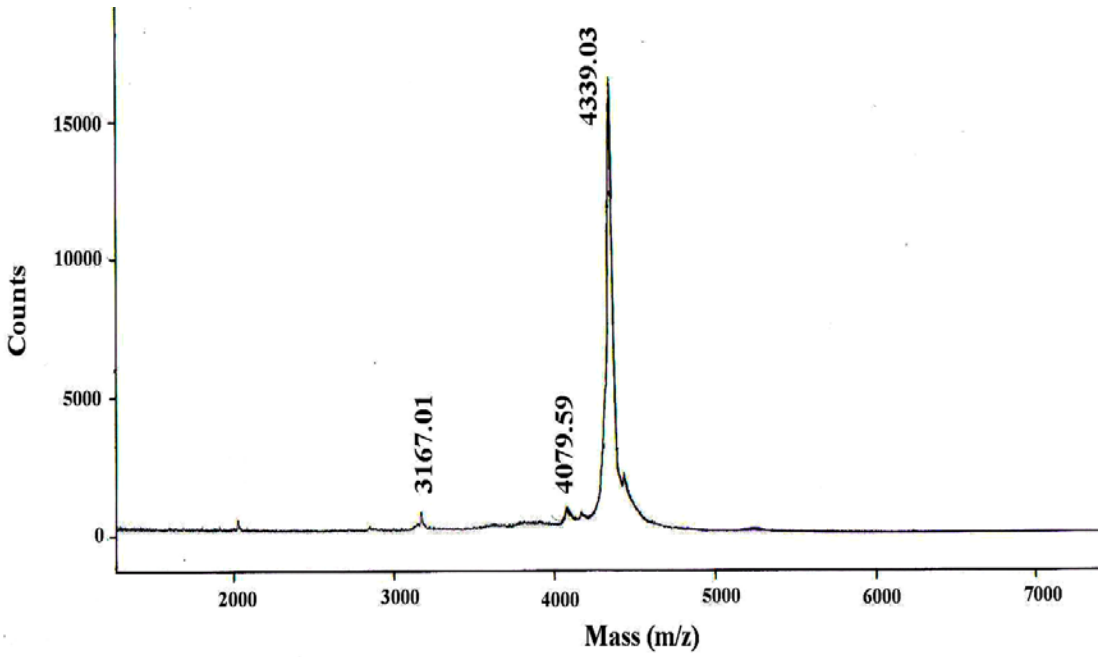

Figure 16 Mass spectrums (MALDI-TOF MS) of HPLC-1-fraction at 21 min

Reverse-phase HPLC-2 of fractions at 20 and 21 min: The bioactive fractions at 20 and 21 min from HPLC-1 were rechromatographed on the same column using gradient 75%A/25%B to 35%A/65%B, which is referred as HPLC-2. The fractions of HPLC-2 were determined antibacterial activities against *Lb. sakei* subsp. *sakei* JCM 1157^T by spot-on-lawn method and confirmed the single peak of MW by ESI-MS. It was found that six fractions, at 11-16 min, displayed inhibition zone and the fraction at 15 min exhibited single peak of MW. Therefore, this fraction was designated FK 15 (Figure 17).

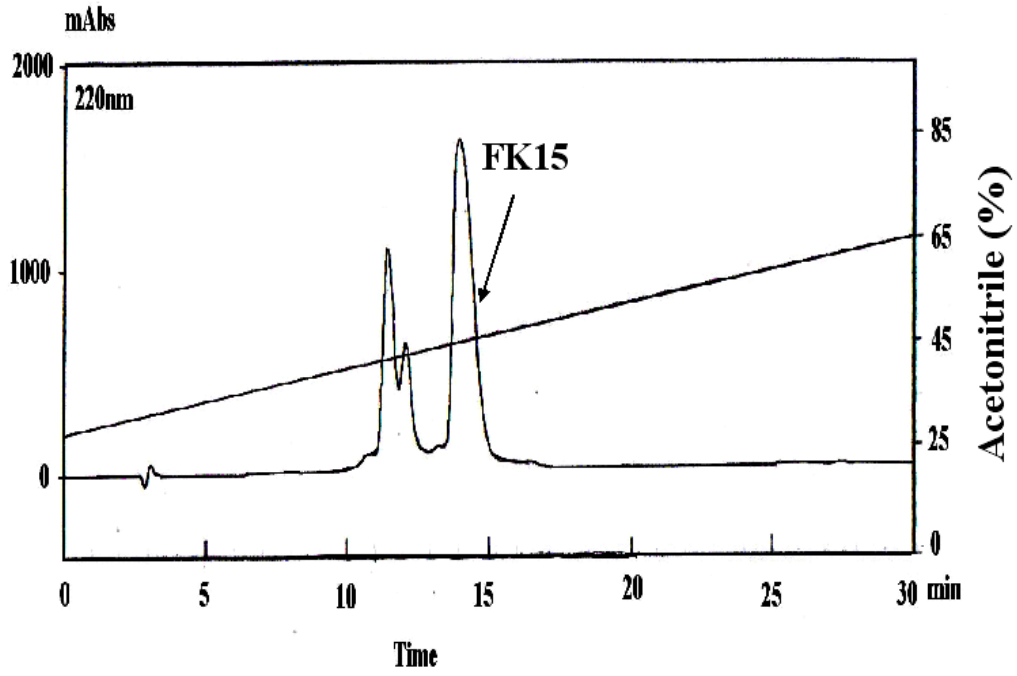

Figure 17 Chromatogram at 220 from RPHPLC-2 of bacteriocin FK15

Molcular weight of bacteriocins FK15: The MW of purified bacteriocin FK15 was analyzed by ESI-MS and corresponded to 4347.32 Da, (Figure 18).

