CHARACTERIZATION AND PURIFICATION OF A BACTERIOCIN PRODUCED BY *LEUCONOSTOC MESENTEROIDES* SUBSP. *CREMORIS*

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ABSTRACT

CHARACTERIZATION AND PURIFICATION OF A BACTERIOCIN PRODUCED BY *LEUCONOSTOC MESENTEROIDES* SUBSP. *CREMORIS*

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In this study, a new bacteriocin isolated from a *Leuconostoc mesenteroides* subsp. *cremoris* strain was purified to homogeneity by pH mediated cell adsorption-desorption, solid phase extraction with Amberlite XAD-16, cation-exchange chromatography and hydrophobic interaction chromatography. The purification resulted in an electrophoretically pure protein that was smaller than 6 kDa as judged by SDS-PAGE. The bacteriocin was found to be very hydrophobic and cationic and could be adsorbed by synthetic calcium silicate due to its cationic and especially hydrophobic nature. It was observed that this bacteriocin was sensitive to α-amylase in addition to proteinase K, trypsine, pepsine and chymotrypsine and had a bactericidal mode of action with a concomitant cell lysis. The results indicated that bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was more stable to combined effect of pH and heat than nisin, lacticin 481, lacticin 3147 and lactococcin G and was bactericidal between pH 2.0-12. It was found that the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was stable to organic solvents and

could be extracted with chloroform containing solvents efficiently for purification.

The bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was found to have a strong inhibitory activity against *Listeria innoqua*, *Listeria monocytogenes*, *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus delbrueckii*, *Lactobacillus cremoris*, other *Leuconostoc meenteroides* strains and gram-negative bacterium *Pseudomonas fluorescens*. Some of the insensitive bacteria were observed to be sensitive when high concentration of the bacteriocin was used.

Keywords: Bacteriocin, protein purification, cell membrane, SDS-PAGE, bacteriolytic.

ÖZ

LEUCONOSTOC MESENTEROIDES SUBSP. *CREMORIS* TARAFINDAN ÜRETİLEN BAKTERİYOSİNİN KARAKTERİZASYONU VE SAFLAŞTIRILMASI

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Bu çalışmada *Leuconostoc mesenteroides* subsp. *cremoris*'den izole edilen yeni bir bakteriyosin pH ile hücre yüzeyine tutunma-salınma, amberlit XAD-16 ile katı-faz ekstraksiyonu, katyon-değişim kromatografisi ve hidrofobik etkileşim kromatografisi ile homojen olarak saflaştırılmıştır. Saflaştırma SDS-PAGE ile molekül ağırlığı 6 kDa dan küçük bulunan elektroforetik olarak saf bir protein ortaya çıkarmıştır.. Bacteriyosinin çok katyonik ve hidrofobik olduğu bulunmuştur ve sentetik kalsiyum silikat tarafından katyonik ve özellikle de hidrofobik doğası nedeniyle adsorbe edilebilmektedir. Bakteriyosinin proteinaz K, tripsin, pepsin ve kimotripsin'e ilave olarak α-amilaz'a da duyarlı olduğu, beraberinde hücre parçalanmasıyla bakterisidal bir etkiye sahip olduğu gözlenmiştir.. Sonuçlar *Leuconostoc mesenteroides* subsp. *cremoris* tarafından üretilen bakteriyosinin pH ve ısının birleşik etkisine karşı nisin, lacticin 481, lacticin 3147 ve lactococcin G den daha dayanıklı olduğunu ve pH 2.0-12 arasında bakterisidal olduğunu ortaya çıkarmıştır. *Leuconostoc mesenteroides* subsp. *cremoris*'in bakteriyosininin organik çözücülere karşı dayanıklı olduğu

ve kloroform içeren çözücüler ile etkili bir biçimde ekstrakte edilebilebildiği bulunmuştur.

Leuconostoc mesenteroides subsp. *cremoris* tarafından üretilen bakteriyosin *Listeria innoqua*, *Listeria monocytogenes*, *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus delbrueckii*, *Lactobacillus cremoris*, diğer *Leuconostoc mesenteroides* suşları ve gram-negatif bakteri *Pseudomonas fluorescens*'e karşı kuvvetli bir inhibitör etkiye sahip olduğu bulunmuştur. Duyarsız bakterilerin bazılarının yüksek konsantrasyonda bakteriyosin kullanıldığı zaman duyarlı oldukları gözlenmiştir.

Anahtar Kelimeler: Bakteriyosin, protein saflaştırma, hücre zarı, SDS-PAGE, bakteriyolitik.

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ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine three phosphate
AU	activity (Arbitrary Unit)
Da	dalton
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
FPLC	Fast performance liquid chromatography
GSP	general secretion pathway
HPK	histidine protein kinase
HPLC	High pressure liquid chromatography
IF	induction factor
kDa	kilodalton
LAB	lactic acid bacteria
min	minute
ml	milliliter
MW	molecular weight
nm	nanometer
NMR	nuclear magnetic resonance
OD	optic density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pI	isoelectric point
PMF	proton motif force
rpm	round per minute
RR	response regulator
SDS	sodium dodecyl sulfate
TEMED	N, N, N', N'-tetramethylethylenediamine

TSB	tryptone soy broth
ΔpH	pH gradient
Δψ	transmembrane potential

CHAPTER 1

INTRODUCTION

1.1. Lactic acid bacteria

Lactic acid bacteria are Gram-positive, non-sporulating microaerophilic bacteria whose main fermentation product from carbohydrate is lactate. The lactic acid bacteria include both cocci (*Lactococcus*, *Vagococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Tetragenococcus*, *Streptococcus*) and rods (*Lactobacillus*, *Carnobacterium*, *Bifidobacterium*). Phylogenetically they are members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria (De Vuyst and Vandamme, 1994). They generally lack catalase, although pseudo-catalase was detected in cultures grown on a low sugar concentration. Glucose is converted mainly to lactic acid (homofermentatives), or to lactic acid, carbon dioxide, ethanol and/or acetic acid (heterofermentatives). The catalase-negative, gram-positive cocci can be divided into two groups: the facultatively anaerobic or microaerophilic bacteria, and the strict anaerobes. Lactic acid bacteria are commonly found in foods, including fermented meat, vegetables, fruits, beverages, and dairy products, in the respiratory, intestinal and genital tracts of humans and animals, in sewage, and in plant materials (De Vuyst and Vandamme, 1994).

The lactic acid bacteria have been used traditionally as starter cultures for the fermentation of foods and beverages since they contribute to flavour and aroma and retard spoilage. The starter cultures present in the food contribute also its preservation

by in situ production of antimicrobial substances such as lactic acid, acetic acid, hydrogen peroxide, bacteriocins etc.

However, production of odours, off-flavours, biogenic amines and slime can be negative factors associated with the growth of lactic acid bacteria. In addition, bacteriocin production by the starter cultures can cause some problems. For example, mixtures of rapidly growing strains of starter cultures for meat fermentation could retard each other's growth via bacteriocin inhibition.

Lactic acid bacteria, especially lactobacilli are believed to play a useful role in the gastrointestinal tract by colonizing there (Gilliland, 1990). Therefore, living cells can be added to foods and feeds and used as probiotics for human and animal consumption. Also, lactobacilli are potentially useful as carrier for oral immunization, since orally administered lactobacilli trigger both mucosal and systemic immune reaction against epitopes associated with these organisms (Gerritse *et al*., 1990). Some important nutritional and therapeutic effects (Gilliland, 1990) attributed to these bacteria are as follows:

a) improvement of nutritional quality of food and feed
b) stabilization of the intestinal microflora, preventing colonization by pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp. and enteropathogenic *E. coli* strains, via adhesion to the intestinal wall and competition for nutrients
c) protection against intestinal and urinary tract infections by producing antibacterial substances
d) tumor suppression via stimulation of the immune system to produce macrophages.

Lactic acid bacteria possess several interesting properties of great economic importance. Many of these properties such as lactose utilization, proteinase activity, bacteriophage defence mechanisms, bacteriocin production and immunity, etc. are

genetically mediated by often unstable and naturally transferable plasmids. Therefore, research is now being focused on the improvement and stabilization of these industrially important features. The ultimate aim is to bring them all together in one or more starter cultures with practical use (Gasson, 1993). Originally, these fermentation characteristics were improved by metabolic engineering and by using classical genetics such as mutation, phenotypic adaptation and selection. Later, in vivo recombination techniques such as conjugation, transposition and transduction as well as protoplast fusion were tried out (Gasson, 1993). In recent years recombinant DNA technology has been applied in the field of lactic acid bacteria (De Vos, 1987). Also, lactic acid bacteria may be successfully used as industrial microorganisms for the synthesis of fine chemicals, pharmaceuticals, and other products useful to humans, since they have several advantages in industrial fermentations:

a) they are ingested and are non-pathogenic;
b) they do not form toxins or toxic products;
c) they are microaerophilic and aerotolerant, requiring a simple fermentation process;
d) they grow rapidly, requiring a short fermentation process;
e) their growth discourages spoilage and contamination with other microorganisms;
f) they can secrete proteins;
g) they can ferment diverse cheap substrates such as milk, whey, plant wastes, and hydrolyzed starch;
h) they have been used in the food industry for years. Consequently, methods for their cultivation on a large scale already exist.

1.2. Antimicrobial potential of lactic acid bacteria

The preservative effect of lactic acid bacteria during the manufacture and subsequent storage of fermented foods is mainly due to the acidic conditions that they bring about in the food during their development. This souring effect is mainly due to the

fermentative conversion of carbohydrates to organic acids with a concomitant lowering of the pH of the food, an important characteristic that leads to an increased shelf-life and safety of the final product. In recent years, it has become clear that the overall inhibitory action of lactic acid acid bacteria is due to more complex antagonistic systems produced by the starter cultures. Lactic acid bacteria are capable of producing and excreting inhibitory substances other than lactic and acetic acid. These substances are antagonistic to a wide spectrum of microorganisms, and thus can make significant contributions to their preservative action. They are produced in much smaller amounts than lactic acid and acetic acid, and include formic acid, free fatty acids, ammonia, ethanol, hydrogen peroxide, diacetyl, acetoin, 2-3-butanediol, acetaldehyde, benzoate, bacteriolytic enzymes, bacteriocins and antibiotics, toxins (many substances traditionally thought of as bacterial toxins because of the action against eukaryotic cells can also be shown to have antibacterial activity), bacteriolytic enzymes (lysostaphin, phospholipase A and hemolysins), bacteriophages and defective bacteriophages, antibiotic substances (gramicidin, valinomycin, and bacitracin that are synthesized by multienzyme complexes) as well as several less well-defined or completely unidentified inhibitory substances (Klaenhammer, 1988; Daeschel, 1989; Piard and Desmazeaud, 1991, 1992). Some of these substances display antagonistic activity towards many food spoilage and foodborne pathogenic microorganisms, including psychotrophic lactobacilli and leuconostocs, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*, etc. (De Vuyst and Vandamme, 1994). The application of appropriate starter cultures for in situ production of antimicrobial compounds in fermented foods and the application of partially purified or pure antimicrobial substances as biological food preservatives in both fermented and non-fermented foods will become much more important in future food preservation. The use of cell-free antimicrobials has advantages over the use of whole cells as a food preservative in terms of minimizing texture and flavor changes, particularly in non-fermented foods. Although the crude fermentation liquor would have greater potential for use than whole cells, the purified antimicrobial substance would be most desirable.

1.3. Bacteriocins

1.3.1. Bacterial interference

Peptide antibiotics such as bacitracin and gramicidin that are synthesized in bacteria by multienzyme complexes or sequential enzyme reactions have not yet achieved widespread application in the treatment of infectious diseases (Kleinkauf *et al*., 1990). However, recent studies of mersacidin and epidermin, ribosomally synthesized peptides of the lantibiotic class, have suggested that they may be at least as effective as some currently used therapeutic agents for the treatment of staphylococcal infections in mice (Limbert *et al*., 1995) and acne in humans, respectively (Ungermann *et al*., 1991).

Ever since the days of Pasteur, it seems that there has been a small subgroup of microbiologists who have stubbornly promoted bacteriotherapy and microbial interference for the treatment and prevention of infectious diseases. The discovery and dramatic success of penicillin, signaling the advent of the antibiotic era, for a time quelled most interest in the possible applications of antagonistic microorganisms to protect the human or animal host against infection. However, one notable exception was the (generally) successful application of the relatively avirulent 502A strain of *Staphylococcus aureus* in the prevention of of serious staphylococcal diseases in neonates and in the treatment of furunculosis (Aly and *et al*., 1982). In recent years, there has been increased concern that because of the widespread overprescribing of antibiotics and consequent increased development of antibiotic resistance, the pharmaceutical industry may no longer be able to develop effective novel antibiotics sufficiently quickly. This concern is now being translated into a resurgence of interest in the implantation into the indigenous microflora of bacteriocin-producing bacterial strains of apparent low virulence that are potentially capable of interfering with colonization and infection by more pathogenic species.

1.3.2. Historical background

Substances currently named bacteriocins comprise a rather ill defined potpourri of proteinaceous molecules that typically first attracted the attentions of researchers because of their physiological capability of interfering with the growth on agar media of certain other, generally closely related bacteria. Until recently, most of the significant progress in bacteriocin research stemmed from investigations of the colicins, those prototype bacteriocins produced by various members of the family *Enterobacteriaceae*, and this resulted in considerable in-depth knowledge of the genetic basis, domain structure, mode of formation, and killing action of these molecules (Pugsley, 1984). However, there now appears to be a remarkable renaissance of research activity centered upon the bacteriocin-like activities of gram-positive bacteria, particularly lactic acid bacteria. Many of these are food grade organisms that are already widely used in the food industry but now offer the further prospect of application to improve food preservation. Another contributing factor has been the burgeoning interest in possible applications of bacterial interference as a strategy for the prevention of certain infectious diseases.

As was the case for so many seminal observations in microbiology, it was Pasteur who, together with Joubert, first systematically recorded an observation of antagonistic interactions between bacteria (Jack *et al*., 1995). In summarizing their findings that ‘‘common bacteria’’ (probably *E. coli*) could interfere with the growth coinoculated anthrax bacilli, either in urine (used as a culture medium) or in experimentally infected animals, they foreshadowed potential practical applications, stating ‘‘These facts perhaps justify the highest hopes for therapeutics.’’ What followed over the ensuing three to four decades was an intensive search for bacteria of relatively low disease potential that could be used in replacement therapy regimens to prevent the establishment of potential pathogens (Jack *et al*., 1995). Anthrax continued to be one of the most popular target diseases in these studies, and various antagonists of the in vivo or in vitro growth of *Bacillus anthracis* were reported, including both gram-

positive (staphylococci, micrococci, and streptococci) and gram-negative (pseudomonads) species. It is not clear how many of these reported interactions can be attributed to antibiotic activities, since in most cases the observations were of a clinical rather than experimental nature and there is no information on the isolation and characterization of any inhibitory chemical substances.

The first clear documentation of the nature of an antibiotic agent produced by *E. coli* was provided by Gratia, who demonstrated in 1925 that strain V (virulent in experimental infections) produced in liquid media a dialyzable and heat-stable substance (later referred to as colicin V) that inhibited in high dilution the growth of *E. coli* ϕ (Jack *et al*., 1995). There followed a period in which a whole series of colicins produced by *E. coli* and closely related members of the *Enterobacteriaceae* were discovered. Interestingly, it later became clear that the unusually small size and heat stability of colicin V set it apart from most of the subsequently isolated colicins, and it now more appropriately seems to fit the description of a microcin (Faith *et al*., 1992).

In recognition of the discovery that antibiotic substances of the colicin type may also be produced by noncoliform bacteria, the more general term ‘‘bacteriocin’’ was joined by Jacob *et al* in 1953. Bacteriocins were specifically defined as protein antibiotics of the colicin type, i.e., molecules characterized by lethal biosynthesis, predominant intraspecies killing activity, and adsorption to specific receptors on the surface of bacteriocin-sensitive cells. Further distinguishing features of the colicins included their relatively high molecular weights and the plasmid association of their genetic determinants.

As a result initially of the influence and efforts of Fredericq (Jack *et al*., 1995), knowledge of the colicins advanced at a great rate and more than 20 different types were identified on the basis of their actions against a set of specific colicin-resistant (generally receptor-deficient) mutants. Fredericq also observed that certain strains of staphylococci produced substances, named by him ‘‘staphylococcins,’’ that were inhibitory to the growth of other staphylococci and some other gram-positive bacteria

but not gram-negative-bacteria. However, attempts to categorize the staphylococcins in a manner similar to the receptor-based colicin classification scheme were not successful (Jack *et al*., 1995).

1.3.3. Physical, chemical and biological properties of bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by microoorganisms belonging to different eubacterial taxonomic branches (Oscáriz *et al*., 2001). The production of small antibiotic peptides is a common defense strategy against bacteria that is displayed not only by microorganisms, but also by animals and plants (Oscáriz *et al*., 2001). Maganins, cecropins and defensins are animal (Lehrer *et al*., 1993; Zasloff, 1987), and thionins are plant (Broekaert *et al*., 1995) antimicrobial peptides. Protease sensitivity is a key criterion in the characterization of an inhibitor as a bacteriocin. Since bacteriocins are by definition proteinaceous substances, they are generally inactivated by an array of proteolytic enzymes (trypsin, α-chymotrypsin, pepsin, proteinase K, etc.). Moreover, the pattern of protease sensitivity may indicate the uniqueness of an isolated bacteriocin, since bacteriocins of different bacteria vary in sensitivity to proteolytic enzymes (De Vusyt and Vandamme, 1994). Conversely, the same pattern of protease sensitivity coupled with an identical spectrum of activity may indicate multiple isolations of the same bacteriocin producer. In addition, the sensitivity to proteolytic enzymes of gastric and pancreatic origin is very interesting with respect to the application of bacteriocins as biological preservative in foods and feeds, since it means that their ingestion will not affect the microbial flora of the gastrointestinal tract (De Vuyst and Vandamme, 1994). However, some bacteriocins seem to be insensitive for certain protease. It is possible that these bacteriocins contain only a minor proteinaceous component. Several bacteriocins contain a carbohydrate, lipid or phosphorous moiety. The presence of such a non-proteinaceous moiety can be verified by the sensitivity of these bacteriocins glycolytic (α-amylase), lipolytic (lipase) and phospholipolytic (phospholipase) enzymes (De Vuyst and Vandamme). Bacteriocin 466 (De Klerk and Smith, 1967) and lactocin 27 (Upreti and Hinsdill,

1973) are typical examples of such lipocarbohydrate protein complexes, whereas caseicin LHS (Dicks *et al*., 1992) and leuconocin S (Lewus *et al*., 1992) behave as glycoproteins. In addition, some bacteriocins contain unusual amino acids (lantibiotics) or several cross-linkages, making the elucidation of their primary structure rather difficult. As an example, the post-translational formation of thioether amino acids during lantibiotic biosynhesis results in the formation of intramolecular rings by monosulfur bridges (Jung, 1991). Such ring structures will result in blank cycles during the automated sequencing by Edman degradation (Schnell *et al*., 1988).