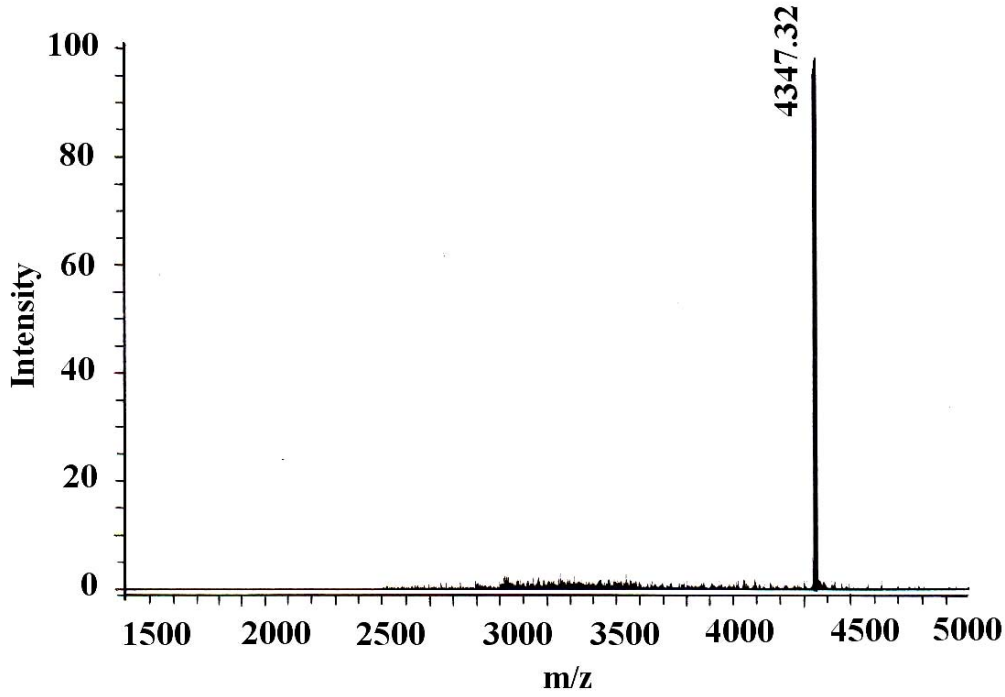

Figure 18 Molecular weight of purified bacteriocin fraction FK15

The efficiency of the purification steps is given in Table 26. The yield recovery Amberlite was about 40 %. At the cation exchange step, the yield recovery was about 2% and specific activity increased approximately 2 times and the purification reached to 2.30 folds. The final purification process obtained two active fractions, FK12 and FK15. The purification of FK12 and FK15 increased of 3.29 and 3.07 folds respectively, but the yield of FK 12 and FK15 recovered only 0.025%.

Table 26 Purification of bacteriocin produced by *Lb. salivarius* K4

sample	Vol. (ml)	protein conc (mg/ml)	activity (AU/ml)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purifi-cation (Fold)
CFS	1000	0.184	3200	3200000	17391.30	100	1
Hydrophobic interaction	100	0.322	12800	1280000	39751.55	40	2.29
Ion-exchange (SP-Sepharose) Fraction FK12	20	0.08	3200	64000	40000.00	2	2.30
RP-HPLC Fraction FK15	1	0.014	800	800	57142.86	0.025	3.29
RP-HPLC	1	0.015	800	800	53333.33	0.025	3.07

5. N-terminal amino acid sequences of bacteriocins FK12 and FK15

Partial N-terminal amino acid sequences of FK 12 and FK 15 were determined by Edman degradation. Thirty-six N-terminal amino acid residues of FK 12 revealed RNSYDYIDSGQFGYDIGNTIANTKFFKRLRHSNQDI, whereas FK15 showed 20 amino acid residues: KRYPNAHGKFLGGLAVGWAC (Figure 19).

These amino acid sequences were analyzed by BLAST program in GenBank. The results showed that partial amino acid sequence of FK12 was 94% homologous with presalivaricin B, which produced by *Lb. salivarius* M7 at accession No. CAB 63109. In addition, partial amino acid sequence of FK 12 was 91% homology to bacteriocin-like prepeptide (ORF3) which produced by *Lb. salivarius* UCC118 at accession No. AAM 61775. FK 15 did not show any significant similarity by BLAST analysis. However, there have been only two reports that *Lb. salivarius* produced bacteriocins. One of them, *Lb.salivarius* UCC118 produced two-peptide bacteriocin, abp 118 α and β. Therefore, bacteriocin FK15 was compared by visual to two-peptide

bactericin, abp 118 α and β. It was found that 75% (manual calculation) of amino acid sequence of bacteriocin FK15 was similar to bacteriocin abp 118 α.

A) Fraction FK12

```
          1                   5                  10                  15
N         Arg-Asn-Ser-Tyr-Asp-Tyr-Ile-Asp-Ser-Gly-Gly-Phe-Gly-Tyr-Asp

                       20                  25                  30
          Ile-Gly-Asn-Thr-Ile-Ala-Asn-Thr-Lys-Phe-Phe-Lys-Arg-Leu-Arg

                       35
          His-Ser-Asn-Gln-Asp-Ile   C
```

B) Fraction FK 15

```
          1                   5                  10
N         Lys-Arg-Tyr-Pro-Asn-Ala-His-Gly-Lys-Phe

                       15                  20
          Leu-Gly-Gly-Lue-Ala-Val-Gly-Trp-Ala-Cys   C
```

Figure 19 Partial N-terminal amino acid sequences of bacteriocins FK12 and FK 15

6. Structural gene analysis of FK 12 and FK 15

To get a complete sequence of the bacteriocins, the structural genes were amplified by PCR. As the previous results revealed that bacteriocin FK 12 and FK 15 were similar to ORF3 and abp 118 α. Therefore, specific primers were designed based on nucleotide sequence of ORF 3 and bacteriocin abp 118 α and β. The genomic DNA from *Lb. salivarius* K4 was amplified by PCR using the designed primers K4 forward and K4 reverse (Table 13). Amplification product of 1.1 kb was obtained and the nucleotide sequences were determined.

The deduced amino acid sequences of bacetriocin FK12 and FK15 revealed 38 and 47 N-terminal amino acid residues, respectively; as the picture shown in Figure 20. In addition, both bacteriocins contained 19 amino acid sequence of leader peptide. According to database searched by BLAST program (GenBank), the structural gene

for bacteriocin FK12 was 96% homology to bacteriocin presalivaricin B at accession No. CAB 63019 and 94% homology to bateriocin-like prepeptide (ORF3) at accession No. AAM 61775. However, only one N-terminal amino acid of FK12 at position 35 was different, bacteriocin FK12 was aspartate whereas presalivaricin B and bacteriocin-like prepeptide (ORF3) were asparagines. In addition, leader peptide amino acid sequence of FK12 at position 6 (from N-terminal) was valine, whereas presalivaricin B and bacteriocin-like peptide were Isoleucine (Figure 21, a and b). The structural gene for bacteriocin in FK15 showed no homology to any bacteriocin sequence by BLAST analysis (NCBI). However, it was found that bacteriocin FK15 was similar to bactericin abp 118 α of 64% identity when analyzed by WU-BLAST2 (EMBL-EBI). Therefore, it is concluded that FK15 is a novel bacteriocin and hence named Salvicin K. Molecular weight of deduced peptides of FK12 and FK15 corresponded to 4434.84 Da and 4346.10 Da, respectively. These molecular weights are highly similar to the molecular weights, which were determined by ESI-MS (4436.46 and 4347.32 Da, respectively).

K4 forward primer

```
1   AGAGCAGATGAAGTGAATAATTATAAGTGTAAATTATAGCTATAATAAATTTTTAAAATT   61

FK12

61  ATGAGGTGTTGAAGTTATGAATAATAATTTTGTACAAGTTGATAAGAAAGAATTGGCACA  121
                        M   N   N   N   F   V   Q   V   D   K   K   E   L   A   H
121 TATAATTGGTGGAAGAAATTCTTATGATTATATAGATAGCGGACAGTTTGGTTACGATAT  181
    I   I   G   G   R   N   S   Y   D   Y   I   D   S   G   Q   F   G   Y   D   I
181 AGGATGTACAATTGCTAATACTAAATTTTTCAAAAGATTAAGACATTCAAATCAGGATAT  241
    G   C   T   I   A   N   T   K   F   F   K   R   L   R   H   S   N   Q   D   I

241 TTGTAGCTAAACTGTTTAGTAATCTTAACATAACCAATTAAGCACAAAATAATGCGTCTT  301
      C   S   *
301 ACGTTAAAATAGTGTTTGAAGCTGAAATGATAATACAACAAAAGGGTAAGGTATGTTTTA  361
361 TAGGTGTTTTAGAAAGGATGTGTTTTATATGCTAAAAAAGTTATGGAATATTATTTGGTT  421
421 AGATGGCGGATTTCTTAAAGGAAATAAGAGATATGTAATCATTCCAATTATCTTTGCAGT  481
481 AGTTCTTCCTCTATCAATGTGGCTATCCGACAACGAAGGAATGTCTTATTTAGAGTATAT  541
541 CCAAGAGCCTAAATGGATAATGTTTGCTGTTTTTTGTTTGGTAAGTGGCAGTACACTACT  601

601 TTATCTATTGGATACTGCAATAAAAGTAAATAAACATATGAAAGGATGATTATCATGAAT  661

    FK15                                                        M   N
661 AAAGAATTTACAGTATTAACAGAAATGGAATTAGTTAAGGTAGACGGTGGGAAACGTTAT  721
    K   E   F   T   V   L   T   E   M   E   L   V   K   V   D   G   G   K   R   Y
721 CCTAATTGTACAGGAAAATTTTTAGGTGGATTAGCAAAAGGAGCAGCATTAGGTGCTATT  781
    P   N   C   T   G   K   F   L   G   G   L   A   K   G   A   A   L   G   A   I
781 TCTGGTGGCGGAGTTCCTGGAGCAGTTATAGGTGGAAATATCGGTATGGTGGCCGGAGCA  841
    S   G   G   G   V   P   G   A   V   I   G   G   N   I   G   M   V   A   G   A
841 ATTTCTTGTTTATAGGAGTGATTAATATGAAAAATTTAGATAAGAGATACACAATTATGC  901
    I   S   C   L   *
901 CAGAAGAAAATCTATCCATAATTGTTGGTGGTAAAAATGGCTATGGTGGAAGTGGAATTC  961
961 GTTGGGTTCACTGTGGAGCAGGTATTGTAAGTGGAGCTTTGATGGGATCTATTGGTGGAA 1021
1021 ACGCATGGGGAGCCGTAGCAGGTGGCATTTCTGGTGGTATAAAAAGTTGTCGTTAAGATG 1081
1081 AGCCTAATGAATTAGAAAATAAGAAATAAATCTATTTTTCTCTTACACTTGACACTACTTGA 1141
                                                    K4 reverse primer
```

Figure 20 Nucleotide sequence of the structural genes of FK12 and FK15

a) FK12

FK12 MNNNF**V**QVDKKELAHIIGG RNSYDYIDSG QFGYDIGCTI ANT**K**FFKRLR HSNQ**D**ICS
PresalivalicinB MNNNF**I**QVDKKELAHIIGG RNSYDYIDSG QFGYDIGCTI ANT**K**FFKRLR HSNQ**N**ICS
Bacteriocin-like peptide MNNNF**I**QVDK KELAHIIGG RNSYDYIDSG QFGYDIGCTI ANT**I**FFKRLR HSNQ**N**ICS

b) FK15

FK15 M**N**KEFTVLTE**M**EL**V**KVDGG KR**Y**PNC**T**GKFLGGL**A**KGAALG**AI**SG**GGVPGAVIGGNIGMV**A**GAISCL**
Abp118 α M**M**KEFTVLTE**C**EL**A**KVDGG KR**G**PNC**V**GNFLGGL**F**AGAAAG**VP**LG**PAGIVGGANLGMVGG**A**LTCL**
Abp118 β MKNLDKRFTIMTEDNLASVNGG KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR

Figure 21 Alignment of amino acid sequences deduced from DNA sequences compare to amino acid sequences in GenBank database (Underline sequence showed the different)

8. Antibacterial spectrum of bacteriocin Salvicin K

It revealed that purified bacteriocin Salvicin K could inhibite several Gram-positive bacteria including *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Enterococcus faecalis* JCM 5803^T, *Bacillus coagulans* JCM 2257^T, *Listeria innocua* ATCC 33090^T and *Brochotrix campestris* NBRC 11547 ^{T}. It did not inhibit *Staphylococcus aureus* and *E.coli* JM109 and other gram negative bacteria listed in Table 27. The highest bacteriocin activity, 3200 AU/ ml, was displayed against *Lb. sakei* subsp. *sakei* JCM 1157^T.