The molecular mass of bacteriocins produced by lactic acid bacteria may vary considerably, ranging from small peptides (e.g. lactocin 481, 1700 Da) to protein-protein and protein-lipid aggregates and macromolecules with a molecular mass in excess of 200 000 Da (e.g. lactocin 27, lactacin B, lactacin F, helveticin J). These aggregates can be disrupted by treatment with detergents or urea; the disruption often results in monomers which are more active than the native bacteriocin complex. For example, polyacrylamide gel electrophoresis of of lactocin 27 in the presence of SDS showed that the active moiety was a protein with a molecular mass of 12 400 rather than 200 000 as determined by size-exclusion chromatography (Upreti and Hinsdill, 1973).

The stability of bacteriocin preparations has often been shown to decrease significantly with increased purification. Also, bacteriocins differ largely with respect to their sensitivity to inactivation by changes in pH and temperature. Most of the bacteriocins produced by lactic acid bacteria are only stable at acid and neutral pH and are inactivated at a pH above 8.0 (nisin, pediocin AcH, leucocin A-UAL 187). Heat resistance is a major characteristic of many bacteriocin produced by lactic acid bacteria and can vary significantly, ranging from 60^{o}C or 100^{o}C for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121^{o}C for 15-20 min (e.g. lactacin B, lactacin F, nisin, etc.). Such heat stability is due to the formation of small globular structures and the occurrence of strongly hydrophobic

regions (e.g. lactacin F, lactococcin A, nisin), stable cross-linkages (e.g. nisin, lacticin 481, lactocin S), a high glycine content (e.g. diplococcin, lactacin F, lactocin 27, lactococcin A), etc. (De Vuyst and Vandamme, 1994).

1.3.4. Bacteriocin classification

Several classification criteria have been used to classify the bacteriocins produced by gram-positive bacteria. According to Jack *et al*. (1995), bacteriocins were classified into four groups based on the presence of disulfide and monosulfide (lanthionine) bonds: (1) antibiotics containing unusual posttranslationally modified amino acids such as dehydroalanine, dehydrobutirine, lanthionine or β-methyl-lanthionine (lantibiotics); (2) antibiotics containing at least one disulfide bridge essential for their activity (cystibiotics); (3) antibiotics with a single –SH residue that should be in a reduced form for the antibiotic to be active (thiolbiotics); and (4) antibiotics without cysteine residues (Table 1.1). Jack *et al*. (1995) stressed that the higher the number of disulfide bonds a bacteriocin has, the wider its activity spectrum tends to be.

According to Klaenhammer (1993), bacteriocins can be classified into four groups on the basis of their molecular mass, thermostability, enzymatic sensitivity, presence of posttranslationally modified amino acids, and mode of action.

Class I bacteriocins: Class I, lantibiotics (lanthionine-containing peptides with antibiotic activity), are small peptides (19-37 amino acids) that have been differentiated from other bacteriocins by their content of didehydroamino acids and thioether amino acids (Jung, 1991). (e.g. nisin, lacticin 481, carnocin UI49, lactocin S). Two subgroups have been defined on the basis of their distinctive ring structures: type A comprises screw-shaped, amphipathic molecules with molecular masses of 1,959 (duramycin) to 4,635 Da (carnocin U149, which is the largest lantibiotic described up to now) (Nes and Tagg, 1996) and with two to seven net positive charges that produce voltage-dependent pores by unspecific interaction with the membrane of the target

cell; and type B consists of more-globular molecules with either no net charge or a net negative charge. Type B includes antibiotics such as mersacidin (Chatterjee *et al*., 1992), actagardin (Jung, 1991), cinnamycin (Kaletta *et al*., 1991), and mutacin A. The latter is produced by *Streptococcus mutans* during the formation of dental plaque (Woodruff *et al*., 1998) and is the most recently described class I, type B bacteriocin. The number of thioether bridges (modified amino acids) present in type A lantibiotics can vary, and members with either three (pep5) (Reis *et al*., 1994), four (epidermin) (Allgaier *et al*., 1986) or five (nisin A and Z, subtilin) (Hurst, 1978; Banerjee and Hansen, 1988) monosulfide bonds have been described.

Class II bacteriocins: Class II, small (<10-kDa), relatively heat-stable (from>30 min at 100^{o}C to 15 min at 121^{o}C), non-lanthionine-containing membrane-active peptides, subdivided into *Listeria*-active peptides with the N–terminal consensus sequence –Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (Class IIa), poration complexes requiring two different peptides for activity (Class IIb), and thiol-activated peptides requiring reduced cysteine residues for activity (Class IIc).

Class II a bacteriocins include Leucocin A, Mesentericin Y105, Mundticin, Piscicolin 126, Bavaricin A, Sakacin P, Pediocin PA-1, Bavaricin MN, Divercin V41, Enterocin A, Enterocin P, Carnobacteriocin BM1, Sakacin A, Carnobacteriocin B2, Bacteriocin 31, and Acidocin A (Ennahar *et al*., 2000). The class IIa is the largest and most extensively studied subgroup of class II bacteriocins that are especially strong inhibitors of the food-borne pathogen *Listeria monocytogenes* (Héchard *et al*., 1992).

Class 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, (Cintas *et al*., 1998), or they may both be necessary for antimicrobial activity (lactococcins Gα/Gβ (Nissen-Meyer *et al*., 1992), lactococcins M/N (Van Belkum *et al*., 1991), and plantaricins EF and JK (Diep *et al*., 1996)).

Class IIc includes all class II bacteriocins that do not fall into class IIa or IIb (Ennahar *et al*., 2000). Ennahar *et al*. (2000) reported that two types of bacteriocins can be distinguished within this group: (a) antibiotics with one or two cysteine residues (thiolbiotics and cystibiotics, respectively), and (b) antibiotics without cysteine (lactococcin A and acidocin B).

Class III bacteriocins: Class III bacteriocins consist of large (>15-kDa), heat-labile (inactivated within 10-15 min at 60-100^{o}C) bacteriocins, e.g. helveticin J, acidophilucin A, lacticin A and B, caseicin 80 (Jack *et al*., 1995). Most of them are produced by by bacteria of the genus *Lactobacillus* (Ennahar *et al*., 2000).

Class IV bacteriocins: This group consists of glycoproteins (lactocin 27), lipoproteins (lacstrepcins) that require non-protein moieties for their activity (Ennahar *et al*., 2000).

Table 1.1. Antibiotic peptides classified according to Jack (1995).

Antimicrobial peptide	Molecular mass (kDa)	Amino acids	Producer microorganism
Lantibiotics			
Actagardine	1.9	19	*Actinoplanes* spp.
Ancovenin	2.0	19	*Streptomyces* spp.
Cinnamycin	2.0	19	*Streptomyces cinnamoneus*
Duramycin	2.0	19	*Streptomyces cinnamoneus*
Epidermin	2.2	22	*Staphylococcus epidermidis*
Gallidermin	2.2	22	*Staphylococcus gallinarum*
Lanthiopeptin	2.0	19	*Streptoverticullum cinnamoneum*
Mersacidin	1.8	19	*Bacillus* sp.
Nisin	3.4	34	*Lactococcus lactis*
Pep5	3.5	34	*Staphylococcus epidermidis*
Subtilin	3.3	32	*Bacillus subtilus*
Cystibiotics			
Pediocin AcH/PA1	4.6	44	*Pediococcus acidilactici* H/PAC 1.0
Leucocin A/UAL 187	3.9	37	*Leuconostoc gelidum* UAL 187
Mesentericin Y 105	3.8	37	*Leuconostoc mesenteroides* Y 105
Sakacin A	4.3	41	*Lactobacillus sake* LB 706
Sakacin P	4.4	43	*Lactobacillus sake* LTH 674
Lactacin F	5.6	57	*Lactobacillus acidophilus* 11088
Carnobacteriocin A	5.1	53	*Carnobacterium piscicola* LV 17 A
Carnobacteriocin BM1	4.5	43	*Carnobacterium piscicola* LV 17 B
Carnobacteriocin B2	4.9	48	*Carnobacterium piscicola* LV 17 B
Cerein 7/8	4.9	56	*Bacillus cereus* Bc7
Thiolbiotics			
Lactococcin B	5.3	47	*Lactococcus lactis* subsp. *cremoris* 9 B4
No cysteine			
Lactococcin A	5.8	54	*Lactococcus lactis* subsp. *cremoris* 9 B4 *L. lactis* subsp. *cremoris* LMG 2130 *L. lactis* subsp. *lactis* bv. *diacetylactis* WM4
Lactococcin M[a]			*L. lactis* subsp. *cremoris* 9B4
Lactococcin N[a]			*L. lactis* subsp. *cremoris* 9B4
Lactococcin Gα[a]			*L. lactis* subsp. *lactis* LMG 2081
Lactococcin Gβ[a]			*L. lactis* subsp. *lactis* LMG 2081

[a]These two pairs of peptides act synergistically

1.3.5. Inhibitory spectrum

Although most bacteriocins produced by Gram-negative bacteria only act on very closely related (gram-negative) species, most bacteriocins of Gram-positive bacteria exhibit activity towards a wide range of Gram-positive species. Klaenhammer (1988)

distinguished two groups of bacteriocins produced by lactic acid bacteria with respect to their inhibitory spectrum. One includes bacteriocins with a narrow inhibitory spectrum, either only against closely related bacteria belonging to the same genus (e.g. diplococcin, lactocin 27, lactacin B, helveticin J) or against other bacterial genera (other lactic acid bacteria, *Clostridium*, *Listeria*, etc.) as well. Mesentericin Y105 are especially active towards *Listeria* spp., and less active or inactive towards lactic acid bacteria. The second class includes bacteriocins with a relatively broad spectrum of activity but again only against Gram-positive bacteria, e.g. nisin, pediocin, and leuconocin S (De Vuyst and Vandamme, 1994). Pediocin PA-1 and enterocin A have been shown to exhibit spectra of activity broader than those of sakacin P and curvacin A, which has been ascribed to the presence of an extra disulfide bond in the two former bacteriocins (Eijsink *et al*., 1998). It has also been speculated that bacteriocins with fewer amino acid residues would tend to have relatively broader antibacterial spectra than those with larger numbers of residues (Jack and Ray, 1995). Generally, nonlantibiotics have narrower spectra of inhibition than lantibiotics (Garneau *et al*., 2002). The bactericidal activity of class IIa bacteriocins seems to be targeting primarily *Listeria* strains. In addition to all species of *Listeria*, species belonging to the following genera have been reported to be sensitive to class IIa bacteriocins: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, *Bacillus* and *Brochothrix* (Ennahar *et al*., 2000).

1.3.6. Secondary and tertiary structures of bacteriocins

Bacteriocins are in general cationic (i.e., they contain an excess of lysyl and arginyl residues) amphipathic molecules. They are usually unstructured in aqueous solution, but have the propensity to form an α-helical structure when exposed to structure promoting solvents such as trifluoroethanol or when mixed with anionic phospholipid membranes (Moll *et al*, 1999). Some peptides form loop structures owing to a

disulphide bridge or a covalent bond. Especially, the presence of intramolecular ring structures is a characteristic feature of lantibiotics (Class I) (Moll, *et al*., 1999)

The structure of several lantibiotics has been elucidated by means of NMR (Van de Kamp *et al*., 1995; Van den Hooven, 1995). Each is an amphiphilic helical peptide with higher degree of flexibility in aqueous solution than in lipophilic solution (Van de Ven *et al*., 1991). Additionally, each has been shown to have potential membrane spanning sequences and a high dipole moment, both of which are necessary to support models for voltage-induced pore formation in lipid membranes (Freund *et al*., 1991). Interestingly, all form alternate faces with hydrophobic residue on the other, while they also show some flexibility in the central region of the peptide (Freund *et al*., 1991). Nisin A has five ring structures termed A, B, C, D and E, of which the last two are intertwined. These ring structures are rigid but interconnected by flexible hinge regions. Nisin A is amphipathic in two ways: First, the majority of the residues in the N-terminal half is hydrophobic and only a single charged residue is present, Lys12, whereas the C-terminal part contains most charged and hydrophilic residues. Secondly, both the first domain, containing ring A, B and C as well as the second domain, containing ring D and E, have a hydrophobic and hydrophilic side (van den Hooven, 1995).

The most precise information on the structure of class II bacteriocins comes from the work of J. C. Vederas, M. E. Stiles and co-workers who have resolved the three-dimensional structures of the 37-residue class IIa bacteriocin leucocin A (Fregeau Gallagher *et al*., 1997) and of the 48-residue class IIa bacteriocin carnobacteriocin B2 (Wang *et al*., 1999). The two structures are similar in that both peptides contain an amphiphilic α-helix spanning from residues 17/18 to residues 31/39 in leucocin A and carnobacteriocin B2, respectively (Eijsink *et al*., 2002). In water or DMSO solution, leucocin A exists as a random coil. In lipophilic media two domains can be identified. First, a three stranded antiparallel β-sheet domain of residue 2-16, linked by a disulphide bridge, and second an amphiphilic α-helix of residue 17-31. The α-helical

part has been suggested to be responsible for the target specificity, whereas the β-sheet exerts the antimicrobial activity (Fregeau Gallagher *et al*., 1997). In contrast to class I bacteriocins, the hydrophobicity of several class II bacteriocins increases gradually from the N-terminal to the C-terminal side of the molecule, while the hydrophobicity of the utmost C-terminal part slightly decreases. This orientation is inverted in comparison to that suggested for nisin (Van den Hooven, 1995).

Current models of pore formation suggest that cationic lantibiotics are attracted to the membrane by ionic interaction. If a sufficiently large membrane potential is present, the peptide adopt a transmembrane orientation, aggregating with their hydrophobic faces toward the bilayer and their charged, hydrophilic faces toward the channel center. When sufficient peptides come together, electrostatic repulsion between the positive charges may be responsible for pushing open the transmembrane channel.

1.3.7. Biosynthesis, processing and secretion of the bacteriocins of lactic acid bacteria

Bacteriocins are synthesized as pre-propeptides, which are processed and transported outside by the dedicated transport machinery or by the sec-dependent mechanism. Cleavage of the leader peptides is performed by specific peptidases or by a proteolytic domain of the dedicated ABC transporter (Nes *et al*. 1996), which recognize highly conserved sequences in the leader peptide. Moreover, the synthesis of lantibiotics, like nisin, necessitates post-translational modification of selected amino acid moieties prior to secretion (De Vuyst and Vandamme 1994). As well as structural and secretion-modification machinery genes, bacteriocin operons always include genes for specific immunity proteins (Jack *et al*., 1995; Nes *et al*., 1996) which protect the producer cells from their own bacteriocins.

Many bacteriocin operons are regulated by a quorum sensing system (Kleerebezem *et al*. 1997). The extracellular accumulation of an induction factor (IF) is sensed by a

two-component signal transduction system consisting of a membrane-located histidine kinase (HK) which phosphorylates a response regulator (RR), which in turn interacts with promoters of structural, biosynthetic and regulatory operons and induces gene expression. Brurberg *et al*. (1997) showed that the induction factors or pheromones are highly specific. That is, they only activate the cognate histidine kinase, encoded by the gene directly downstream of the pheromone gene. Bacteriocins and pheromones do show some common characteristics and, in fact, some pheromones do show some (minor) bacteriocin activity (Eijsink *et al*., 1996; Anderssen *et al*., 1998). However, this activity is almost neglectable compared to the activities of real bacteriocins.

Quorum sensing-based regulation of bacteriocin production in LAB is not restricted to class II bacteriocins but is also often found in conjunction with the production of lantibiotics. One major difference between the lantibiotics and class II bacteriocins is that regulation of the former does not rely on a dedicated (non-bacteriocin) peptide pheromone; instead, the bacteriocin itself acts as the pheromone (Kuipers *et al*., 1995). It has been found that the IF of the plantaricin operons in *Lactobacillus plantarum* C11, PlnA, has bacteriocin-like activity (Anderssen *et al*., 1998) and that bacteriocin operons in *Carnobacterium piscicola* LV17B can be induced by carnobacteriocin CB2 and by a bacteriocin-like IF (Cnbs) whose gene is organized in an operon with cnbK (HK) and cnbR (RR) (Quadri *et al*., 1997).

The lantibiotic nisin autoregulates its own production (Kuipers *et al*. 1995). In *Lactococcus lactis* N8 (a nisin Z producer) both *nisZBTCIPRK* and *nisFEG* operons are induced by nisin (Quiao *et al*., 1996). In nisin A producers, two inducible promoters are located upstream of *nisA* and *nisF*; a third promoter is located upstream of nisR (Kuipers *et al*., 1995). In many class II bacteriocins, the IF, HK and RR are organized in an autoinducible regulatory operon and the IF is a bacteriocin-like peptide which may have no inhibitory activity (Nes *et al*., 1996). It is suggested that the three component system could be first triggered as a consequence of an excess in IF concentration occurring through slow accumulation, as a consequence of cell growth

(Nes *et al*., 1996). It is reported that the IF may function as a cell density signal (Nilsen *et al*., 1998; Eijsink *et al*., 1996). Some scientists suggest that signal-transducing system for class IIa bacteriocins may mediate the response to an environmental signal (Hühne *et al*., 1996; Axelsson and Holck, 1995). Nilsen *et al*. (1998) suggested that environmental factors may affect binding of the IF to the HK or the balance of phosphorylation required for the activation of the RR. For example, Brurberg *et al*. (1997) showed how medium composition and access to oxygen affect quorum sensing-based production of sakacin P. Diep *et al*. (2000) showed that quorum-sensing-based production of sakacin A is highly temperature dependent. These authors showed that the Bac^+ phenotype was lost only at growth temperatures between 33 and 35^0C (and not at e.g. 30^0C). The phenotype could be restored by adding synthetic pheromone (whose amino acid sequence was known from genetic studies). Another example illustrating the interference of growth conditions with quorum sensing-based bacteriocin production comes from the work of Nilsen *et al*. (1998) on the production of enterocins by *Enterococcus faecium* CTC492. It was shown that the effects of certain growth conditions (e.g. pH, presence of salt) on bacteriocin production could be counteracted by adding the pheromone, EntF.