Table 27 Bacteriocin activity of Salvicin K against indicator strains

Indicator strains	Bacteriocin activity (AU/ml)
Lactic acid bacteria	
Lactococcus lactis subsp. *lactis* ATCC 19435^T	-
Lactococcus lactis subsp. *cremoris* TUA 1344L	-
Lactobacillus plantarum ATCC 14917^T	-
Pediococcus pentosaceus JCM 5885	-
Lactobacillus sakei subsp. *sakei* JCM 1157^T	3200
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	
Enterococcus faecalis JCM 5803^T	100
Other gram positive bacteria	
Bacillus subtilis JCM 1465^T	-
Bacillus circulans JCM 2504^T	-
Bacillus coagulans JCM 2257^T	200
Bacillus cereus JCM 2152^T	-
Listeria innocua ATCC 33090^T	200
Micrococcus luteus IFO 12708	-
Brochotrix campestris NBRC 11547 ^{T}	200
Gram negative bacteria	
Escherichia coli JM109	-

9. Effect of proteolytic enzyme, pH and heat on bacteriocin Salvicin K

Salvicin K was completely inactivated with proteinase K treatment. Bacteriocin activity of Salvicin K was stable at various pH at 30°C for 2 hr. Salvicin K was heat tolerant up to 100°C for 5 min, but decreased by 50 % extended heating at 100°C for 30 min and 121°C for 15 min, (Table 28).

Table 28 Effect of proteolytic enzyme, pH and heat on bacteriocin, Salvicin K, activity against *Lb. sakei* subsp. *sakei* JCM 1157^T

Treatment	Bacteriocins activity (AU/ml)
Proteolytic enzyme activity	
Control (untreated)	3200
Proteinase K	0
pH	
Control (no incubated , dilute pH6)	200
3	200
4	200
5	200
6	200
7	200
8	200
9	200
10	200
Temperature	
Control (untreated)	3200
100°C 5 min	3200
100°C 30 min	1600
121°C 15 min	1600

10. Mode of action of bacteriocin on *Lb. sakei* subsp. *sakei* JCM 1157^T

10.1 Mode of action of partially purified bacteriocin

The mode of action of the partially purified bacteriocin produced by *Lb. salivarius* K4 against *Lb. sakei* subsp. *sakei* JCM 1157^T was investigated. Partially purified bacteriocin from Amberlite concentration at final concentration of 100 AU/ml was added into *Lb. sakei* subsp. *sakei* JCM 1157^T. The bacteriocin showed the

bactericidal activity against *Lb. sakei* subsp. *sakei* JCM 1157^T. The optical density (OD 600 nm) and cell number by plate count method (10^7 CFU/ml) decreased less than 10 after the culture was exposed to bacteriocin for 6 hr, whereas, the control strains increased the viable cell count at the same period (Figure 22).

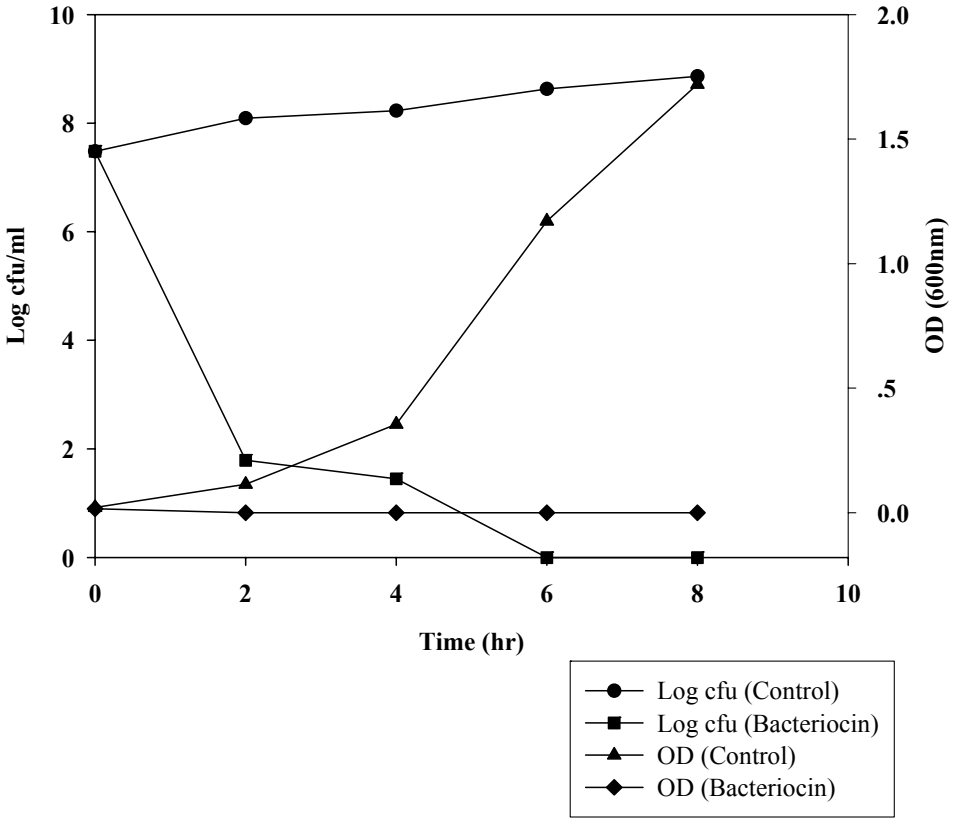

Figure 22 Mode of action of partially purified bacteriocin produced by K4

10.2 Mode of action of bacteriocin Salvicin K

When compared to those of both MRS control broth (without acetonitrile or bacteriocin) and MRS broth with acetonitrile, the purified bacteriocin with a final concentration of 100 AU/ml in MRS broth was found to inhibite *Lb. sakei* subsp. *sakei* JCM 1157^T (Figure 23). Viable cell count in the broth was completely destroyed after the culture was exposed to bacteriocin within 2 hr. The optical density (600 nm) of *Lb. sakei* subsp. *sakei* JCM 1157^T culture in the same broth trended to

reduce when further incubated up to 8 hr, while the population of *Lb. sakei* subsp. *sakei* JCM 1157^T in both control MRS broth and broth with acetonitrile increased by 3-4 log cycles (by OD) in comparison to the initial counts.

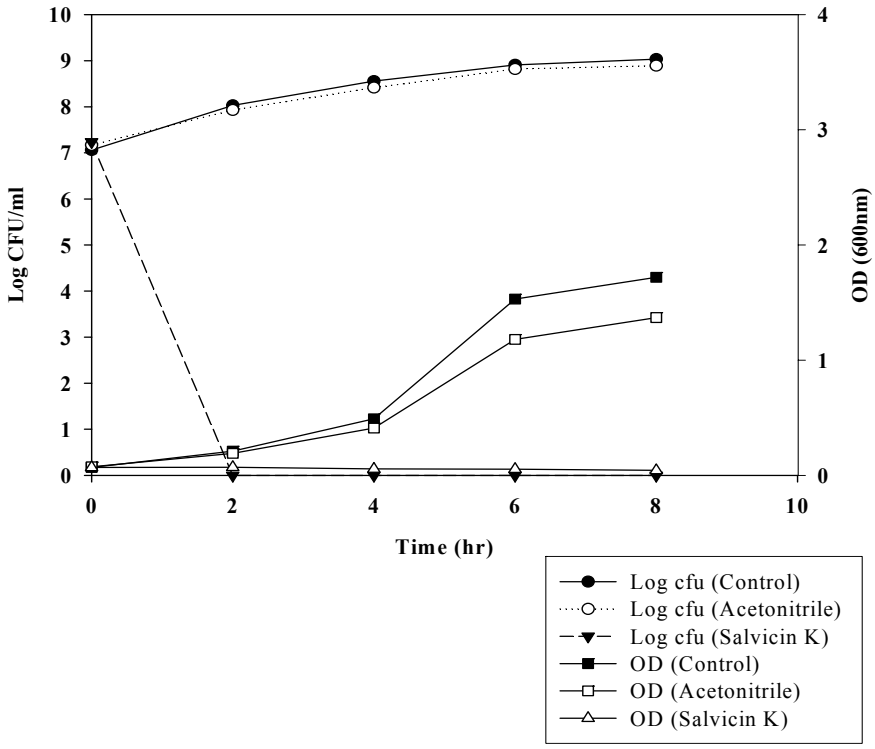

Figure 23 Mode of action of bacteriocin Salvicin K

DISCUSSION

About 300 LAB colonies were isolated from chicken intestinal content and their viable cell counts were determined to be 7.00, 7.37, 8.34 and 8.72 log cfu/g. Previous study by Smith (1965) showed that LAB count in 4 different sections of small intestine and ceaca of chicken were 8.0, 8.2, 8.2, 8.6 and 8.7 log cfu/ml. Number of LAB in duodenum and jejunum in this experiment were less than those from Smith (1965), which explained by many factors affected to a number of LAB flora such as health, age, and diet (Fuller, 1989).

Screening of these LAB for their abilities to produce bacteriocins resulted two isolates, K4 and K7, which were identified as *Lb. salivarius*. LAB are accounted for an important part of the intestinal microflora of chicken and contribute to maintain the ecological balance among the different microorganisms (Zacconi *et al*., 1999). *Lactobacillus* colonization of the chicken intestinal system takes place soon after hatching, during food ingestion. The species mainly represented were reported to be *Lactobacilli* (Sarra *et al*., 1992), important inhabitants of the intestinal tract of man and animal (Klaenhammer, 1995).

It has been reported that *Lb. salivarius* was found as normalflora in chicken intestines (Sarra *et al*., 1992). Several studies also supported that *Lb. salivarius* often found in chicken intestine. *Lb. salivarius* A23 was isolated from chicken intestines (Zacconi *et al*., 1999). Paco *et al,* (2003) isolated *Lactobacillus salivarius subsp. salicinius* species from chicken intestines.

CFS produced by K4 and K7 showed antibacterial activities against only Gram-positive bacteria but did not inhibit Gram-negative bacteria. Gram-negative bacteria are generally insensitive to bacteriocins from LAB because of their protective outer membrane, which is a constituent of the cell envelop in all Gram-negative bacteria, providing them with efficient permeability barrier against macromolecules and hydrophobic substances (Messens and De Vuyst, 2001). However, it has been reported that LAB isolated from chicken intestines produced bacteriocin-like activity

against Gram-negative bacteria such as *Salmonella sp.* and *E.coli* (Nitisinprasert *et al*., 2000).

Some bacteriocins showed inhibitory spectrum against other lactobacilli or taxonomically-related Gram-positive bacteria, whereas some, such as bulgaricin, acidolin, lactocidin or acidophilin, were active against a wide spectrum of Gram-positive or Gram-negative bacteria (Boris *et al*., 2001).

It has been reported that another strain, *Lb. salivarius* UCC118, exhibited wide spectrum of activity. Its CFS displayed antibacterial activities against both Gram-positive and Gram-negative bacteria such as *B. subtilis*, *B. cereus*, *B. thuringiensis*, *E. faecalis*, *E. faecium*, *L. monocytogenes*, *Staphylococcus aureus*, *Pseudomonas fluorescens* and only one strain Gram-negative (Dunne *et al*., 1999). In addition, bacteriocin Salivaricin B which produced by *Lb. salivarius* M7 was active against many closely related LAB strains, including *Lb. fermentum, Lb. plantarum, Lb. acidophilus* and against many more distantly related Gram-positive bacteria such as *L. monocytogenes L2, Streptococcus faecalis, Staphylococcus epidermis* and *Staphylococcus aureus* (Cataloluk and Gürakan, 2003).

LAB are able to inhibit the growth of other microorganisms by excretion of metabolite products such as organic acids, hydrogen peroxide, diacetyl and bacteriocin (Huot *et al*., 1996). However, these results were not from acidic effect as CFS was adjusted to neutralize to get rid of the acidic effect. The antibacterial activities of CFS produced by K4 and K7 were inactivated by proteolytic enzymes, indicating that it has proteinaceous structure being a bacteriocin. (Vaughan *et al*., 2001).