Environmental factors may affect several critical parts of the regulatory mechanism, e.g., basal expression of genes involved in pheromone production and sensing, the effectiveness of pheromone secretion and processing, or the affinity of the histidine kinase sensor for the pheromone. For example, pH or presence of salt was shown to affect the interaction between the pheromone and its receptor, since the high net charge of the pheromone (Kleerebezem and Quadri, 2001) indicates that this interaction involves electrostatic forces.

The sensing of its own growth, which is likely to be comparable to that of competing bacteria, would make sense from an ecological point of view. Firstly, this would ensure that defence against related bacteria is reinforced when the competition for nutrients is getting tougher. Secondly, cell-cell communication ensures

synchronization of bacteriocin production by cells of the same strain. Thirdly, the requirement of a quorum ensures that the bacterium only starts investing energy in bacteriocin production if there are sufficient other (similar) producers around; thus, the effort of combating competitors is shared and the potential impact of this effort is increased (Eijsink *et al*., 2002).

Induction is not the only factor influencing the expression of bacteriocin operons: carbon source regulation and the level of cell-adhered nisin have been shown to influence nisin synthesis (De Vuyst and Vandamme 1992) and catabolite repression has been claimed to operate in the regulation of plantaricin C production (Barcena *et al*., 1998). There are also some reports of induction of bacteriocin production caused by cells and extracts of sensitive strains (Barefoot *et al*., 1994).

Bacteriocins are first formed as ribosomally synthesized precursors or prepeptides, which appear not to be biologically active and contain N-terminal extension or leader sequence. Subsequent cleavage of the prepeptide at specific processing site removes the leader sequence from the antimicrobial molecule concomitantly with its export to the outside of the cell (Håvarstein *et al*., 1995). The leader peptide's removal during transmembrane translocation is accomplished by the same protein that is associated with the bacteriocin transport (Nes *et al*., 1996; Håvarstein *et al*., 1995). These leaders are believed to serve as signal peptides for the processing and the secretion of bacteriocins. One important feature of these leaders in class IIa bacteriocins is the presence of two glycine residues in the C-terminus, at positions -2 and -1 relative to the processing site. Secretion of class IIa bacteriocins are carried out 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. While all other class IIa bacteriocins investigated appear to be secreted via a dedicated transport system, the newly characterized enterocin P has been shown to be synthesized without a double-glycine

leader, but with a typical *sec*-type signal peptide, thereby being processed and exported by the GSP (general secretory pathway) (Cintas *et al*., 1997).

A model developed for the lantibiotic subtilin has suggested that folding due to thioether bonding occurs following translocation of unfolded molecules through the membrane. It was argued that folding prior to export might provide a barrier to traversal of the membrane by known mechanisms (Hansen *et al*., 1991).

1.3.8. Genetics of bacteriocin production

The DNA sequences of a large number of bacteriocin genetic loci have been determined during the past decade. These studies have revealed great variation in the way in which bacteriocin loci are organized (Eijsink *et al*., 2002). While bacteriocin production is often correlated with the presence of a plasmid, genes encoding several class IIa bacteriocins have been shown to be located on chromosome fragments (Hühne *et al*., 1996; Quadri *et al*., 1997). Yet, whether plasmid-borne or chromosomal, the structural gene for bacteriocins and its surrounding regions reveal one to three operon-like structures, which may be divergently transcribed and are involved in the bacteriocin production and extracellular translocation, the immunity of the producers, and in some cases, the regulation of bacteriocin synthesis (Nes *et al*., 1996).

The bacteriocin structural gene encodes a precursor peptide that is secreted and processed by either a dedicated machinery or via the *sec*-dependent pathway. In the case of two-component bacteriocins (class IIb); two structural genes follow each other, as is the case PlnEF and Pln JK (Eijsink *et al*., 2002).

Different secretion mechanisms employ different types of leader peptides that normally can be recognized and classified relatively easily (Eijsink *et al*., 2002). Two dedicated secretion mechanisms have been described for peptide bacteriocins. One has been found for nisin and a few other lantibiotics. These class I bacteriocins are

produced with a characteristic leader that is cleaved off by a dedicated serine protease encoded by a gene that is co-transcribed with other genes in the bacteriocin locus (McAuliffe *et al*., 2001). The most common dedicated secretion machinery found with respect to both class I and class II bacteriocins employes so-called double-glycine leader peptides (Håvarstein *et al*., 1994, 1995). Bacteriocin precursors that contain these leader peptides are secreted and processed by a dedicated ABC-transporter and a so-called accessory protein. The ABC transporter contains an extra N-terminal domain that has proteolytic activity and that cleaves off the leader concomitantly with export (Håvarstein *et al*., 1995). The processing and secretion of bacteriocin precursors with a double-glycine leader peptide depends on the presence of two co-transcribed genes, which usually are designated T and E. Gene T encodes the dedicated ABC-transporter (Håvarstein *et al*., 1995). For transcriptionally regulated bacteriocins, additional open reading frames (ORFs) are present in the vicinity of the structural gene, always in the same order, and form a putative three-component signal-transduction autoregulatory cassette encoding a possible induction factor (IF), a histidine protein kinase (HPK) and a response regulator (RR) (Ennahar *et al*., 2000).

It is becoming increasingly clear that lactic acid bacteria produce several bacteriocins and contain a variety of bacteriocin genes that are scattered over the chromosome and plasmids (Franz *et al*., 2000; Møretro, 2000). One of the best examples is the work on strain *Carnobacterium piscicola* LV17. This strain produces at least three bacteriocins, involving genes at three different locations (two on plasmids, one on the chromosome) (Franz *et al*., 2000).

A degree of care is required when assigning bacteriocin determinants to a particular genomic location. This is well illustrated by the case of the genes for nisin biosynthesis in *Lactococcus lactis* subsp. *lactis* which, because of their transmissible and curable nature (Gasson, 1984; Tsai and Sandine, 1987), were thought to be plasmid encoded. More recent molecular analysis has established that these genes are located on the chromosome (Dodd *et al*., 1990) and are carried by a large conjugative transposon

(Horn *et al*., 1991; Rauch and De Vos, 1992). Genetic analysis of nisin has progressed rapidly over recent years with a number of research groups investigating the biosynthesis of this post-translationally modified peptide.

1.3.9. Mode of action

A widely accepted hypothesis for the mode of action of bacteriocins is that the bacteriocin acts in two steps, involving adsorption of the bacteriocin to specific or non-specific receptors on the cell surface resulting in cell death (Tagg *et al*., 1976). Bacteriocins of lactic acid bacteria have bactericidal effect on sensitive cells, all or not resulting in cell lysis (Van Belkum *et al*., 1991). Lactocin 27 (Upreti and Hinsdill, 1975), leucocin A-UAL 187 (Hastings and Stiles, 1991) and leuconocin S (Lewus *et al*., 1992) have been reported to act bacteriostatically. However , the designation of lethal versus static effect is dependent on aspects of the assay system, including the number of arbitrary units, the buffer or broth, the purity of the inhibitor, and the indicator species and cell concentrations used (De Vuyst and Vandamme, 1994). The degree of activity of bacteriocins against sensitive bacteria and, indeed, the range of apparently sensitive species can sometimes be substantially increased by testing either at particular pH values (Zajdet *et al*., 1985) or in the presence of chemical agents that weaken cell wall integrity (Stevens *et al*., 1991). Some relatively resistant strains can usually be detected within a generally sensitive species, and various typing schemes have been devised which are based on either determination of the inhibitory activity of the test strains against standard sets of indicator bacteria or evaluation of the sensitivity profiles of the test strains when exposed to sets of bacterial strains known to produce different bacteriocin-like agents (Von Tersch and Carlton, 1983). Interpretation of spectra of inhibitory activity in terms of specific bacteriocin activities can sometimes be difficult if the producer strains release more than one bacteriocin-like agent (Van Belkum *et al*., 1992) or if the inhibitory activities of other metabolic products such as acids and hydrogen peroxide are not eliminated (Tagg *et al*., 1976) Several other general observations may be made which apply to the antibacterial activities of the

low-molecular-weight bacteriocins: (I) within a given species, some strains may be sensitive and others may be resistant to a particular bacteriocin; (ii) a strain that appears to be sensitive to a bacteriocin may also have some cells in the population that are resistant to it; (iii) a strain can be sensitive to one bacteriocin while being resistant to a similar type of bacteriocin; (iv) cells of a strain producing one bacteriocin can be sensitive to another bacteriocin;(v) although the spores of a strain whose cells are sensitive to abacteriocin are resistant to that bacteriocin, they become sensitive following germination; (vi) under normal conditions, gram-negative bacteria are not sensitive to bacteriocins produced by gram-positive bacteria (Jack *et al*., 1995).

Despite the widespread use of nisin as a biopreservative, until recently relatively little is known about the mechanism by which it is able to kill sensitive cells. The energy-transducing cytoplasmic membrane is the primary target of nisin. The lantibiotic nisin has, like Pep5, been shown to inhibit the biosynthesis of DNA, RNA, protein, and polysaccharides and the treated cells have no sufficient energy to perform biosynthetic processes (Sahl, 1985). It was demonstrated that nisin and epidermin use undecaprenylpyrophosphoryl-MurNAc(pentapeptide)-GlcNAc, the so-called lipid II primarily as a docking molecule for formation of highly effective, targeted pores (Brötz *et al*., 1998; Wiedemann, *et al*., 2001). Cells that were depleted of lipid II by preincubation with the lipopeptide ramoplanin are less sensitive to nisin and epidermin (Brötz *et al*., 1998). These data suggest that lipid II acts as a kind of docking molecule. Similarly, carnocin U149 might require a docking molecule for activity. The high sensitivity of nisin producing strains to carnocin U149 was suggested to be related to a protein, NisP (Stoffels *et al*., 1994). Simultaneously, cell wall biosynthesis in inhibited through arresting the precursor in the membrane. In addition, nisin is able to form non-targeted pores (Moll *et al*., 1996) and, particularly in staphylococci, it activates cell wall hydrolyzing enzymes (Bierbaum and Sahl, 1985; Bierbaum and Sahl, 1987). Nisin and Pep5 are able to release two cell wall hydrolyzing enzymes, an N-acetylmuramoyl-L-alanine amidase and an N-acetylglucosaminidase, which are strongly cationic proteins binding to the cell wall via electrostatic interactions with the

negatively charged teichoic-, teichuronic-, and lipoteichoic acids; tight binding to these polymers keeps the autolysins inactive. The cationic peptides displace enzymes from the cell wall intrinsic inhibitors by a cation exchange-like process, resulting in apparent enzyme activation and rapid cell lysis (Bierbaum and Sahl, 1987). Electron-microscopic studies have shown that activation of the autolytic enzymes appears to be predominant in the area of the cell wall septum between two daughter cells (Bierbaum and Sahl, 1991). It is suggested that following membrane depolarization by pore formation, any damage in the region of septum caused by induction of autolytic enzymes can not be repaired. In addition, since the pores formed in the membrane are not sufficiently large to allow efflux of high-molecular mass components, enhanced osmoinduced influx of water through pores and the resulting increase in osmotic pressure will encourage subsequent cell lysis (Bierbaum and Sahl, 1991). Thus, its antibiotic activity is based on a multiplicity of activities which may combine differently for individual target bacteria and explain the range of sensitivities of various bacterial species. The N-terminal part of nisin is essential for binding to the membrane bound cell wall precursor lipid II and the resulting inhibition of the peptidoglycan synthesis. However, the positively charged C-terminus of nisin is still important for the initial binding to the anionic cell wall polymer and thus for antimicrobial activity in vivo.

The low-molecular-weight bacteriocins of gram-positive bacteria generally appear to be membrane active. The lantibiotic subgroup of bacteriocins tends to differ from the other groups in the voltage dependence of their membrane insertion. Nisin does not require a membrane receptor but requires an energized membrane for its activity, which appeared to be dependent on the phospholipids composition of the membrane (Jack *et al*., 1995). In contrast, the bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent protein-mediated manner.. Both the lantibiotic and non-lanthionine-containing bacteriocins affect membrane permeability barrier by forming water-filled membrane channels or pores by barrel-stave mechanism Benz *et al*., 1991). Barrel-stave poration complexes

have been proposed to be formed between one, two, or possibly even more species of amphipathic peptides, resulting in ion leakage, loss of proton motive force, and cell death. Also, prior to the formation of pores, all of the non-lanthionine-containing bacteriocins interact with membrane-associated receptor proteins, in contrast to the antibiotic-type bacteriocins, which appear to have no such requirement (Schüller *et al*., 1989).

Studies with artificially energized cytoplasmic membrane vesicles of *B. subtilus* show that nisin, Pep5, subtilin, and SA-FF22 induce the efflux of preaccumulated radiolabelled amino acids (Jack *et al*., 1994 and Kordel *et al*., 1989). Similar studies of the action of nisin on liposomes have also confirmed that nisin dissipates membrane potential by increasing membrane permeability. Nisin and Pep5 form channels 1 nm in diameter, subtilin forms channels of 2 nm in diameter, and SA-FF22 channels are 0.5 to 0.6 nm in diameter. The mean pore lifetimes of these lantibiotics were rather high, in some cases up to 30 s (Benz *et al.*, 1991). In general, non-lanthionine-containing bacteriocins also exert their bactericidal action by destabilizing the cytoplasmic membrane of sensitive cells. Treatment of whole cells with low concentrations of Pediocin PA-1 or AcH (Bhunia *et al*., 1991) or Pediocin JD (Christensen and Hutkins, 1992) increases the permeability of the cytoplasmic membrane, as determined by the increased influx of small molecules and efflux of UV-absorbing materials from the cytoplasm. Moreover, fluorescence spectroscopy of the tryptophan residues of pediocin PA-1 has demonstrated that the peptide associate with phospholipid bilayers prior to insertion and pore formation and that pore formation possibly occurs in conjunction with a protein receptor in the cytoplasmic membrane of sensitive cells (Chikindas *et al*., 1993).

The mechanism of action of lactococcin A against both whole cells and membrane vesicles prepared from bacteriocin sensitive cells has been studied extensively (Van Belkum *et al*., 1991). In immune cells, dissipation of proton motive force (PMF) and increase in membrane permeability occurred only at very high concentrations of

lactococcin A. Treatment of cytoplasmic membrane vesicles of sensitive cells with low concentrations of lactococcin A inhibits both influx and efflux of leucine, again suggesting that dissipation of PMF and loss of permeability barrier of the membrane had resulted from lactococcin treatment. This increase in membrane permeability was also found to occur in a voltage-independent manner. In addition, treatment of membrane vesicles from immune cells with low concentration of lactococcin A did not inhibit either PMF-driven uptake or efflux of leucine, indicating that the immunity factor in these cells acts at the level of the cytoplasmic membrane. It was also observed that membrane vesicles from lactococcin A-resistant cells (including *Clostridium actobutylicum*, *B. subtilus*, *E. coli*) and liposomes derived from phospholipids purified from sensitive cells of *L. lactis* (and thus containing membrane-associated proteins) were not affected by lactococcin A. The high degree of specificity of lactococcin A action could be taken to indicate that its action is exerted through interaction with membrane-associated binding sites in the sensitive cells. Electron-microscopic investigation of sensitive cells following treatment with lactococcin A revealed no loss of membrane integrity, supporting the theory that pore formation without loss of membrane integrity lactococcin A is the cause of membrane permeabilization and cell death.

Similarly, mesentericin Y105 was shown to inhibit transport of both leucine and glutamate by dissipating membrane potential and to induce efflux of preaccumulated amino acids in sensitive cells of *Listeria monocytogenes* cells (Maftah *et al*., 1993). Moreover, mesentericin Y105 also inhibited ADP-stimulated respiration and ATP synthesis in rat liver mitochondria and adenine nucleotide translocase in beef heart mitochondria through pore formation in the energy-transducing membranes. These latter results would suggest that if receptor proteins in the membrane are associated with the action of the non-lantibiotic bacteriocins, mitochondria may also have receptors for mesentericin Y105 and these receptors may not be specific for bacteriocins. A mannose PTS permease (EII t^{Man}) has been proposed to be a target molecule for mesentericin Y105 (Dalet *et al*., 2001; Héchard *et al*., 2001) and leucocin

A (Ramnath *et al*., 2000). Besides *Listeria* strains have been shown to be cross-resistant to various subclass IIa bacteriocins while they were still sensitive to nisin, a class I bacteriocin (Rasch and Knochel, 1998). A recent study strongly points out that leucocin A actually requires an interaction with a chiral receptor at the surface of the target cell to be active (Yan *et al*., 2000). The D-enantiomer of leucocin A is not active against 10 different strains, whereas all of them are sensitive to the natural leucocin A. Taken together these results strongly indicate that EII t^{Man} could be the chiral receptor needed for bacteriocin interaction at the surface of target cells (Héchard and Sahl, 2002). Accordingly, EII t^{Man} is proposed to be a target molecule for all class IIa bacteriocins in *Listeria monocytogenes*, *Enterococcus faecalis* and likely in other sensitive bacteria (Héchard and Sahl, 2002). On the other hand, several class IIa bacteriocins are able to permeabilize the liposomes made of lipids from target cells, supporting the notion that a proteinaceous target is not necessary for activity. Taking into account these contradictory results, the following model for subclass IIa bacteriocin mode of action can be proposed (Héchard and Sahl, 2002). The bacteriocin first interacts with a target molecule, possibly the EII t^{Man} mannose permease. This interaction is absolutely required for in vivo activity against whole bacterial cells, probably favouring the forthcoming interaction of the bacteriocin with the cytoplasmic membrane. In that case, the structure and the expression level of the target molecule could influence the activity of the bacteriocin. The possible interaction of the bacteriocin with EII t^{Man} could also lead to switch this permease to an open state and could thereby participate to the permeabilization of the target cell. Secondly, the bacteriocin may interact with the cytoplasmic membrane leading to both pore formation or localized disruption of the membrane. This second step may not require a target molecule and may be mostly dependent on electrostatic and hydrophobic interactions with the membrane, explaining why bacteriocins could be active against liposomes or phospholipid vesicles (Héchard and Sahl, 2002).

The bactericidal effect of low concentrations of lactococcin B on sensitive cells has also been found to be produced by dissipation of PMF and loss of the permeability

barrier function of the membrane (Venema *et al*., 1993). In addition, the membrane functions of strains carrying specific immunity protein genes were not affected by lactococcin B even at high concentrations, while the insensitivity of sensitive cell membrane-derived liposome vesicles again indicates the probable presence of protein receptors for lactococcin in the membrane.

Since cell death seems to occur via destabilization of the cytoplasmic membrane, the bacteriocin molecules must cross the cell wall before interaction with the membrane. Many bacteriocins have been reported to have greater bactericidal activity at low pH. The peptides carry both positive and negative charges with a net positive charge below pH 7. As the pH drops below 6, the net positive charge increases due to His, and as the pH drops below 4, the net charge due to Asp and Glu residues also decreases. It has been suggested that the pH-induced alterations in net charge may facilitate translocation of some bacteriocin molecules through the cell wall (Ray *et al*., 1993). Also, both non-lanthionine-containing bacteriocins and lantibiotic molecules are adsorbed to the cell surfaces of gram-positive bacteria, irrespective of their being bacteriocin producer, nonproducer, resistant, or sensitive. The degree of bacteriocin adsorption is pH dependent, with a maximum at about pH 6.0 and a minimum at or below pH 2.0 (Yang *et al*., 1992). The molecular components on the cell surfaces of gram-positive bacteria to which bacteriocin molecules are adsorbed are thought to include teichoic and lipoteichoic acids (Bhunia *et al*., 1991).

Several reports indicates that although nisin is not active against gram-negative bacteria, membrane vesicles of derived from gram-negative cells are sensitive to it (Gao *et al*., 1991). This points out that the resistance of gram-negative bacteria to bacteriocins may be due to the relative impermeability of their lipopolysaccharide layer. Both gram-negative and resistant gram-positive bacteria can be made sensitive to pediocin AcH and nisin following exposure to sublethal stresses (Stevens *et al*., 1991). Hence, it has been suggested that adsorption of bacteriocin molecules can induce a change in cell wall barrier functions only in sensitive gram-positive bacteria,

rendering the wall more permeable to the bacteriocin. No such changes occurs following adsorption to bacteriocin-resistant cells (Kalchayanad *et al*., 1992).

1.3.10. Resistance and immunity to bacteriocins

Several factors may contribute to making a cell resistant towards bacteriocins such as the composition and structure of both cell wall and cellular membrane. In addition, certain receptors that are essential for bacteriocin action may be lacking or mutated. Also, the presence of aspecific proteases may reduce bacteriocin effectiveness.

It is reported that bacteriocin sensitive populations includes resistant cells with structural modifications, which cause them spontaneously to emerge in case of expose to bacteriocin (Noerlis and Ray, 1993). The mechanisms implicated in bacteriocin resistance involve structural and physiological changes. In the case of nisin, *Listeria monocytogenes* resistance has been attributed to changes in the fatty acid composition (Mazzotta and Montville, 1997) and the phospholipid composition (Ming and Daeschel, 1995). Change in the fatty acid composition was reported to render the membrane less fluid by which preventing the insertion of nisin molecules (Ennahar *et al*., 2000). As far as class IIa bacteriocins are concerned, the occurrence of naturally tolerant or resistant *L. monocytogenes* strains has been observed to be more frequent than with nisin (Rasch and Knøchel, 1998). It has been commented that, over time, the occurrence of naturally tolerant or resistant strains of *L. monocytogenes* may result from a sustained exposure to bacteriocin in natural environments (Larsen and Nørrung, 1993).

Bacteriocin producer strains having an immunity gene appear to produce specific immunity proteins that prevent pore formation by the bacteriocin. Also, immunity genes have been observed in lactic acid bacteria that do not produce bacteriocins at all (Hühne *et al*., 1996). It appears that lactic acid bacteria contain varying numbers of immunity genes that provide varying degrees of protection towards varying subgroups

of bacteriocins and that may also vary in terms of expression levels (Eijsink *et al*., 2002). This could play a major role in determining differences in bacteriocin sensitivity between closely related strains and, thus, in determining the target cell specificity of an individual bacteriocin (Eijsink *et al*., 2002). Very little is known about the mode of action of these immunity proteins (Nes and Holo, 2000). For class II bacteriocins, most experimental work has been done on small immunity proteins belonging to one-peptide bacteriocins. Venema *et al*. (1995) showed that the C-terminal part of the lactococcin A immunity protein is exposed to the exterior of the cell. Most results in the literature indicate that such interactions do not occur in contrast to the above observation. For example, there are no indications that bacteriocins actually bind to immunity proteins (Quadri *et al*., 1995). Also, it has been shown that sensitive cells are not protected from bacteriocin action by adding immunity protein to the culture medium (Nissen-Meyer *et al*., 1993). It was shown that while the expression of the immunity protein CbiB2 of carnobacteriocin B2 within the cells provided immunity against carnobacteriocin B2 applied externally, the addition of CbiB2 to the culture medium failed to protect sensitive cells, confirming that CbiB2 is not an extracellular protein (Quadri *et al*., 1995). These observations suggest that class IIa bacteriocin immunity proteins would be free intracellular molecules, which would prevent bacteriocin action at the membrane site indirectly via a membrane-bound protein (Ennahar *et al*., 2000).

The incidence of nisin resistance, either naturally or acquired, has been described for a range of organisms. The process by which these strains confer resistance to nisin is not known, but involves a mechanism distinct from that of nisin self-immunity exhibited by nisin producing strains. There have been a number of reports in which nisin resistance in lactococcal strains have been associated with plasmid DNA (McKay and Baldwin, 1984; Klaenhammer and Sanozky, 1985; von Wright *et al*., 1990). The most advanced genetic analysis of this form of resistance has been provided by Froseth and McKay (1991) who have identified and sequenced a gene (*nsr*) encoding a 319 amino acid protein. It has been predicted that this protein, which contains a hydrophobic

region at the N-terminus, is located in the membrane (Froseth and McKay, 1991), although how it blocks nisin function has yet to be elucidated. The *nsr* gene, isolated from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, confers nisin resistance at a level one tenth of that of nisin immunity exhibited by nisin producing strains indicating that the two mechanisms are dissimilar. Furthermore, in hybridizations nsr sequences displayed no significant homology to genomic digests of a nisin producing strain (Froseth and McKay, 1991).

1.3.11. Factors affecting bacteriocin production in lactic acid bacteria

As the production of bacteriocins is dependent on the growth and physiological activity of the producing bacteria, the amount of bacteriocin produced is proportional to the amount of biomass. Bacteriocin production in LAB is growth-associated: it usually occurs during the growth phase (Parente *et al*., 1997) ceases at the end of growth phase. A decrease of bacteriocin titer usually follows. This may be attributed to adsorption on producer cells or to degradation by specific or non-specific proteases. Adsorption to producer cells has been shown for most bacteriocins. Since adsorption of bacteriocins to cells is maximal at pH 5.5-6.5 (Yang *et al*., 1992) and decrease at low pH, it is not surprising that no reduction of bacteriocin titer is sometimes observed in fermentations without pH control (Yang and Ray, 1994). Although bacteriocin production is growth associated, because of the complex interaction of the many factors with affect bacteriocin production and desorption, maximization of growth does not necessarily result in maximization of bacteriocin production. Although an approximately linear relationship was obtained between bacteriocin production rate and growth rate for some bacteriocins in both batch and continuous fermentations (Parente *et al*., 1994), a strongly non-linear relationship has been observed for nisin in both batch (Kim *et al*., 1997) and continuous culture (Meghrous *et al*., 1992) and for plantaricin C in continuous culture (Bárcena *et al*., 1998).

A given bacteriocin can be produced by several strains or species. It has been found that nisin production varied among different strains, while pediocin AcH showed less variation (Yang and Ray, 1994). For nisin differences among strains were due to expression level and activity of maturing enzymes and, to a lesser extent, to nisin immunity.

Bacteriocin production is deeply affected by type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and inhibitors.

Bacteriocins can be produced from media with different carbohydrate sources. Nisin Z can be produced from glucose, sucrose and xylose by *Lactococcus lactis* IO-1 (Matsusaki *et al*., 1996) but better results were obtained with glucose (4000 IU ml^{-1}) compared to xylose (3000 IU ml^{-1}). Glucose, followed by sucrose, xylose and galactose were the best carbon sources for the production of pediocin AcH in an unbuffered medium (Biswas *et al*., 1991). Sucrose was found to be a better carbon source than glucose for enterocin 1146 production (Parente and Ricciardi, 1994). Both anions (phosphate) and cations (Mg^{+2}, Ca^{+2}) affect bacteriocin production and their effect is strain specific.

Tween 80 has been found to increase the production of some bacteriocins (Parente and Hill, 1992; Daba *et al*., 1993). However, Tween 80 may simply have the effect of preventing bacteriocin adsorption on polypropylene and glass surfaces thus increasing apparent bacteriocin titers. Additional of ethanol (1% v/v) increased amylovorin L471 (De Vuyst *et al*., 1999) production. The stimulatory effect was attributed to gene expression, prevention of bacteriocin aggregation and yield of bacteriocin per unit biomass produced under stress conditions.

1.3.12. Screning of bacteriocin producing strains

There are many techniques for the detection of bacteriocin producing strains. These techniques are subdivided into two main groups, direct and indirect screening methods and are based on the diffusion of bacteriocins through solid or semi-solid culture media to inhibit the growth of sensitive indicator strain (De Vuyst and Vandamme, 1994). In direct or deferred antagonism methods consist of the spot-on-the-lawn and flip-streak methods. In the spot-on-the-lawn method, the putative bacteriocin producer is spotted on an agar medium and incubated overnight to develop colonies. Next, the colonies are overlayered with a sensitive indicator organism and incubated to develop zones of inhibition. In the flip-streak method, the putative bacteriocin producing strain is streaked on to the medium, incubated and a bacteriocin sensitive strain is streaked perpendicular to the first on the reverse side of the agar (De Vuyst and Vandamme, 1994). In the direct method (Tagg *et al*., 1976), both the putative bacteriocin producer and the indicator organism are incubated simultaneously to develop zones of inhibition early in the growth phase of the bacteriocin producer organism. In the well-diffusion method (Tagg and McGiven, 1971) supernatants from the putative bacteriocin producing organism are placed in the wells cut into the agar inoculated with a sensitive microorganism. The density of the indicator microorganism is an important factor for the sensitivity of the method. Also, it is important to test several indicator microorganisms while screening strains for bacteriocin production.

The clear inhibition zones on the indicator lawn around the colonies or wells can indicate bacteriocin production. However, there are other antagonistic substances produced by lactic acid bacteria in addition to bacteriocins including organic acids, hydrogen peroxide, etc. Bacteriophages may also be responsible for inhibition zones. Therefore, some precautions must be taken to avoid inhibition by other substances in the early stages of screening process. The use of pH-neutralized, catalase-treated supernatants of the producer organism can eliminate a possible inhibitory effect on the indicator organism due to lactic acid and hydrogen peroxide, respectively. Hydrogen

peroxide production can also be eliminated by anaerobic inhibition (De Vuyst and Vandamme, 1994).

Growth inhibition by contaminating phages can be avoided using the flip plate assay of Kékessy and Piguet (1970), which involves an inversion of the agar plate before overlayering with the indicator organism in order to put an agar barrier between the bacteriocin producing cells and indicator cells. The improved deferred sandwich method of Héchard *et al*. (1990) and examination of clear inhibition zone on a spot deferred antagonism assay plate for the presence of lytic bacteriophages (Lewus *et al*., 1991) may further confirm inhibition due to the release of bacteriocins. Moreover, bacteriocin inhibition will result in the formation of diminishing zones of inhibition after spotting samples diluted to extinction, and will not result in individual plaque formation as is the case in bacteriophage inhibition.

1.3.12.1 Detection specific bacteriocin-producing lactic acid bacteria by colony hybridization and dot blot

Although bacteriocin activity can easily be assayed, purification and amino acid sequencing is the only method available to determine exactly which bacteriocin is being expressed by a particular strain. Obviously, such an approach cannot be used for routine screenings because of the specialized equipment and personnel required Martínez *et al*., 1998).

DNA techniques, such as polymerase chain reaction (PCR) or DNA hybridization, are widely used to detect specific sequences and therefore, they could be applied for the identification of genes belonging to the operon or gene cluster of a specific bacteriocin. Although the detection of such genes does not guarantee its expression, the development of specific probes and/or primers would be very useful when searching for a novel bacteriocin. Those strains with the potential to produce bacteriocins whose biosynthesis is encoded in genes already sequenced could easily be discarded. In

addition, specific probes and primers would be a valuable tool for studying the behaviour of bacteriocinogenic LAB used as starter cultures in food fermentations, especially when studying combinations of strains that produce different bacteriocins (Martínez *et al*., 1998).

Martinez *et al*. (1998) developed a colony hybridization method for detecting lactic acid bacteria encoding specific bacteriocins. Specific PCR-generated probes were used to detect colonies of pediocin PA-1, lactococcin A, enterocin AS-48, nisin A and lacticin 481 producing strains. The probes were shown to be sensitive and specific for sequences belonging to the structural genes of the respective bacteriocins.

1.3.13. Methods for the measurement of bacteriocin activity

The methods used for assessing the efficiency of antimicrobial compounds have largely been developed to encounter the needs of clinical microbiology (Skyttä and Sandholm, 1991). In the early years no efforts were made to standardize the methods and therefore the methodology used has been somewhat inconsistent. Various modifications of agar diffusion assays, however, as well as test procedures in liquid media are widely used and partly standardized for testing the activity of antimicrobial agents. More recently the demand for rapid but still reproducible and reliable methods has strongly increased (Skyttä and Sandholm, 1991).

The methods used for the screening of food antimicrobials may be divided into end-point and descriptive methods (Davidson and Parish, 1989). The most widely used endpoint method is the agar diffusion assay although its limitations are generally recognized. Since the size of the inhibition zone is dependent on the rate of diffusion misleading results may be obtained if the agent to be tested is hydrophobic and will not diffuse into the agar. Furthermore, the endpoint methods scarcely provide information of the effects of the compounds on the growth kinetics of the microorganism (Skyttä and Sandholm, 1991). In descriptive tests sampling or automated recording of

microbial growth is carried out at timed intervals. Consequently, one can evaluate the effects of of an antimicrobial over a longer period of time due to the kinetic recording of the results. Various growth curve parameters can be used to describe the inhibitory effects. The extension of the lag phase is probably the most widely used parameter. It has been shown that in a food system, even a slight delay of a lag phase may have an important influence on the shelf life. Other parameters, such as end-absorbance (Mattila, 1987), slope, which is a gradient of the exponential growth phase (Adams and Hall, 1988), and area under the growth curve have also been used (Mattila and Sandholm, 1989).

Quantitative measurements of bacteriocin activity are essential for most of the activities with respect to their characterization. Several methods have been used for the quantitation of the lantibiotic nisin (De Vuyst and Vandamme, 1994); due to its importance as food preservative, standard units and methods have been defined for this substance and a commercial standard preparation is available. Methods for the measurement of bacteriocin activity are usually derived from those of antibiotics. Modifications (spot, well, disc diffusion) of agar diffusion assay (Pucci *et al.*, 1998; Harding and Shaw, 1990) and photometric (Geis *et al*., 1983; Zezza *et al*., 1983) methods have been widely used. Methods based on conductance and bioluminescence have been used less frequently (Giraffa *et al*., 1990; Reid *et al*., 1990).

The criteria used for the determination of inhibitory effects by turbidometry are based on the changes of visible turbidity and is carried out by the measurement of absorbance, i.e., optical density of the indicator culture in liquid medium. One of the most basic turbidometric applications is to measure the end absorbance value after a specified incubation period in parallel in the cultures with and without the bacteriocin. Mørtvedt and Nes (1990) applied a scale for measuring bacteriocin activity where a unit of activity was defined as a reciprocal of the highest dilution of a 20-µl sample resulting in a 50% decrease of maximum absorbance after an overnight incubation at 30^0C. Furthermore, turbidometric methods have been widely used for the quantitative

determination of minimal inhibitory concentration (MIC). MIC is often defined simply as the lowest concentration of the substance to be tested which results in no visible turbidity after 24 h of incubation (Davidson and Parish, 1989). Besides the quantification of overall antimicrobial activity, the turbidity measurements carried out at constant intervals may simultaneously provide information on dose response effects for example, loss of viability and rate of lysis in the indicator strain culture as well as data on the effects of various physicochemical factors. (Skyttä and Sandholm, 1991)

Skyttä and Mattila-Sandholm (1991) used automated turbidometry for assessing the antimicrobial efficacy of bacteriocin-like inhibitors produced by *Pediococcus damnosus* and *Pediococcus pentosaceus* against *Pseudomonas aeruginosa*, *Pseudomonas fragi*, *Salmonella enteritidis* and *Salmonella infants*. The growth of the indicator organisms was kinetically monitored throughout the incubation period and various growth curve parameters were used as quantitative indicators of growth inhibition. The results showed that a kinetic method based on automated turbidometry provides a sophisticated tool for the determination of bacteriocin activity. A number of growth curve parameters were applicable for the quantification of inhibitory effects. However, the area value after 24 h appeared to describe most effectively the overall inhibitory effects since all the other growth curve parameters were simple end-point values giving information solely at a single point of time. The authors reported that the area reduction rate scale used provides a much more accurate method of measurement for the determination of potency than ever can be achieved by the millimeter scale used in agar diffusion tests.

Since standard solution of bacteriocins is generally not available, critical dilution assays (CDA) are used with arbitrary units to express activity. The titer of the bacteriocin solution is defined as the reciprocal of the lowest amount of bacteriocin solution causing a definite inhibition of the indicator strain (Eugenio *et al*., 1995). This method is semi-quantitative, provides only a discontinuous scale for activity and always requires that a complete series of dilutions is carried out to estimate activity.