The bacteriocin production of K4 and K7 were found in exponential phase and the maximum of bacteriocin activity was occurred in late exponential phase. However, the bacteriocin activity decreased when cell entered at stationary phase. Since the production of bacteriocins is dependent on the growth and physiological

activity of the producing strains, the amount of bacteriocin released into the medium is correlated with the quantity of biomass produced. Almost all bacteriocins from lactic acid bacteria display primary metabolite kinetics (Zamfir *et al*., 2000). The production of bacteriocin is growth-associated because production occurs during mid-exponential phase and increase to reach a maximal level at the end of the exponential phase or the beginning of the early-stationary phase where the maximal biomass was observed. Bacteriocin production completely stopped when cell entered the stationary phase (Cheigh *et al.*, 2002). Other reports supported that the loss of bacteriocin activity may be due to degradation by endogenous protease induced during the growth phase and/or the adsorption of bacteriocin on the surface of the producer (Messens *et al*., 2003; Onda *et al*., 2003), a limited immunity of producer cells. One suggests that immunity is based either on the complex formation between the mature bacteriocins molecules and the immunity peptides or on the interaction of immunity peptides and limited cell receptor sites (Zamfir *et al*., 2000).

The results found that the maximum bacteriocin activity was found at 30°C. In the case of K4 and K7, the bacteriocin activity decreased at 37 and 42°C at stationary phase. Zamfir *et al*., (2000) reported that prolong the exponential growth phase provided enough energy source and results in higher biomass amounts and, because of its growth-associated production kinetics, higher bacteriocin titers.

Optimal pH for bacteriocin production were 5.5-7 for K4 and 5.5-10 for K7. Several studies reported that the optimal pH for bacteriocin production was usually 5.5-6, often lower than optimal for growth (Cheigh *et al.*, 2002). In addition, due to a low pH and hence accumulating lactic acid levels or an exhausted energy source, cell growth ceases or stops and so does bacteriocin production. Since immunity of the bacteriocin producer cells is based on the production of immunity peptides, the gene of which are co-transcribed with the bacteriocin structural genes. Its level will also decrease once bacteriocin production stops. Consequently, pH and lactic acid production influence both cell growth and bacteriocin production kinetics (Zamfir *et al*., 2000).

In addition, bacteriocin production decreased in the presence of increasing salt concentrations. Neysens *et al*. (2003) reported that NaCl interfered both with cell growth and bacteriocin production. Bacteriocin production was negatively affected by NaCl because the amount of biomass formed was lower, as well as the apparent rate of bacteriocin in activation. Bacterial metabolism is sensitive to salt exhibits specific ionic and water binding properties. The latter effect is of utmost importance because the addition of salt to the fermentation liquor leads to a decrease in a_w. Decreases in a_w below to the optimum values for growth result in a linear decrease of the growth rate.

Bacteriocin activity of CFS K4 was stable at wide pH range 3-10 and it was heat tolerant. The stability was similar to another bacteriocin-producing *Lb. salivarius* (Cataloluk and Gürakan, 2003). Heat stability is another major feature of low molecular-weight bacteriocins (Oscáriz and Pisabarro, 2001). These properties would be useful for food industrial processing under pasteurization condition. However, bacteriocin activities of K7 were found to be stable at pH range 4-7. However, higher activities were obtained at high pH range 8-10 and at low pH to 3. It seems that such activities were rather different from other bacteriocins exhibiting activities at low pH. It has been reported that many bacteriocins displayed greater antibacterial activities at low pH values (pH 5 and below) than at physiological pH, because a higher amount of bacteriocin molecules are available at low pH values. At lower pH values, the solubility are often increased, less aggregation of hydrophobic peptides occurs, and less bounding of bacteriocins to cell surface takes place. Also, hydrophilic bacteriocins may have an enhanced capacity to pass through hydrophilic regions of the cell surface of the sensitive target bacteria (Messens and De Vuyst, 2002).

It has been reported that the complex pattern of monosulfide and disulfide intramolecular bonds helps in the stabilization of secondary structures by reducing the number of possible unfolded structures (entropic effect). From a structural point of view, the effect of the intramolecular bonds is additive, and the higher their number, the higher the global stability of peptide. It was observed that most supernatants of bacteriocin-producing strains were resistant to autoclaving conditions and heat

treatment (100-121°C). However, some bacteriocins produced by *Lactobacillus* strains (helveticin J) were inactivated by 10-15 min treatment of 60-100°C (Oscáriz and Pisabarro, 2001).

The profile of bacteriocin production of K4 and K7 indicated that K4 produced higher yield of bacteriocins than K7 did. In addition, K4 showed broader spectrum of antibacterial activity than K7 did. Therefore, for the further purification study, only K4 was selected.

The purification processes involving Amberlite hydrophobic adsorption, followed by cation-exchange chromatography and RP-HPLC has been successful in the purification of bacteriocins from *Lb. salivarius* K4. The antibactrial activity of CFS was found to increase after hydrophobic adsorption, suggesting that bacteriocin is strongly hydrophobic, which is typical of many bacteriocins (Onda *et al*., 2003). The purification by RP-HPLC resulted in two bacteriocins, FK12 and FK15. The recoveries (% yield) of both bacteriocins were decreased particularly in cation-exchange and RP-HPLC. Many authors reported high loses of bacteriocin during purification processes (Bogovič Matijašić and Rogels, 1998).