Although CDA are easy to use and suffice for most applications, they are of little value when statistical comparisons on quantitative measurements of bacteriocin activity are to be carried out. When agar diffusion assays and photometric methods are based on a dose /response curve to estimate bacteriocin activity, a continuous scale for activity is obtained. Both agar diffusion and photometric methods for the quantitation of activity of antimicrobials have their drawbacks and the lack of standard solutions make the construction of dose/response curves difficult (Eugenio *et al*., 1995).

1.3.14. Applications of bacteriocins

There are various preservation techniques used to avoid food poisoning and spoilage, such as reduction in temperature, water activity, pH in addition to preservatives such as antimycotic (e.g., natamycin), inorganic (sulfate, nitrite) and organic compounds (propionate, sorbate, benzoate) that are retard microbial growth (Garneau *et al*., 2002). Also, there are other technologies to restrict access of microorganisms to food products such as pasteurization, sterilization, packaging and aseptic processing. With the increasing demand for more natural and microbiologically safe food products, new preservation methods have been developed.

The production of a bacteriocin with a broad spectrum of antibacterial activity against other genera of lactic acid bacteria can be an important property for starter cultures and is of special interest in controlled lactic acid fermentation of meat products and plant materials such as vegetables and silage. The use of bacteriocinogenic lactic acid bacteria as starter cultures or bacteriocins as food additives may be an effective approach to control food spoilage and foodborne pathogenic microorganisms such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*, etc. (Okereke and Montville, 1991) The use of bacteriocins to inhibit pathogens is especially attractive in products such as minimally processed refrigerated meats where high acidity is undesirable and which rely solely on refrigeration as a means of preservation. *L. monocytogenes* and *C. botulinum* are of

particular concern in these products, because the former will grow at refrigerated temperatures and heat resistant spores of the latter would survive a minimal heat process and germinate, grow, and produce toxin in temperature-abused food. The use of bacteriocins in heat-processed foods may reduce the thermal process intensity and both improve the nutritional and organoleptic properties of the food and minimize the heat processing cost.

Many lactic acid bacteria have important roles in the production of fermented foods, and some of these bacteria have been shown to be capable of inhibiting the growth of a wide variety of food spoilage organisms (Stiles and Hastings, 1991). The classic example of a commercially successful naturally produced inhibitory agent is nisin. Known since 1928 to be produced by some *Lactococcus lactis* isolates and structurally characterized in 1971 as a lanthionine-containing peptide (Jack *et al*., 1995), nisin and nisin-producing strains have had a long history of application in food preservation, especially of dairy products (Hurst, 1991). Recognition that nisin may be produced by *Lactococcus. lactis* strains while they are naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested has led the U.S. Food and Drug Administration to accord GRAS (generally recognized as safe) status to nisin. Nisin has been employed for over 50 years and is currently approved in more than 60 countries for use in cheese, liquid egg products, canned vegetables, pasteurized dairy food products and salad dressing (Garneau *et al*., 2002).

The major spoilage problem in the brewing industry is associated with lactic acid bacteria and nisin has been investigated as a novel protective strategy. The fact that the primary beer fermentation relies on yeasts which are quite insensitive to nisin is a particular advantage over the situation in dairying where both the starter organisms and the spoilage agents are nisin sensitive gram-positive bacteria. Nisin has also proved effective in bottle conditioned beers (Ogden, 1987) and in pasteurized products it may allow a reduction in the intensity of heat treatment with analogous advantages to those in the canning industry. Additional applications have been investigated in both the

wine making (Radler, 1990; Daeschel *et al*., 1991) and the distilling industries (Henning *et al*., 1986). The capacity to protein engineer the nisin molecule may also provide variants that are more potent and effective in a wider range of physical environments or against a wider range of spoilage and food-poisoning organisms (Gasson and De Vos, 1994).

Enterocin was shown to control the growth of *Listeria monocytogenes* in chicken breast, ham, and pork. Lacticin 3147 protects cottage cheese and natural yoghurt against *L. monocytogenes* and *Bacillus cereus* (Garneau *et al*., 2002). Lacticin 3147 has been shown to have potential for the prevention of mastitis which is one of the most costly diseases in dairy cows (Ryan *et al*., 1998; Ryan *et al*., 1999).

In addition to bacteriocins with antibacterial activity, antiviral and antitumor activity of some bacteriocins has been discovered. Enterocin CRL35 is a class IIa bacteriocin from *Enterococcus faecium* CRL35 that has been shown to exert antiviral activity against thymidine-kinase positive and deficient strains of herpes simplex (HSV) type 1 and 2 in Vero and BHK-21 cells (Wachsman *et al*., 1999). Studies showed that virus adsorption and penetration are not affected. Instead, a late step of virus multiplication is prevented.

Watanabe and Saito (1980) studied the cytotoxicity of pyocin S2 produced by *Pseudomonas aeruginosa* M47 to virally transformed mammalian cells, human malignant cells and normal cells in the same species. Pyocin S2 inhibited the growth of not only tumor cells but also normal cells. Hill and Farkas-Himsley (1991) also reported that a partially purified bacteriocin from *Escherichia coli* HSC10 is toxic to KHT cells growing in vivo as micro metastases.

Hetz *et al*. (2002) studied the cytotoxic effect of Microcin R492 produced by *Klebsiella pneumoniae* on HeLa cells. At low (5µg/ml) and intermediate (10µg/ml) concentrations, microcin E492 induced biochemical and morphological changes

typical of apoptosis, such as cell shrinkage, DNA fragmentation, extracellular exposure of caspase activation and loss of mitochondrial membrane potential. Induction of apoptosis by microcin E492 was associated with the release of calcium from intracellular stores, probably after microcin-triggered ion-channel formation.

1.4. Recovery and purification of bacteriocins

The methods applied to bacteriocin purification are those which are generally used in protein purification.

1.4.1. Prerequisites for purification

The assay method used to follow purification needs to be rapid, simple, and not necessarily very accurate. Unfortunately, such methods are conspicuous by their absence. Simple, inaccurate methods exist but speed is difficult to achieve in a biological assay.

A rapid method for determining protein is also required so that specific activity (i.e. units bacteriocin per mg protein)-which gives a measure of purity-may be estimated. This need is fulfilled by the method of determining absorption at 280 nm with a spectrophotometer. Inaccuracies are introduced by the presence of nucleic acids but can be partially corrected for if measurements are taken at both 260 nm and 280 nm and a formula such as the one given below applied (Mayr-Harting *et al*., 1972):

$$\text{Protein (mg/ml)} = 1.45\ E_{280} - 0.74\ E_{260}$$

Alternatively protein may be estimated by a calorimetric method using the Folin phenol reagent (Lowry *et al*., 1951). It is also useful to adopt the practice of drawing up a table indicating the progress of the purification, including information of the

following type: volume (ml), concentration (units/ml), total activity (%), and degree of purification.

1.4.2. Concentration and removal of small molecules

Unfortunately, it is not always possible to obtain a sufficiently concentrated source of bacteriocin and hence large initial volumes of solution are required which means that a first concentration and purification step is necessary. Generally speaking, during concentration and purification the temperature should be kept as low as possible and the pH carefully controlled.

Ultrafiltration membranes have been used for concentration and purification of some bacteriocins by testing a variety of cut-off values. However, if large volumes have to be dealt with this method is impractical. These methods are very useful where they follow a concentration process such as ammonium sulphate precipitation or fractional absorption by which initial large volumes may be easily reduced. Ammonium sulphate precipitation is frequently used as a preliminary concentration step and achieves in addition some purification. Precipitation with ammonium sulphate also dissociates bacteriocin aggregates and results in high specific activities.

Researchers have been familiar with pellicle formation when performing ammonium sulphate precipitation of cell free supernatant containing bacteriocin of lactic acid bacteria. When crude cell free culture supernatant made up to 35-40% ammonium sulphate saturation, a surface pellicle containing the bacteriocin is recovered. Contreras *et al.* (1997) achieved Lactobin A purification from *Lactobacillus amylovorus* LMG P-13139 by ammonium sulphate precipitation of culture supernatant brought to 35% saturation, methanol-chloroform (1:2, vol/vol) extraction and reverse phase chromatography on PepRPC HR 5/5. SDS-PAGE of floating pellicle samples revealed that only small amounts of extraneous proteins were present in this fraction. Bacteriocin activity could be localized by overlayering the denaturing SDS-PAGE gel

with a lawn of indicator organisms. However, no distinct banding pattern apart from one large spot streaking from 4.5 kDa to approximately 6.5 kDa could be observed. Also this floating pellicle fraction was eluted as a smearing peak that did not correspond to the sharp peaks obtained from reverse phase chromatography. This chromatography result and detection of fatty acids with Oil O Red staining indicated the formation of large lactobin A aggregates due to hydrophobic interaction of lactobin A with fatty acids. Examination of the pellicle fraction revealed that chain length of the fatty acids in this fraction showed perfect homology with the composition of Tween 80, a non ionic detergent present in MRS broth. Tween 80 constituted the most important component of the lactobin A micelles. A simple one-step methanol-chloroform extraction removed most fatty acid contamination from pellicle fraction and distinct banding pattern of reverse phase chromatography was obtained.

When purifying bacteriocins of lactic acid bacteria, ammonium sulphate precipitation is used before cation-exchange or hydrophobic interaction chromatography. If cation-exchange chromatography is to be performed, dialysis or desalting is obligatory in order to reduce the ionic strength of bacteriocin solution. Another approach for this purpose is to dilute bacteriocin solution with distilled water or a suitable buffer.

Bacteriocin solutions can also be concentrated with vacuum evaporation, freeze drying, and organic solvents including chloroform, chloroform-methanol, ethanol and acetone. Which of these methods is chosen will depend on the volume and nature of the starting material, the apparatus available, and the sequence of the purification procedures. Following this initial step the impure material is subjected to a suitable combination of some of the purification methods given below.

1.4.3. Fractional precipitation

Ammonium sulphate is the reagent most frequently used to bring about precipitation and some examples where it has been used are cited above. The reason for the choice

of ammonium sulphate is that it is highly soluble and the solubility does not vary much with the temperature. It has, however, the disadvantage that concentrated solutions are acidic and hence the pH must be adjusted with either ammonium hydroxide or sulphuric acid if pH alteration during fractionation is to be avoided. Precipitation may also be brought about by lowering the dielectric constant of the bacteriocin solvent by addition of organic solvents. It is essential to carry out precipitation by organic solvents at low temperature since a considerable amount of heat is generated when solvents such as ethanol are added to water.

Pediocins from *Pediococcus acidilactici* NCIM 2292, *P. pentosaceous* NCIM 2296 and *P. cerevisiae* NCIM 2171 were partially purified by fractional precipitation with cold acetone (Jamuna *et al*., 2004). Pediococcus cells were cultured in 1-l unbuffered TGE broth and cell free supernatant was concentrated to one-tenth of the original volume in a rotary vacuum evaporator and cooled to below 0^{o}C for precipitation in ice-cold acetone. Ice-cold acetone was gently added with constant stirring to attain 60% saturation and the mixture was incubated for 10 min at temperatures below 0^{o}C. Precipitated major broth proteins were removed by centrifugation in a Sorvall refrigerated high-speed centrifuge. The supernatant was subjected to rotary vacuum evaporation to remove acetone, adjusted to pH 5.0, and concentrated to one-fifth of the culture concentrate. The sample was then precipitated with 80% acetone, the precipitate was reconstituted in distilled water, and the residual acetone was removed by rotary evaporation, the pH of bacteriocin preparation was adjusted to 5.0 and activity was measured thereafter.

1.4.4. Fractional adsorption

Although this method is widely used in enzyme purification it has been applied to the purification of bacteriocins in a relatively few instances. Janes *et al*. (1998) developed a rapid extraction for nisin, pediocin RS2, leucocin BC2, lactocin GI3, and enterocin CS1 from concentrated freeze-dried crude culture supernatants by adsorption onto acid

or alkaline rice hull ash or silicic acid. Rice hull ash is a co-product of rice milling and is composed primarily of silica and carbon (Proctor and Palaniappan, 1990). Analysis of rice hull silica by infrared spectroscopy indicated it was similar to silicic acid (Proctor *et al*., 1995). A lipase from *Candida cylindracea* was immobilized effectively onto Rice hull ash (Tantrakulsiri *et al*., 1997) Bacteriocins were adsorbed onto Rice hull ash or silicic acid by a pH-dependent method and desorbed by decreasing the pH to 2.5 or 3.0 and heating at 90^0C for 5 min. The maximum adsorption and optimal pH range for different bacteriocins were as follows: nisin, 97% at pH 7.0; lactocin GI3, 94% at pH 6.0; pediocin RS2 97% at pH 8.0 to 9.0; leucocin BC2, 88% at pH 9.0; and enterocin CS1, 94% at pH 5.0. The desorption level of lactocin GI3 or enterocin CS1 from the surfaces of both Rice hull ash and silicic acid was 94%, while the desorption level of pediocin RS2 and leucocin BC2 was 50% or less. Nisin was desorbed readily from silicic acid (91%) but not from Rice hull ash (50% or less). The adsorption of bacteriocins onto Rice hull ash and silicic acid increased with the increasing concentration of bacteriocins. Analysis of the desorbed bacteriocins after dialysis and sodium dodecyl sulfate-16% polyacrylamide gel electrophoresis showed a single band that gave a single inhibition zone when overlaid with *Lactobacillus plantarum* for detection of lactocin GI3, enterocin CS1, and nisin. It is indicated that adsorption of bacteriocins onto Rice hull ash and silicic acid was due to a multilayer of protein-protein weak hydrogen bonds that were influenced by electrostatic interactions. Rice hull ash appeared useful for extraction, concentration, and partial purification of the five bacteriocins.

1.4.5. Fractional denaturation

Differences in the relative stability of some bacteriocins enable partial purification to be achieved by heat treatment. For example, the minor contaminating fraction in the crude colicin produced by *Shigella sonnei* P9 (O) can be destroyed by pasteurization at 65^0C for 20 min leaving it pure with respect to colicin E2 (Mayr-Harting *et al*., 1972).

1.5. Purification of bacteriocins of lactic acid bacteria

The characterization of bacteriocins is dependent on their purification. Elucidation of their biochemical structure requires homogeneity as well as an adequate yield of protein. Purification of bacteriocins, especially those produced by lactic acid bacteria, has proven to be a difficult task. Problems encountered during purification are related to the tendency of such molecules to associate with other molecular substances, their hydrophobicity, etc. Additionally, since they comprise such a heterogeneous group of substances, specific purification protocols need to be devised for each bacteriocin. This explains why only few bacteriocins have been purified to homogeneity: nisin (Berridge *et al*., 1952; Cheesman and Berridge, 1957), pediocin AcH (Bhunia *et al*., 1988), lactacin F (Muriana and Klaenhammer, 1991), carnocin U149 (Stoffels *et al*., 1992), curvacin A (Tichaczek *et al*., 1992), sakacin P (Tichaczek *et al*., 1992), mesentericin Y105 (Hechard *et al*., 1992), sakacin A (Holck *et al*., 1992) and lactococcin G (Nissen-Meyer., 1992).

The research group of Nes devised a scheme for the purification of low-molecular weight bacteriocins of lactic acid bacteria, including four steps: (i) ammonium sulfate precipitation; (ii) cation exchange chromatography; (iii) hydrophobic interaction chromatography; (iv) reverse-phase high performance liquid chromatography.

The application of cation-exchange and hydrophobic interaction chromatography is convenient since bacteriocin of most lactic acid bacteria posses surplus of positively charge amino acids and strong hydrophobic nature. Yang *et al*. (1992) developed a purification protocol based on the property that bacteriocins of lactic acid bacteria are adsorbed on the cells of producer strain. Dextranicin 24 (Revol-Junelles and Lefebvre, 1996) from *Leuconostoc mesenteroides* subsp. *dextranicum* strain J24, pediocin AcM (Elegado *et al*., 1997) from *Pediococcus acidilactici* M, leucocins A-, B- and C-TA33a (Papathanasopoulos *et al*., 1998) from *Leuconostoc mesenteroides* TA33a were purified by pH mediated cell adsorption-desorption method and reverse-phase

chromatography. Wu *et al*. (2004) purified a bacteriocin from *Pediococcus pentosaceus* ACCEL by pH mediated cell adsorption-desorption and ion-exchange FPLC. Bovicin 255 was purified from a rumen *Streptococcus* spp. by pH mediated cell adsorption-desorption method, ammonium sulphate precipitation, reverse-phase and gel filtration chromatography on fast protein liquid chromatography system (Whitford *et al*., 2001).

The cationic and hydrophobic nature of bacteriocins of lactic acid bacteria is used for their recovery from complex fermentation broths which contain high levels of peptides (10-30 g l^{-1} compared to a bacteriocin concentration of 10-100 mg l^{-1}). Laboratory purification procedures include an ammonium sulphate precipitation step, followed by various combinations of ion-exchange and hydrophobic interaction chromatography, with a final RP-HPLC purification step (Parente and Ricciardi, 1999). Chromatography may be replaced by preparative isoelectric focusing (Venema *et al*., 1997) and immunoaffinity chromatography has been reported for one-step purification of nisin A (Suárez *et al*., 1997). Although these procedures may provide excellent results in terms of purification and yield, they are not suitable for large scale bacteriocin recovery and purification.

Bacteriocins can be adsorbed on HIC (hydrophobic interaction chromatography) and cation exchange resins. Nisin and carnocin U149 have been purified to homogeneity with a simple two step protocol based on adsorption on HIC and cation exchange resins (Stoffels and *et al*., 1993). Octyl sepharose or phenyl sepharose are predominantly used for hydrophobic interaction chromatography. Amberlite XAD-16 (Casaus *et al*., 1997) and Sep-Pak C_8 cartridges (Héchard *et al*., 1999) were also used as hydrophobic adsorbents for a variety of bacteriocin of lactic acid bacteria. Amberlite XAD-16 was used in the purification of Carnocin UI49 from *Carnobacterium piscicola* (Stoffels *et al*., 1993) and of enterocin B from *Enterococcus faecium* T136 (Casaus *et al*., 1997).

As indicated previously, Rice hull and silicic acid were tested for extraction and purification of nisin, pediocin RS2, leucocin BC2, lactocin GI3, and enterocin CS1 (Jane *et al*., 1998). Based on SDS-PAGE of desorbed bacteriocins, it was found that lactocin GI3, enterocin CS1 and nisin were pure.

Diatomite calcium silicate can also be used for isolation of bacteriocins from lactic acid bacteria. Nisin, pediocin PO2, brevicin, and piscicolin126 were extracted from fermentation broth by adsorption onto Micro-Cel (a food grade diatomite calcium silicate anticaking agent) and subsequent desorption with SDS (Coventry *et al*., 1996). Adsorption of nisin, pediocin, piscicolin and brevicin onto Micro Cel E was relatively specific. The specificity of adsorption allowed substantial degree of purification of these bacteriocins based on nitrogen determination. The specificity of adsorption of these bacteriocins was reported to be due to the hydrophobic properties of the peptides. Unfortunately, the removal of SDS (by cold precipitation) was only partial (60-70%).

A simple, two-step purification protocol based on detergent (Triton X-114) phase partitioning and cation-exchange chromatography was applied for the purification of divercin V41 (Métivier, *et al*., 2000).

Li *et al*. (2001) optimized the conditions for nisin extraction in PEG/Salt aqueous two-phase systems. The PEG/Salt aqueous two-phase systems have hydrophobicity differences between the two-phase systems. Since nisin is a hydrophobic polypeptide, it was partitioned to the PEG-rich top phase. This procedure was reported to be an economic approach in large-scale nisin recovery.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains and media

Bacteriocin producing *Leuconostoc mesenteroides* subsp. *cremoris* used in this study was isolated by Dr. Yurdugül from Kavaklıdere wines. *Enterococcus faecalis* LMG 2602 was used as indicator microorganism in all assays. The other indicator microorganisms used are given in 2.4 and propagated for 16 h in the following media, and at the temperatures indicated; *Lactobacillus* (MRS, 37^0C), *Leuconostoc* (MRS, 30^0C) *Staphylococcus, Listeria*, *Pediococcu*s, *Bacillus*, *Lactococus, Klebsiella, Salmonella* and *E. coli* (Tryptone soy broth or M17, with 0.5 % glucose, 30^0C).

2.2. Bacteriocin activity assay

Leuconostoc mesenteroides subsp. *cremoris* was grown for 16 h in MRS broth at 30^0C. The culture was centrifuged at 12 000 rpm for 30 min at $+4^0$C and heat treated to inactivate proteases before it was applied. The serially two-fold dilutions of the culture supernatant were assayed for bacteriocin activity by the spot-on-the lawn technique. Five µl of supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* was spotted onto the surface of indicator lawn. *Enterococcus faecalis* LMG 2602 was routinely used as the indicator culture. Indicator lawns were prepared by adding 0.1 ml of overnight culture to 10 ml of M17 soft agar (0.75 %). The contents of the tubes were mixed by vortex and poured over the surfaces of pre-poured agar plates. Serial two-fold dilutions

were carried out in MRS broth used for the producer strain *Leuconostoc mesenteroides* subsp. *cremoris*. The bacteriocin titer was defined as the reciprocal of the highest two-fold dilution showing complete inhibition of the indicator lawn and was expressed in activity units (AU) per ml of culture media. The titer of the bacteriocin solution, in AU/ml, was calculated as (1000/d) D, where D is the dilution factor and d is the dose.

2.3. Production of bacteriocin

1 l MRS broth was inoculated with 0.1 % (v/v) *Leuconostoc mesenteroides* subsp. *cremoris* and propagated for 16 hours with an initial pH of 6.5-7.0 at 30^0C. Following incubation, culture fluid was heated for 80^0C at 10 min to inactivate proteases. Then, cells were removed by centrifugation at 12 000 rpm for 30 min.

2.3.1. Effect of pH on the bacteriocin production of *Leuconostoc mesenteroides* subsp. *cremoris*

100 ml each of MRS broth was adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, and inoculated (0.1 %) with an overnight culture of *Leuconostoc mesenteroides* subsp. *cremoris*. Following incubation at 30^0C for 16 h, bacteriocin activity was measured.

2.3.2. Effect of temperature on the bacteriocin production of *Leuconostoc mesenteroides* subsp. *cremoris*

100 ml each of MRS broth was adjusted to an initial pH of 6.5-7.0 and inoculated (0.1 %) with an overnight culture of *Leuconostoc mesenteroides* subsp. *cremoris*. The bacteriocin production studies were carried out at 10, 15, 20, 25, 30 and 37 ^{0}C.

2.4. Activity spectrum of the bacteriocin

The following microorganisms were employed as indicator: *Lactobacillus plantarum* LP73, *Lactobacillus plantarum* Z11L, *Lactobacillus plantarum* HÜ (Hacettepe University), *Lactobacillus salivarius* M7, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus plantarum* 80B, *Lactobacillus plantarum* DSM 20246 and *Lactobacillus plantarum* DSM 20174 were kindly provided by Assoc. Prof. Dr. Candan Gürakan, Food Engineering Department, Middle East Technical University. *Lactobacillus plantarum* RSSK 10, *Lactobacillus delbrueckii* subsp*. lactic* RSSK 498, *Lactobacillus plantarum* RSSK 675, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923, *Lactobacillus cremoris* RSSK 708 and *Listeria monocytogenes* were provided from Refik Saydam Type Culture Collection, Ankara.

Lactobacillus fructivorans, Lactobacillus delbrueckii subsp. *delbrueckii*, *Lactobacillus casei* NRRL 21, *Lactobacillus casei* NRRL 22, *Lactobacillus casei* NRRL 23, *Lactobacillus casei* NRRL 334, *Lactobacillus casei* NRRL 441, *Bacillus subtilus*, *E. coli* O157:H7, *Klebsiella pneumoniae Salmonella enteritidis* and *Streptococcus thermophilus* were from Food Engineering Department of Middle East Technical University.

Lactobacillus sake NCDO 2714 (LMG 2313), *Lactobacillus plantarum* LMG 2003, *Enterococcus faecalis* LMG 2602, *Listeria innoqua* LMG 2813, *Lactococcus lactis* LMG 3113, *Lactococcus lactis* LMG 2088, *Lactococcus lactis* LMG 2907, *Lactococcus lactis* LMG 2908, *Lactococcus lactis* LMG 2909, *Lactococcus lactis* LMG 2910, *Lactococcus lactis* LMG 2911, *Lactococcus lactis* LMG 2912, *Lactococcus lactis* SIK-83, *Lactococcus lactis* IL-1403, *Lactococcus lactis* subsp. *cremoris* LMG 2132, *Pseudomonas fluorescens* LMG 3020, *Bacillus cereus* LMG 2732, *Pediococcus pentosaceus* LMG 2001, *Staphylococcus aureus* LMG 3022, *Staphylococcus carnosus* LMG 2709, *E. coli* LMG 3083 and *Salmonella enterica*

typhimurium SL 1344 LMG 3085 were kindly provided by Prof. Dr. Mustafa Akçelik, Department of Biology, Ankara University.

Salmonella paratyphi B 33653, *Salmonella paratyphi* B 33656, *Salmonella paratyphi* B 34323, *Salmonella paratyphi* B 38359, *Salmonella paratyphi* B 38470, *Salmonella paratyphi* B 38518, *Salmonella enteritidis* 38055, *Salmonella enteritidis* 38383, *Salmonella enteritidis* 33280, *Salmonella choleraesuis* 34088, *Salmonella choleraesuis* 21, *Salmonella paratyphi* A 2961 and *Salmonella paratyphi* B 4620 were provided from Department of Microbiology of GATA (Gülhane Military Medical Faculty).

2.4.1. Effect of high concentrations of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* against insensitive species and strains

Bacteriocin containing culture supernatant fluid (1 liter) of *Leuconostoc mesenteroides* subsp. *cremoris* was concentrated by ammonium sulphate precipitation and tested against insensitive gram-positive and gram-negative bacteria with the spot-on-the-lawn method. Proteins were precipitated by the slow addition of ammonium sulphate to the supernatant, with 80% saturation and stirring at $+4^0$C overnight. The precipitate was collected by centrifugation at $+4^0$C for 30 min at 12 000 rpm. The pellet was resuspended in 20 ml 0.05 M phosphate buffer, pH 6.5. Ammonium sulphate precipitation was performed with 40%, 50%, 60% and 70% saturation previously. However, these fractions did not recover all the bacteriocin in the supernatant except for 80% saturation.

2.4.2. Comparison of bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* with other bacteriocins on common indicators

Bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* was compared with lacticin from *Lactococcus lactis* LMG 2907, lacticin 3147 from *Lactococcus lactis* LMG 2910, *Lactococcus lactis* LMG 2911, *Lactococcus lactis* LMG 2912, lacticin

481 from *Lactococcus lactis* LMG 3113, lactococcin G from *Lactococcus lactis* LMG 2088 and nisin from *Lactococcus lactis* SIK-83 on common indicator bacteria. A strain for which the highest dilution factor of a bacteriocin preparation caused a clear inhibition zone was considered to be the most sensitive.

2.5. Sensitivity of the bacteriocin to enzymes

The following enzymes (1mg/ml) were dissolved in the appropriate buffers as follows: Proteinase K, 0.05 mol/l sodium acetate, pH 6.2; trypsin, 0.04 mol/l Tris-hydrochloride, 0.1 mol/l CaCI2, pH 6.2; lysozyme, 50 mmol/l potassium phosphate, pH 6.3; pepsin, 0.2 M citrate buffer, pH 6.0; alkali serine protease and chymotrypsin, 0.02 mol/l Tris-HCI, pH 7.8; α-amylase, 0.03 mol/l sodium acetate buffer, pH 6.0 and catalase, 0.05 M phosphate buffer, pH 6.0. Controls included buffer; buffer and enzymes; buffer and heat-inactivated enzymes; and buffer plus bacteriocin. Bacteriocin-enzyme reaction mixtures and all controls were incubated at 37^0C for 2 h, after which titers of the bacteriocin were determined.

2.6. Sensitivity of the bacteriocin to dissociating agents

Crude bacteriocin was treated with the following reagents: 0.1 % Tween 80, 1.0 % Tween 80, 6 M Guanidine hydrochloride, 8 M urea, 1 % SDS, 0.1 % SDS, 5 %SDS, 0.2 % β-mercaptoethanol, 2 % β-mercaptoethanol, 0.2 % β mercaptoethanol + 0.1 % SDS, 2.0 % β-mercaptoethanol + 1.0 % SDS, 1.0 % Triton X-100, 0.1 % Triton X-100

Controls included bacteriocin or detergent in 50 mmol/l sodium phosphate buffer, pH 6.0. All samples and controls were incubated at 37^0C for 6 h for bacteriocin activity.

2.7. Effect of heat treatment and pH on bacteriocin activity

2.7.1. Effect of heat treatment on bacteriocin activity

One ml each of crude bacteriocin (1600 AU/ml) was heated at 45, 50, 60, 70^0C and 100^0C. Samples were removed at 0, 5, 10, 20, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115 and 120 min, and titred for loss of activity on *Enterococcus faecalis* LMG 2602.

2.7.2. Effect of pH on bacteriocin activity

To test the influence of pH, the cell free culture supernatant was adjusted to various pH values ranging from 2 to 12 (from approximately 4.8 to 4.0, 3.0, 2.0 with HCI and to 5.0, 60, 7.0, 8.0, 9.0, 10, 11, 12 with NaOH) and incubated at room temperature for 5, 15, 30, 45, 60 min and for 4h. MRS broth (pH range 2.0-12) served as a control.

2.8. Effect of organic solvents on the bacteriocin activity

Crude bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was incubated at room temperature for 2 h with the following organic reagents at a final concentration of 10 % and 25 % (v/v): methanol, hexane, toluene, petroleum ether, ethanol, acetonitrile, isoamylalcohol, 2-propanol, formaldehyde, acetone, di-ethylether, 1-butanol, chloroform. Thereafter, all solvents were evaporated at 45^0C and the remaining bacteriocin activity was titred.

2.9. Extraction of the bacteriocin with organic solvents

Various organic solvents including chloroform, chloroform-methanol (2:1, v/v), methanol, n-propanol, chloroform-isopropanol (2:1, v/v) hexane, isoamylalcohol,

di-ethylether, petroleum ether, toluene, 1-butanol were added to supernatant containing the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* in 1:1 ratio. Culture supernatant fluid (100ml) was stirred vigorously for 20 min with organic solvents. When propanol was used for the extraction, 50 g/l NaCI was added to the mixture in order to obtain phase separation. Phase separation was achieved by centrifugation (12 000 rpm for 30 min). The organic phase and the aqueous phase were collected and solvent removed by evaporation at 45^0C. The residue from the organic phase was resuspended in an amount of water equal to the starting volume of the original supernatant fluid. Bacteriocin activity of both preparations was determined.

Extraction of the bacteriocin with chloroform and chloroform-methanol was studied further with 1 liter of culture supernatant of *Leuconostoc mesenteroides* subsp. *cremoris.* Bacteriocin activity was assayed for the solvent and aqueous phases, interfacial layer between the two phases and the pellet at the bottom and sides of the centrifuge tube. The solvent phase was evaporated and the sediments were dissolved in 1-2 ml of distilled water. The bacteriocin activity in the aqueous phase was tested directly. Sediments collected at the solvent interface and at the bottom of the centrifuge tubes were resuspended in 1-2 ml of water and bacteriocin activity was tested.

2.10. Recovery and partial purification of the bacteriocin by diatomite calcium silicate

Diatomaceous earths (calcium silicate) have been tested for the partial purification of piscicolin 126, nisin, pediocin PO2 and brevicin 286 by Coventry *et al*. (1996). They determined the optimal conditions for desorption of piscicolin 126 and applied to other bacteriocins.

2.10.1. Adsorption of the bacteriocin

Micro-Cel E, obtained from Celite Corporation, Lompoc, California, was used as the adsorbent for the extraction of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* from the culture supernatant. Micro-Cel E was added (1 %, wt/vol) to culture supernatant of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* (1600 AU/ml, pH 4.8) and mixed by magnetic bar for 30 min at room temperature during adsorption. The suspension was centrifuged at 12 000 rpm for 20 min at room temperature. The supernatant was assayed for the bacteriocin activity. The pelleted residue was washed three times to get rid of unbound proteins. Micro-Cel E was suspended in sterile distilled water (1 % wt/vol) and tested against the indicator microorganism *Enterococcus faecalis* LMG 2602.

2.10.2. Desorption agents and solutions

The Micro-Cel adsorbed bacteriocin was desorbed for 60 min at room temperature with 1 % (wt/vol) of various surfactant solutions (Tween 80, Triton X-100, SDS) in distilled water, 1 % disodium EDTA, 1 % urea, phosphate buffer (0.2 M, pH 7.0) with various NaCI concentrations and distilled water with final pH range of 2.0-12 and organic solvents (methanol, chloroform, hexane, toluene, isoamylalcohol, ethanol, acetonitrile). Organic solvents were used undiluted, and evaporated after the desorption. Other desorption solutions were dissolved at the concentrations indicated. Desorption solutions at the indicated concentrations and pHs were tested against the indicator bacterium as control.

2.10.3. Desorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* from Micro-Cel E

The pelleted Micro-Cel adsorbing the bacteriocin was resuspended in a volume of desorption agent equal to original volume of supernatant. The suspension was

stirred with a magnetic bar for 1 hour at room temperature, and the Micro-Cel was pelleted by centrifugation at 12 000 rpm for 20 min at room temperature. The supernatant obtained was filter-sterilized (pore size, 0.45 μm) and assayed for the bacteriocin activity against *Enterococcus faecalis* LMG 2602. The Micro-Cel residue was resuspended in a volume of sterile distilled water equal to the original volume and assayed for the bacteriocin activity again the same indicator microorganism.

The effect of pH (2 to12) on the desorption of the bacteriocin from Micro-Cel was determined. The pH of the Micro-Cel adsorbed bacteriocin suspension in sterile distilled water was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 12 by 10 M NaOH and HCI. After desorption for 60 min at room temperature, Micro-Cel was pelleted by centrifugation and the pH of the supernatant was adjusted to pH 5.0-5.5 since bacteriocins of lactic acid bacteria are stable at acidic pHs. Next, assay of the bacteriocin activity was performed.

2.11. Mode of action of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* and adsorption of the bacteriocin onto indicator cells

2.11.1. Preparation of the bacteriocin

Leuconostoc mesenteroides subsp. *cremoris* was grown in 1 liter MRS broth at 30^0C for 16 h for the production of the bacteriocin. Cells were removed from culture fluid at 12 000 rpm for 30 min. Crude bacteriocin was precipitated from the cell-free supernatant by ammonium sulphate (80%). This precipitate was dissolved in 20 ml sterile distilled water and dialyzed by Sephadex G-25 column. One arbitrary unit (AU) of the bacteriocin was defined as 5 μl of the highest dilution of crude preparation showing a clear zone of growth inhibition on the lawn of sensitive cells of *Enterococcus faecalis* LMG 2602.

2.11.2. Assay of adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*

Adsorption of the bacteriocin to indicator cells was studied by the procedure employed by Bhunia *et al*. (1991). Generally, the cells were grown to a concentration of 2 x 10^8 cfu/ml (OD at 600 nm about 0.7) in 15 ml tubes in M17, MRS or TS broth at 30^0C. 1 ml culture was taken and the cells were removed by centrifugation and washed twice with sterile 5 mmol/l phosphate buffer (pH 6.0). Next, these cells were resuspended to the original volume (1 ml) with the same buffer. 1ml cell suspension was mixed with 0.1 ml bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* (6400 AU). The mixture was incubated at room temperature for 15 min and the cells were removed by centrifugation (10 000 rpm for 5 min). The bacteriocin activity in the supernatant was determined. Controls consisted of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* mixed with 1 ml of buffer without the cells. The percentage of the bacteriocin retained in the samples with respect to the control was calculated.

2.11.3. Determination of loss of viability of indicator cells after incubation with the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*

1 ml volume of each sensitive indicator strain (*Enterococcus faecalis* LMG 2602, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923, *Listeria innoqua* LMG 2813 and *Lactococcus lactis* SIK-83) from exponential growth phase was transferred to duplicate sterile bottles containing 80 ml of broth to obtain a culture with an O.D. of 0.6 or 0.8, and incubated at 30^0C. *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 was grown in MRS broth with an initial pH of 6.8. The other three strains were grown in M17 broth. When the OD values reached 0.6-0.8, one of the duplicate bottles for each culture received 1 ml of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* such that the final bacteriocin concentration in bottle would be 300 AU/ml. Controls consisted of cell suspensions

without bacteriocin. After incubation the colony forming units were counted and the percentage of cell viability was determined with respect to the control.