The analysis of N-terminal amino acid sequence by Edman degradation showed that partial amino acid sequence of FK12 was homologous with presalivaricin B (Cataloluk, 2001) and bacteriocin-like prepeptide (ORF3) (Flynn *et al*., 2000) whereas FK 15 showed no homology to any bacteriocins by BLAST analysis. In addition, the deduced nucleotide complete peptide of FK12 revealed that it was synthesized as a precursor protein of 57 amino acids and had a leader peptide of 19 amino acids. Whereas, the nucleotide sequence of FK15 was synthesized as a precursor protein of 66 amino acids and had leader peptide of 19 amino acids. Both leader peptides contained double glycine (Gly-Gly) at position -1 and -2. After BLAST analysis, it was found that FK12 was homologous with presalivaricin B produced by *Lb. salivarius* M7 (Cataloluk, 2001) and also homology to bacteriocin-like peptide (ORF 3) of *Lb. salivarius* UCC 118 (Flynn *et al*., 2000). From previous reports, two strains of *Lb. salivarius* produced different bacteriocins. *Lb. salivarius*

M7 produced bacteriocin presalivaricin B and *Lb. salivarius* UCC118 produced two-peptide bacteriocin, abp118 α and β. However, gene cluster of *Lb. salivarius* UCC 118 had shown amino acid sequence of bacteriocin-like prepeptide (ORF3) similar to presalivaricin B but it was not reported as antimicrobial agent.

Deduced nucleotide complete peptide of FK15 showed no homology to any bacteriocins by BLAST analysis (NCBI) but it was 64% similar to abp118 α when used WU-BLAST2 program (EMBL-EBI). Since *Lb. salivarius* UCC 118 produced two-peptide bacteriocin abp 118 α and β, the amino acid sequence was compared with bacteriocin FK15 and indicated that there were some same sequences which were found both in bacteriocin FK15 and abp 118 α. The complete amino acid sequence of bacteriocin FK15 was MNKEFTVLTEMELVKVDGG KRYPNCTGKFLGGLAKG AALGAISGGGVPGAVIGGNIGMVAGAISCL while that of bacteriocin abp 118 α was MMKEFTVLTECELAKVDGG KRGPNCVGNFLGGLFAGAAAGVPLGPAG IVGGANLGMVGGALTCL. It was found that N-terminal amino acid sequence of bacteriocin FK15 showed high homology to bacteriocin abp 118 α while C-terminal was completely different. Moreover, the leader peptide of nucleotide deduced complete peptide was very similar to that of abp 118 α. However, the molecular weight of bacteriocin FK15 (4347.32 Da) was different from bacteriocin abp 118 α (4076 Da). Therefore, it can be considered bacteriocin FK15 as a novel bacteriocin, and hence named Salvicin K.

Class II bacteriocins comprise a very large group of heat-stable unmodified peptide bacteriocins (O'Sullivan *et al*., 2002) with molecular masses smaller than 10 kDa (Oscáriz and Pisabarro, 2001). None of the bacteriocins in this class displays any post-translational modification beyond the cleavage of an 18-21 amino acid leader region from the pro-bacteriocin molecule. All are small, between 36 and 57 amino acids after loss of leader peptides (Hill, 1995). These results showed that bacteriocin FK12 and Salvicin K belong to class II bacteriocin as small heat peptide (<10 kDa), heat-stable, *Listeria*-active peptide. Moreover, cationic and hydrophobic peptides have been reported to be first synthesized as prepeptides with leader peptides of the double-glycine type (Ennahar *et al*., 2000, Van Belkum and Stiles, 2000., Eijsink *et*

al., 2002), which were also found in both FK12 and Salvicin K. The Gly-Gly was found at the -1 and -2 position before the processing site. More feature of class II LAB bacteriocin preceding the double glycine motif is the two-isoleucine residues at position -3 and -4. However, leader peptide of Salvicin K did not contain two-isoleucine residues at position -3 and -4. In addition, this sequence revealed an apparent class-II bacteriocin, which did not contain the YGNGV. Flynn *et al*, (2000) reported that *Lb. salivarius* UCC 118 produced two-peptide bacteriocin Abp 118 α and β, which is class IIb bacteriocins whose activity depends on the complementary activity of two peptides. Abp 118 α peptide possesses inhibitory activity when concentrated, and this activity is enhanced by the presence of the abp 118 β peptide, which on its own has no associated bacteriocin activity. Abp 118 α and β showed broad spectrum of antibacterial activity, since it is capable inhibiting against *Bacillus* , *Listeria*, *Enterococcus* and *Staphylococcus* species without an apparent antibacterial activity towards related LAB, with the exception of *Lb. fermentum* KLD. However, Salvicin K showed antibacterial activities against LAB including, *Lb. sakei*, *Leuconostoc, Enterococcus* and Gram-positive bacteria such as *Listeria* and *Br. Campestris*, but it showed no antibacterial activities against *Staphylococcus sp*. and other Gram-negative bacteria. Moreover, Salvicin K demonstrated a bactericidal mode of action against *Lb. sakei* subsp. *sakei* JCM 1157^T which was cell lysis as OD (600nm) of samples were decreased. These properties suggested that Salvicin K has potential as a natural food preservative.

As described before, bacteriocin FK 12 was similar to presalivaricin B and Cataloluk and Gürakan (2003) had reported its characteristic details. Therefore, only bacteriocin Salvicin K, which is novel bacteriocin, will be further studied.

Detail characterization of purified Salvicin K indicated that it has similar physicochemical stability like CFS. Proteinase K completely inactivated the purified Salvicin K, indicating that it has protein structure and thus identified as bacteriocin (Vaughan *et al.,* 2001, Klaenhammer, 1988). In addition, Salvicin K was heat tolerant at high temperature and stable at pH 3-10 that would be useful for food industrial

processing under pasteurization condition. In addition, heat-stability is another major feature of low molecular-weight bacteriocin (Oscáriz and Pisabarro, 2001).

Since the CFS showed high antibacterial activities than Salvicin K, it is possible that bacteriocin Salvicin K may have synergistic activities with other bacteriocins or other substances which may contain in CFS. Therefore, synergistic activities should be studied in the future.

This is the first report that describes the production and characterization of bacteriocin Salvicin K produced by *Lb. salivarius* existing in chicken intestine.

CONCLUSION AND RECOMMENDATION

Two bacteriocin-producing strains K4 and K7 were isolated from chicken intestines. These two isolates displayed antibacterial activities against only Gram-positive bacteria. Both isolates were identified as *Lb. salivarius*, therefore, the isolates were designated *Lb. salivarius* K4 and *Lb. salivarius* K7. The bacteriocins produced from both isolates were heat tolerant and stable at pH at 3-10.