2.11.4. Adsorption of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* onto various species and strains

All of the strains employed in determining the activity spectrum of the bacteriocin were tested for their capacity to adsorb the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. To determine adsorption of the bacteriocin, cells of the indicator bacteria were incubated in a suitable broth (in 15 ml) to late exponential phase (approximately O.D. 0.7). 1 ml culture was taken and the cells were removed by centrifugation (10 000 rpm for 5 min), washed twice in a sterile 5 mmol/l phosphate buffer (pH 6.0) and resuspended to the original volume (1 ml) in the same buffer. 1 ml of cell suspension was mixed with 0.1 ml of the bacteriocin. The mixture was incubated at room temperature for 15 min and the cells were removed by centrifugation. The bacteriocin activity in the supernatant (unbound bacteriocin) was measured by the method mentioned above.

2.11.5. Effect of temperature on the adsorption of the bacteriocin

Enterococcus faecalis LMG 2602 was used to determine effect of temperature on the adsorption of the bacteriocin. Cells were resuspended in 1 ml phosphate buffer (5 mmol/l; pH 6.0) after centrifugation and washing and waited for at these temperatures: 0, 20, 30, 40, 50, 60, 70 and 80^0C. Bacteriocin was added to each tube and the tubes were incubated at respective temperatures for 15 min. Following centrifugation (10 000 rpm for 5 min) the supernatants of these bacteria were assayed for the unbound bacteriocin.

2.11.6. Effect of pH on the adsorption of the bacteriocin

Enterococcus faecalis LMG 2602 was suspended in 5 mmol/l phosphate buffer at pH 2.0 to 9.0. The pH was adjusted with HCI and NaOH to maintain pH. Bacteriocin was added to each tube and the tubes were incubated at the respective pHs for 15 min at room temperature. Following centrifugation (10 000 rpm for 5 min) the supernatants were assayed for the bacteriocin activity.

2.11.7. Effect of salts on the adsorption of the bacteriocin

Enterococcus faecalis LMG 2602 was grown 16 h at 30^0C. 1 ml culture was removed by centrifugation at 10 000 rpm for 5 min, washed and resuspended in 1ml sterile salt solutions including NaCI (100 mmol/l), phosphate buffer (100 mmol/l), KCI (100 mmol/l), $MgCI_2$ (100 mmol/l), Tris-HCI (100 mmol/l) , Na-Acetate (100 mmol/l) and EDTA (100 mmol/l). After the addition of the bacteriocin (0.1 ml), the suspensions were incubated at 30^0C for 1 h. Following centrifugation (10 000 rpm for 5 min) the supernatants were assayed for the bacteriocin activity.

2.11.8. Effect of detergents on the adsorption of the bacteriocin

Whole cells of *Enterococcus faecalis* LMG 2602 were grown 16 h at 30^0C. 1 ml culture was removed by centrifugation at 10 000 rpm for 5 min, washed and resuspended in 1ml sterile phosphate buffer containing 1 % SDS, 1 % Triton X-100, 1 % Tween 80 and 4 mol/l guanidine-HCI . The mixtures were heated to 60^0C for 15 min as reported by Bhunia *et al*. (1991) and the suspensions were centrifuged. Next, the pellets were washed twice with phosphate buffer (5mmol/l, pH 6.0) and resuspended to 1 ml with the same buffer. After the addition of the bacteriocin (0.1 ml), the suspensions were held at room temperature for 1 h. Following incubation, the cells were removed by centrifugation and the supernatant assayed for the unbound bacteriocin activity. Controls consisted of untreated cells.

2.11.9. Effect of organic solvents on the adsorption of the bacteriocin

Cells (1ml) of *Enterococcus faecalis* LMG 2602 were suspended in ethanol, chloroform, methanol, butanol, isopropanol, acetone and hexane (80 %) and held at room temperature for 30 min. The solvents were removed by centrifugation and air drying. The pellets were resuspended in 1 ml phosphate buffer (5 mmol/l, pH 6.0) and mixed with 0.1 ml bacteriocin preparation. The suspensions were held at room temperature for 1 h. At the end, the cells were removed by centrifugation and the supernatant assayed for the bacteriocin activity. Controls were untreated cells.

2.11.10. Effect of enzymes on the adsorption of the bacteriocin

Cells of *Enterococcus faecalis* LMG 2602 were grown 16 h at 30^0C. 1 ml culture was removed by centrifugation at 10 000 rpm for 5 min, washed and incubated with lysozyme (1mg/ml in 0.005 mol/l Tris-HCI, pH 7.0)), trypsin (1mg/ml in 0.005 mol/l phosphate buffer, pH 6.0), pepsin (1 mg/ml in 0.2 M citrate buffer, pH 6.0), chymotrypsin (1 mg/ml in 0.02 mol/l Tris-HCI, pH 7.8), proteinase K (1mg/ml in 0.05 mol/l sodium acetate buffer, pH 6.2), α-amylase (1mg/ml in 0.03 mol/l sodium acetate buffer, pH 6.0). Samples were incubated at 37^0C for 1 h and centrifuged at 10 000 rpm for 5 min. The pellets were washed twice with 5 mmol/l phosphate buffer (pH 6.0) to remove enzymes and assayed for the bacteriocin activity. Controls were untreated cells.

2.11.11. Measurement of lysis of sensitive cells by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*

Sensitive strains of *Enterococcus faecalis* LMG 2602, *Listeria innoqua* LMG 2813, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923, *Lactococcus lactis* SIK-83 were used as indicator bacteria. 1 ml volume of each sensitive indicator strain from exponential growth phase was transferred to duplicate sterile bottles containing 80

ml of broth to obtain a culture with an OD of 0.6 or 0.8, and incubated at 30^0C. *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 was grown in MRS broth with an initial ph of 6.8. The other three strains were grown in M 17 broth. When the OD values reached 0.6-0.8, one of the duplicate bottles for each culture received 1 ml of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* such that the final bacteriocin concentration in bottle would be 300 AU/ml. The cultures were incubated up to 240 min (4 h) and at selected intervals OD values were measured.

2.12. Purification of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris*

Purification of the bacteriocin was performed by various combinations of pH mediated cell surface adsorption-desorption and chromatographic methods including cation exchange chromatography, hydrophobic interaction chromatography and size-exclusion chromatography. In addition some other combinations of methods, namely, a) chloroform-methanol extraction (2:1, v/v) of the pellicle from ammonium sulfate precipitation (40%), b) Micro Cel E mediated adsorption-desorption fraction and cation-exchange chromatography on carboxy methyl cellulose of this fraction was controlled for purity.

However, the sequential chromatographic protocol for the purification of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* consisted of pH mediated cell adsorption-desorption, hydrophobic interaction chromatography on Amberlite XAD-16, cation exchange chromatography on Macro-Prep High S, hydrophobic interaction chromatography on phenyl Sepharose CL-4B and size-exclusion chromatography on Sephadex G-50 column.

2.12.1. Ammonium sulfate precipitation plus chloroform-methanol extraction approach

Bacteriocin containing culture fluid was obtained from 1liter cultures after 16 h fermentation at 30^0C when the pH had dropped to approximately 4.8 by centrifugation (12 000 rpm, 30 min, 4^0C). The supernatant portion was adjusted to 6.5 with 10 M NaOH. This crude supernatant was made up to 40 % ammonium sulphate and held overnight at 4^0C with stirring. The sample was centrifuged at 12 000 rpm fro 30 min at 4^0C and the surface pellicle was recovered. Bacteriocin containing pellicle was resuspended in 50 mM sodium phosphate buffer (pH 6.5). This crude bacteriocin fraction was treated with 25 volume of a mixture of chloroform-methanol (2:1, v/v) for 1 h at 4^0C. The resulting fine-grained white precipitate was collected by centrifugation for 30 min at 12 000 rpm at 4^0C and air-dried and dissolved in 10 ml of 10 % 2-propanol/0.1 % TFA. It was considered as partially purified bacteriocin.

2.12.2. pH mediated cell adsorption-desorption of the bacteriocin

The cell mediated adsorption-desorption method of Yang *et al*. (1992) was performed to purify the bacteriocin. Fresh culture of *Leuconostoc mesenteroides* subsp*. cremoris* was inoculated 0.1 % in 1 liter MRS broth and incubated for 16 h at 30^0C The *Leuconostoc mesenteroides* subsp. *cremoris* culture was heated to 80^0C for 15 min to inactivate the proteases. The pH of the culture was adjusted to 6.0 by addition of 10 M NaOH solution. It was stirred for 2 h at room temperature to allow the bacteriocin to adsorb onto bacterial cells, which were collected by centrifugation for 30 min at 12 000 rpm at 4^0C. Also, the same experiments was performed by employing 6-20 liter cells of *Leuconostoc mesenteroides* subsp. *cremoris* to adsorb the bacteriocin content of 1 liter supernatant to remove all the bacteriocin found in 1 liter supernatant. To remove impurities and unbound proteins, the cells were washed three times by resuspension in 1000 ml of 5 mM

sodium phosphate buffer (Washing was carried out 3000 ml buffer in total), recentrifugation at 12 000 rpm for 30 min at 4^0C and draining off the buffer washings. The cells were resuspended in 250 ml of 100 mM NaCI solution, and the pH was adjusted to 2.0 by gradual addition of HCI. The pH 2 extract was stirred in cold room (4^0C) overnight, centrifuged at 12 000 rpm for 30 min at 4^0C. The supernatant fluid was filtered through a 0.22 μm sterile disposable filter. The pH of the bacteriocin extract was adjusted to 5.5-6.0 and was desalted by Sephadex G-25 (Fraction I).

2.12.3. Solid-Phase extraction on Amberlite XAD-16

20 g of Amberlite XAD-16 was added to 250 ml pH mediated cell adsorption-desorption fraction. The sample was gently agitated at 4^0C for 2 h. The matrix was washed with 100 ml of distilled water and 200 ml of 40% (v/v) ethanol in distilled water, and the bacteriocin activity was eluted with 150 ml of 70% (v/v) 2-propanol in distilled water containing 0.1% trifluoroacetic acid (TFA) (pH 2.0). The eluate was evaporated to 5-10 ml by boiling in hot-plate. The sample was diluted to 100 ml with 0.02 M Sodium phosphate buffer (pH 5.7) (Fraction II).

2.12.4. Cation-exchange chromatography

Purification of the bacteriocin by cation-exchange chromatography was done by adding 25 ml of Carboxymethyl cellulose or Macro prep High S cation exchanger gel slurry equilibrated in 20 mM sodium phosphate buffer (adjusted previously to pH 5.7) to the bacteriocin extract obtained from Amberlite XAD-16 extract (Fraction II). The pH of the mixture was adjusted to 5.7 again after addition of the bacteriocin extract. The mixture was held under stirring for 60 min. The supernatant was removed, and the sedimented gel slurry was loaded on a 2 x 20 cm glass column. The gel was washed with 20 bed volumes of 20 mM sodium phosphate buffer. Elution of the adsorbed bacteriocin was carried out in a stepwise manner

through 100 ml of 20 mM sodium phosphate buffer containing 2.0 M NaCI (Fraction III).

2.12.5. Hydrophobic interaction chromatography on Phenyl-Sepharose

Fraction obtained from cation exchange chromatography with 0.02 M phosphate buffer (pH 5.7) containing 2.0 M NaCI was applied to a 13 ml Phenyl-Sepharose CL-4B column equilibrated with 0.02 M phosphate buffer (pH 5.7) containing 2.0 M NaCI. The column was then washed with 5 bed volume of buffer and bacteriocin activity was eluted with 65 ml of 70% ethanol and 30% buffer with a linear gradient and 2 ml fractions were collected. The eluted fractions were assayed for bacteriocin activity. Active fractions were pooled and concentrated by ethanol precipitation and activity against indicator organism was checked. (Fraction IV).

2.12.6. Size-exclusion (gel filtration) chromatography

Concentrated fraction obtained from pH mediated cell surface adsorption-desorption fraction (fraction I) subjected to size-exclusion chromatography on Sephadex G-50 using a 1.5 x 90 cm glass column. The column was equilibrated and eluted with 50 mM sodium phosphate buffer pH 5.7 containing 0.15 M NaCI. The absorbance at 280 nm was monitored and 3 ml fractions were collected. Each fraction was assayed for bacteriocin activity. Fractions demonstrating inhibitory activity against *Enterococcus faecalis* LMG 2602 were pooled and concentrated by ethanol precipitation or acetone precipitation. The precipitate was dissolved in a minimal volume of sodium phosphate buffer, pH 5.7 or distilled water.

2.12.7. Micro Cel mediated adsorption for the recovery and purification of the bacteriocin

Adsorption of the bacteriocin to Micro Cel and subsequent desorption by SDS was performed for the recovery and purification as explained in 2.10.

2.12.8. Ethanol precipitation of the bacteriocin

9 volumes of cold ethanol 100% was added to 1 volume of bacteriocin solution and mixed. The mixture was left at 20^0C overnight and centrifuged for 30 min at 12 000 rpm at 4^0C. Supernatant was discharged carefully and the pellet retained. The pellet was air-dried to eliminate any ethanol residue and dissolved in a minimal volume of sterile distilled water.

2.12.9. Acetone precipitation of the bacteriocin

4 volumes of cold acetone was added to 1 volume of bacteriocin solution and mixed. The mixture was left at -20^0C overnight and centrifuged for 30 min at 12 000 rpm at 4^0C. Supernatant was discharged carefully and the pellet retained. Sample was air-dried to eliminate any acetone residue and dissolved in a minimal volume of sterilized distilled water.

2.13. Estimation of molecular weight by SDS-PAGE

Active fractions from different purification methods containing bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* were pooled and concentrated by ethanol precipitation. The concentrated samples were loaded on SDS polyacrylamide gels. Molecular weight markers for peptides ranging from 6.5 to 180 kDa (Sigma) were used as a Standard. After electrophoresis the gels were divided. One half containing the sample and molecular weight markers was stained.

The other half of the gel was fixed immediately for 2 h in fixing solution (Appendix E). It was then wahed in several volumes of distilled water for 3 to 4 h. The gel was placed in a large sterile glass perti-dish and overlaid with 40 ml of soft tempered agar seeded with 400 µl indicator strain. The plate was incubated overnight at 30^0C and examined for a zone of inhibition (Bhunia *et al*., 1987).

Materials

Protein molecular weight standards; acrylamide(30%)/ bisacrylamide (0.8%); 4X Tris-CI/SDS (pH 8.8); 10% (w/v) ammonium persulfate; 10% (w/v) SDS; TEMED (N,N,N',N'-tetramethylethylenediamine); isobutyl alcohol (water-saturated); 4X Tris-CI/SDS (pH 6.8); 2X SDS/sample buffer; 1XSDS/electrophoresis buffer; Bromphenol Blue solution; 0.75-mm spacers; teflon combs; 25 and 100 µl syringes with flat-tipped needles; 0.45-µm filters.

Pouring the separating gel

1. Mix the separating gel solution by adding 30% acrylamide 70.8% bisacrylamide, 4X Tris-CI/SDS, pH 8.8, and water to a 50 ml flask. Add 50 µl of 10% ammonium persulfate and 10 µl TEMED to the solution and gently swirl to mix.
2. Using a Pasteur pipette, transfer the separating gel solution to the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~1.5 to 2 cm from the top of the shorter glass plate.
3. Using another Pasteur pipette, slowly cover the top of the gel with a layer of isobutyl alcohol by gently squirting the isobutyl alcohol against the edge of one of the spacers.
4. Allow the separating gel to polymerize fully.

Pouring the stacking gel

5. Pour off completely the layer of isobutyl alcohol. Rinse the separating gel top three times with distilled water.

6. Prepare a 5% stacking gel solution by adding 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4X Tris-CI/SDS, pH 6.8, and 3.05 ml water. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED to the solution and gently swirl to mix.

7. Using a Pasteur pipette, slowly allow the stacking gel solution to trickle into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~ 3 cm.

8. Insert a Teflon comb into the layer of stacking gel solution and allow the stacking gel solution to polymerize 30-45 min at room temperature.

Loading the gel

9. Dilute the protein to be analyzed at least 1:1 (v/v) with 2X SDS/sample buffer in a microcentrifuge tube and boil for 1 min at 100^0C. If the sample is aprecipitated protein pellet, dissolve the protein to be separated in 50 to 100 µl SDS/sample buffer and boil 1 min at 100^0C. After boiling, cool the sample to room temperature, and centrifuge for a few seconds to remove particulate matter.

10. Carefully remove the teflon comb without tearing the edges of the polyacrylamide wells.

11. Using a Pasteur pipette, fill the wells with 1X SDS/electrophoresis buffer.

12. Place the upper buffer chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour 1X SDS/electrophoresis buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower buffer chamber.

13. Partially fill the upper buffer chamber with 1X SDS/electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.

14. Using a Hamilton syringe with a flat-tipped needle, load the protein samples into the wells by carefully applying the sample as a thin layer at the bottom of the wells.

15. Fill the remainder of the upper buffer chamber with additional 1X SDS/electrophoresis buffer.

Running the gel

16. Connect the power supply to the anode and cathode of the gel apparatus and run at 100 V.

17. After the Bromphenol Blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

18. Remove the upper buffer chamber and the attached sandwich.

19. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels.

20. Carefully slide the spacers out from the edge of the sandwich along its entire length and remove the gel from the lower plate.

Staining of Protein Bands

1. Place the gel in a small plastic box and cover with Coomassie Brilliant Blue R-250 solution (Appendix E). Agitate slowly for overnight.

2. Pour off the staining solution and cover the gel with a solution of methanol/water/acetic acid (40%:50%:10% v/v/v) (Appendix E). Agitate slowly for 30 min.