The purification of bacteriocins producing by *Lb. salivarius* K4 was finally achieved by RP-HPLC and two active fractions designated FK 12 and FK15 were obtained. Partial amino acid sequence and complete sequence of FK12 were homologous with presalivaricin B 94 and 96%, respectively. In addition, FK 12 also showed homology to bacteriocin-like peptide 91 and 94%, respectively whereas both sequences of FK15 showed no homology to any bacteriocins at the GenBank database. However, complete amino acid sequence of FK15 was 64% similar to abp 118 α by WU-BLAST2 (EMBL-EBI). Therefore, it can be concluded that FK15 is a novel bacteriocin and hence named Salvicin K. The studies on the properties of purified Salvicin K indicated that Salvicin K was heat tolerant and stable at wide pH range of 3-10. In addition, it was completely digested by proteolytic enzyme. Moreover, Salvicin K demonstrated a bactericidal mode of action against *Lb. sakei* subsp. *sakei* JCM 1157^{T}.

Since these two isolates were screened from chicken intestines, they may have probiotic potentials to use in poultry industry. Moreover, the bacteriocins from them may have application in the food industry, and therefore further studies are needed to be conduceted.

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APPENDIX

1. Calculation of bacteriocin activity (AU) (Contreras *et al*., 1997)

Total activity (AU) = Bacteriocin activity (AU) X volume (ml)

Specific activity (AU/mg) = $\frac{\text{Bacteriocin activity (AU/ml)}}{\text{Protein concentration (mg/ml)}}$

% Yield (%total activity recovered) = $\frac{\text{Total activity of purified protein (AU)}}{\text{Total activity of crude bacteriocin (AU)}}$ X 100

2. Composition of media

2.1 Luria-Bertani (LB) Broth

Bactotryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Distrilled water into	1000 ml

Autoclave 121°C 15 min

2.2 SOB broth

Bactotryptone	20 g
Yeast extract	5 g
1 M Sodium chloride	10 ml
1 M Potassium chloride	10 ml
Distrilled water	1000 ml

Autoclave 121°C 15 min, then added 10 ml of 2 M M^{2+}(1M $MgCl_2$ and 1M $MgSO_4$) which is filtering by filter (0.45 μm)

2.3 SOC broth

SOC broth is SOB broth which is added sterile glucose at final concentration 0.02 M (1 ml of 2 M glucose into 100 ml of SOB).

3. Composition of buffer

3.1 TE

10 mM Tris-HCL pH 7.6
1 mM EDTA pH 8

3.2 TBE

45 mM Tris-borate
1 mM EDTA pH 8

Appendix Table 1 Profile of growth and bacteriocins production of K4 at 30°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.100	6.040	0
2	0.127	7.030	0
4	0.286	7.310	100
6	0.640	7.920	200
8	1.540	8.120	800
10	3.030	8.940	1600
12	4.100	8.630	1600
14	4.600	8.810	1600
16	5.650	8.730	3200
20	9.500	8.840	3200
24	10.500	8.860	3200

Appendix Table 2 Profile of growth and bacteriocins production of K4 at 37°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.0760	7.017	100
2	0.141	7.093	200
4	0.570	7.903	400
6	2.100	8.839	800
8	4.500	8.878	1600
10	6.920	9.121	1600
12	7.520	9.049	1600
14	7.600	9.267	1600
16	7.900	9.225	1600
20	9.950	9.000	1600
24	9.500	9.079	1600

Appendix Table 3 Profile of growth and bacteriocins production of K4 at 42°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.0185	7.130	0
2	0.167	7.190	200
4	0.585	7.966	200
6	2.340	8.869	800
8	5.080	8.925	1600
10	7.050	9.146	1600
12	7.590	9.140	1600
14	7.650	9.274	1600
16	7.400	8.146	1600
20	10.250	8.813	1600
24	11.000	8.973	800

Appendix Table 4 Profile of growth and bacteriocins production of K7 at 30°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.029	6.398	0
2	0.081	6.716	0
4	0.095	6.833	0
6	0.193	7.164	0
8	0.416	7.736	0
10	0.590	8.236	400
12	2.420	8.681	1600
14	3.690	8.716	800
16	4.390	8.767	800
20	4.300	8.924	800
24	4.400	8.272	800

Appendix Table 5 Profile of growth and bacteriocins production of K7 at 37°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.038	6.748	0
2	0.110	6.763	0
4	0.328	7.462	0
6	1.620	8.544	800
8	4.010	8.796	800
10	4.660	8.728	800
12	4.640	8.690	800
14	4.650	8.703	400
16	5.040	8.371	400
20	5.100	8.537	400
24	5.750	8.863	400

Appendix Table 6 Profile of growth and bacteriocins production of K7 at 42°C

Time	OD (600nm)	Log cfu/ml	Activity (AU/ml)
0	0.038	6.490	0
2	0.082	6.690	0
4	0.422	7.560	0
6	1.540	8.530	400
8	3.450	8.720	800
10	4.100	8.690	800
12	4.410	8.700	800
14	4.480	8.790	800
16	4.670	8.790	800
20	7.250	8.610	800
24	7.500	8.480	800

Appendix Table 7 Mode of action of partially purified bacteriocin produced by *Lb. salivarius* K4

Time	OD (control)	OD (bacteriocin)	Log cfu (control)	Log cfu (bacteriocin)
0	0.021	0.016	7.480	7.480
2	0.114	0	8.090	1.790
4	0.355	0	8.230	1.450
6	1.170	0	8.630	0
8	1.720	0	8.860	0

Before = before treated with bacteriocin

Appendix Table 8 Mode of action of Salvicin K produced by *Lb. salivarius* K4

Time	OD (control)	OD (Acetonitrile)	OD (Salvicin K)	Log cfu (control)	Log cfu (Acetonitrile)	Log cfu (SavicinK)
0	0.069	0.074	0.069	7.060	7.170	7.236
2	0.210	0.191	0.070	8.029	7.929	0
4	0.492	0.410	0.056	8.556	8.415	0
6	1.530	1.180	0.054	8.908	8.200	0
8	1.720	1.370	0.044	9.033	8.892	0

0 = before treated with bacteriocin

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