3. Discard destaining solution and replace with fresh solution. Repeat until the gel is clear except for protein bands.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Detection of the bacteriocin activity

Bacteriocin activity of the cell-free supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* was tested by spot-on-the lawn method. Activity was measured as the reciprocal of the highest two-fold dilution showing antimicrobial activity. According to to the observations, 1/8 dilution of the crude supernatant is the highest dilution showing antimicrobial activity against indicator strain that is *Enterococcus faecalis* LMG 2602, which was used in all measurements for bacteriocin activity. Bacteriocin activity of cell-free supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* (crude extract) was calculated as:

$$8 \times 200 = 1600 \text{ AU/ml.}$$

AU= Arbitrary Unit; 8 = Reciprocal of the highest dilution (1/8); 200 = 1000 µl / 5 µl (conversion factor)

An example to the titration of the bacteriocin activity of the fraction obtained by ammonium sulphate precipitation of the supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* of was given in Figure 3.1. As seen from the Figure 3.1, the highest dilution showing a clear inhibition zone on the indicator lawn is 1/128; the least two

fold dilution that did not bring about a clear inhibition zone (1/256) was not illustrated on the lawn. According to the above formula, the corresponding bacteriocin activity calculated as 128 X 200 = 25600 AU/ml.

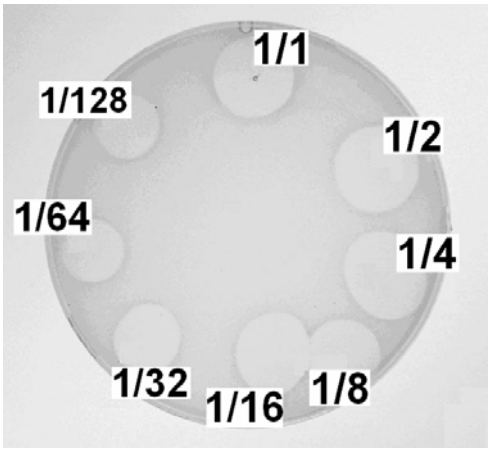


Figure 3.1. Titration of the bacteriocin activity of the pellet after ammonium sulfate precipitation.

3.2. Effect of pH on the bacteriocin production from *Leuconostoc mesenteroides* subsp. *cremoris*

The effect of initial medium pH on cell growth and bacteriocin production is shown in Table 3.1. As can be seen from Table 3.1, *Leuconostoc mesenteroides* subsp. *cremoris* produced bacteriocin between pH 5.5 and 7.0. Nevertheless, no bacterial growth and bacteriocin production were detected under uncontrolled conditions at pH 5.0, 4.5 and 4.0. The highest bacteriocin activity was recorded in MRS broth adjusted to pH 5.5, 6.0, 6.5, and 7.0.

Table 3.1. Effect of pH on the production of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* at 30^0C.

pH	Activity (AU/ml)
4.0	0
4.5	0
5.0	0
5.5	1600
6.0	1600
6.5	1600
7.0	1600

3.3. Effect of temperature on the bacteriocin production from *Leuconostoc mesenteroides* subsp. *cremoris*

Growth and bacteriocin production occurred at temperatures between 20 and 37^0C. The producer organism resulted in 800 AU/ml bacteriocin activity at 20 and 25^0C. When the producer cultivated at 30 and 37^0C, it resulted in 1600 AU/ml bacteriocin activity (Table 3.2). Consequently, temperature of 30^0C and an initial pH of 6.5-7.0 was chosen in all cultivations of *Leuconostoc mesenteroides* subsp. *cremoris* for the maximum production of the bacteriocin.

Table 3.2. Effect of temperature on the production of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* with an initial pH of 6.5-7.0.

Temperature (^{o}C)	Activity (AU/ml)
10	0
15	0
20	800
25	800
30	1600
37	1600

3.4. Activity spectrum of the bacteriocin

Table 3.3 shows sensitivity of the various strains to the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* as measured with the spot-on-the–lawn method. The antibacterial spectra of the activity of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was assayed against 62 indicator strains (of which 19 gram-negative strains) belonging to the 13 genera including lactobacilli (plantarum, casei, sake, acidophilus, delbrueckii, salivarius, and cremoris), lactococci (lactis and cremoris), Bacilli, Pediococcus, Enterococci, Listeria, Leuconostoc, Staphylococci, *Salmonella enteritidis*, *Salmonella paratyphi* B, *Salmonella choleraesuis*, *Salmonella enterica*, Esherichia, Klebsiella, Streptococci and Pseudomonas (Table 3.3). *Lactococcus lactis* strains was not inhibited by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* apart from *Lactococcus lactis* SIK-83 and *Lactococcus lactis* LMG 2909, which are strongly inhibited. None of the *Lactobacillus casei* strains and *Lactobacillus plantarum* RSSK 10 was inhibited by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. *Staphylococcus aureus* LMG 3022, *Bacillus subtilus* and *Streptococcus thermophilus* were not inhibited by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. Other strongly inhibited bacteria were *Enterococcus*

faecalis LMG 2602, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923, *Lactobacillus plantarum* RSSK 675, *Lactobacillus cremoris* RSSK 708, *Lactobacillus delbrueckii* subsp. *lactic* RSSK 498, *Pseudomonas fluorescens* LMG 3020, *Bacillus cereus* LMG 2732 (a spore-forming bacterium), *Listeria monocytogenes* and *Listeria innoqua* LMG 2813. Anti-*Listeria* activity is a distinguishing characteristic for most pediocin-like bacteriocins (Class II a bacteriocins) produced by *Leuconostoc* spp. The food-borne pathogen *Listeria monocytogenes* has been associated with many cases of listeriosis.

Lactobacillus plantarum LMG 2003 and *Lactobacillus sake* LMG 2313 (NCDO 2714) were weakly inhibited sensitive bacteria. It is interesting that the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* inhibited the growth of *Pseudomonas fluorescens* LMG 3020, a gram-negative bacterium. It is known that generally bacteriocins of lactic acid bacteria do not inhibit the growth of gram-negative bacteria.

Salmonella enteritidis, *E. coli* O157:H7, *Klebsiella pneumoniae*, *Salmonella paratyphi* B 33653, *Salmonella paratyphi* B 33656, *Salmonella paratyphi* B 34323, *Salmonella paratyphi* B 38359, *Salmonella paratyphi* B 38470, *Salmonella paratyphi* B 38518, *Salmonella enteritidis* 38055, *Salmonella enteritidis* 38383, *Salmonella enteritidis* 33280, *Salmonella choleraesuis* 34088, *Salmonella choleraesuis* 21, *Salmonella paratyphi* A 2961, *Salmonella paratyphi* B 4620 were also employed as indicator organisms to test the effect of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* on gram-negative bacteria. The results showed that bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* did not inhibited any of these gram-negative bacteria. As indicated above, *Pseudomonas fluorescens* LMG 3020 was the sole gram-negative organism inhibited by the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris.*

The bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* displayed a narrow inhibitory spectrum towards other lactic acid bacteria. *Lactobacillus delbrueckii* strains were found to be more sensitive to the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* than other lactobacilli. Bacteriocins produced by lactic acid bacteria are inhibitory towards closely related species and strains. There is a close relationship between leuconostocs and *Lactobacillus delbrueckii* group while the *Lactobacillus casei* and Pediococcus group are more distantly related to leuconostocs as shown in Figure 3.2. Bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* was also tested on the producer organism itself and it was found that the producer strain was not inhibited. This situation indicates that the producer organism have an immunity protein against its own bacteriocin. It was reported that immunity proteins for class II bacteriocins are soluble and mainly located in the cytoplasm (Eijsink *et al*., 1998)

Some of the lantibiotics such as nisin and some of the non-lanthionine-containing bacteriocins such as pediocin AcH have been shown to act on several species of gram-negative bacteria provided that the integrity of the outer membrane is disrupted. The outer membrane of gram-negative bacteria prevents molecules like bacteriocins, antibiotics, dyes and detergents from reaching the cytoplasmic membrane (Nikaido and Vaara, 1987) and acts as a permeability barrier for the cell. It was shown that gram-negative bacteria are not generally sensitive to nisin (Hurst, 1981; Spelhaug and Harlander, 1989). The outer membrane protects the cell by excluding nisin although cytoplasm membrane is susceptible (Kordel *et al*., 1989).

Many bacteriocins of lactic acid bacteria have a rather narrow host range against gram-positive bacteria, i.e. they produce antibacterial action against a few related strains or species. Also, sensitive strains differ greatly in their sensitivity to bacteriocin, and even in the population of a highly sensitive strain there are cells that are resistant to bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. At the bacteriocin concentration tested, some strains appeared to be

resistant to the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. For the indicator strains sensitive to bacteriocin, sensitivities to bacteriocin appeared to vary considerably from one strain to another.

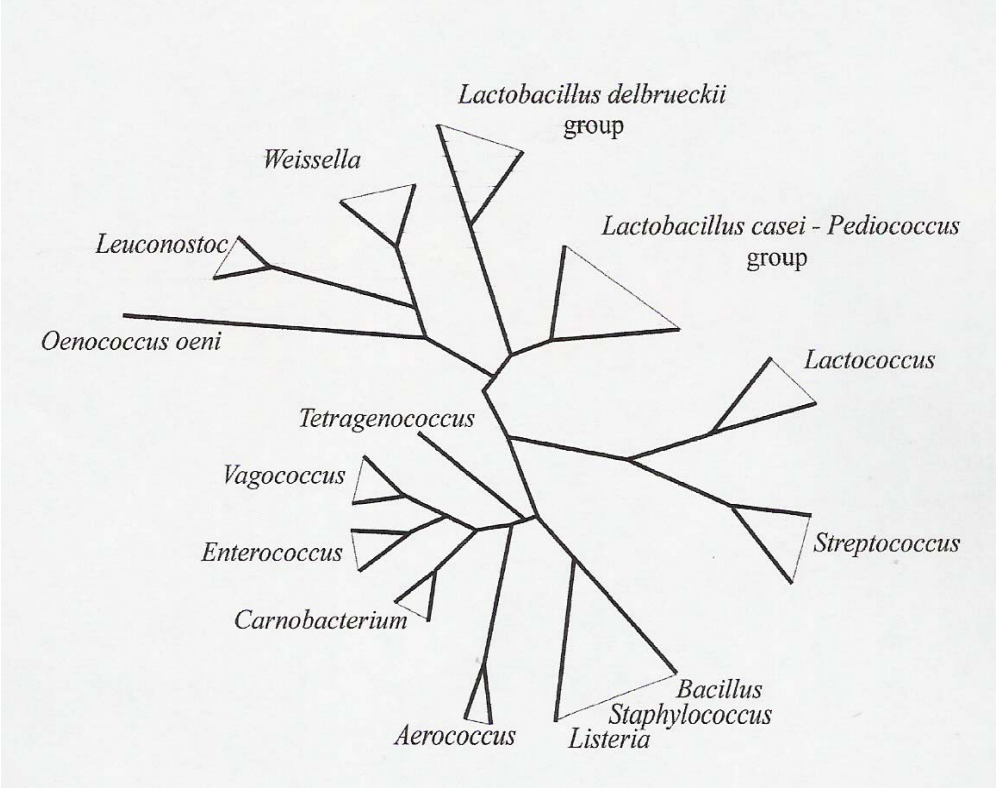


Figure 3.2 Schematic, unrooted phylogenetic tree of the LAB, based on the 16S rRNA gene sequences (Axelsson, 2004).

Table 3.3. Activity spectrum of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*.

indicator		indicator	
Lactobacillus casei NRRL 21	-	*Leuconostoc mesenteroides* subsp. *cremoris* RSSK923	+
Lactobacillus casei NRRL 22	-	*Leuconostoc mesenteroides* subsp. *cremoris* (producer)	-
Lactobacillus casei NRRL 23	-	*Bacillus subtilus*	-
Lactobacillus casei NRRL 334	-	*Bacillus cereus* LMG 2732	+
Lactobacillus casei NRRL 441	-	*Pseudomonas fluorescens* LMG 3020	+
Lactobacillus acidophilus ATCC 4356	-	*Enterococcus faecalis* LMG 2602	+
Lactobacillus plantarum LP73	-	*Pediococcus pentosaceous* LMG 2001	-
Lactobacillus plantarum Z11 L	-	*Listeria innoqua* LMG 2813	+
Lactobacillus plantarum HÜ	-	*Listeria monocytogenes*	+
Lactobacillus plantarum DSMZ 20246	-	*Staphylococcus aureus* LMG 3022	-
Lactobacillus plantarum DSMZ 20174	-	*Staphylococcus aureus*	-
Lactobacillus plantarum RSSK 675	+	*Staphylococcus carnosus* LMG 2709	-
*Lactobacillus plantaru*m RSSK10	-	*E. coli* O157:H7	-
Lactobacillus plantarum 80B	-	*E.coli* LMG 3083	-
Lactobacillus plantarum LMG 2003	-+	*Klebsiella pneumoniae*	-
Lactobacillus delbrueckii subsp. *delbrueckii*	+	*Streptococcus thermophilus*	-
Lactobacillus delbrueckii subsp. *lactic* RSSK 498	+	*Salmonella paratyphi* A 2961	-
Lactobacillus cremoris RSSK 708	+	*Salmonella paratyphi* B 33653	-
Lactobacillus fructivorans	+	*Salmonella paratyphi* B 33656	-
Lactobacillus salivarius M7	-	*Salmonella paratyphi* B 34323	-
Lactobacillus sake LMG 2313 (NCDO 2714)	-+	*Salmonella paratyphi* B 38359	-
Lactococus lactis LMG 2907	-	*Salmonella paratyphi* B 38470	-
*Lactoc*occus *lactis* LMG 2908	-	*Salmonella paratyphi* B 38518	-
Lactococcus lactis LMG 2909	+	*Salmonella paratyphi* B 4620	-
Lactococcus lactis LMG 2910	-	*Salmonella enteritidis*	-
Lactococcus lactis LMG 2911	-	*Salmonella enteritidis* 38383	-
Lactococcus lactis LMG 2912	-	*Salmonella enteritidis* 38055	-
Lactococcus lactis SIK 83	+	*Salmonella enteritidis* 33280	-
Lactococcus lactis IL-1403	-	*Salmonella cholerasuis* 34088	-
Lactococcus lactis subsp. *cremoris* LMG 2132	-	*Salmonella choleraesuis* 21	-
Lactococcus lactis LMG 3113	-	*Salmonella enterica typhimurium* LMG 3085	-
Lactococcus lactis LMG 2088	-		

+, presence of activity; -, absence of activity; -+,presence of weak activity

3.4.1. Activity spectrum of the bacteriocin at high concentrations of the bacteriocin

Table 3.4 shows the effect of high concentration of the crude bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* against species or strains which were resistant to the crude bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. These indicator bacteria were selected from resistant strains indicated in the Table 3.1 to test whether these bacteria might be inhibited by employing high concentrations of the bacteriocin obtained by ammonium sulphate precipitation (80% saturation) of the 1 liter culture fluid of *Leuconostoc mesenteroides* subsp. *cremoris* and dissolving the pellet in 20 ml of 0.05 M (pH 6.5) sterile phosphate buffer (800 000 AU/ml). Of the resistant indicator strains, *Lactobacillus plantarum* Z11L, *Lactobacillus plantarum* 73 *Lactobacillus plantarum* Hacettepe University, *Lactobacillus plantarum* DSM 20246, *Lactobacillus plantarum* 80B, *Lactobacillus salivarius* M7, *Lactobacillus acidophilus* ATCC 4356, *Lactococcus lactis* LMG 2908, *Pediococcus pentosaceus* and the weakly inhibited *Lactobacillus sake* LMG 2313 (NCDO 2714) were strongly inhibited. *Lactobacillus plantarum* LMG 2003 (weakly inhibited by the crude supernatant), *Lactococcus lactis* LMG 3113, and *Lactococcus lactis* IL-1403 were weakly inhibited by the high comcentration of the bacteriocin. The remaining insensitive gram-positive and gram-negative strains were insensitive to high concentrations of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. Therefore, activity spectrum of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* became broader with high concentration of the bacteriocin compared to supernatant; with the use of concentrated bacteriocin, the number of sensitive strains increased from 15 to 26 out of 43 gram-positive bacteria. However, the increased bacteriocin activity had not changed the sensitivities of gram-negative bacteria. The high concentrations of the bacteriocin may have resulted in transmembrane pores increased in size. For example, it was found that the transmembrane pores produced by pediocin AcH increased in size with increasing pediocin concentration (Ray, 1992).

The inhibition of insensitive strains to bacteriocin at high concentrations was also reported for lactococcin A. It was reported that dissipation of PMF (Proton Motive Force) and increase in membrane permeability occurred only at very high concentrations of lactococcin A (Van Belkum *et al*., 1991). In addition, it was shown that the membrane functions of strains carrying specific immunity protein genes were not affected by lactococcin B even at high concentrations, while the insensitivity of sensitive cell membrane-derived liposome vesicles indicated the probable presence of protein receptors for lactococcin B in the membrane (Venema *et al*., 1993). The possible mechanism of bacteriocin resistance of some gram-positive bacteria has been suggested to be associated with the barrier properties of cell wall.

Table 3.4. Effect of high concentration of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* against resistant indicators.

Resistant indicators	Inhibition with high conc. of bacteriocin from *L. mesenteroides* subsp. *cremoris*
Lactobacillus sake LMG 2313 (NCDO 2714)	+
Lactobacillus casei NRRL 21	-
Lactobacillus casei NRRL 22	-
Lactobacillus casei NRRL 23	-
Lactobacillus casei NRRL 334	-
Lactobacillus casei NRRL 441	-
Lactobacillus plantarum RSSK 10	-
Lactobacillus plantarum Z11 L	+
Lactobacillus plantarum HÜ	+
Lactobacillus plantarum 80B	+
Lactobacillus plantarum DSM 20246	+
Lactobacillus plantarum LP73	+
Lactobacillus plantarum LMG 2003	-+
Lactobacillus acidophilus ATCC 4356	+
Lactobacillus salivarius M7	+
Lactococcus lactis LMG 2911	-
Lactococcus lactis LMG 2908	+
Lactococcus lactis LMG 2088	-
Lactococcus lactis LMG 2910	-
Lactococus lactis LMG 2907	-
Lactococcus lactis LMG 3113	-+
Lactococcus lactis LMG 2912	-
Lactococcus lactis IL-1403	-+
Lactococcus cremoris LMG 2132	-
Pediococcus pentosaceus LMG 2001	+

Table 3.4. (Continue)

Resistant indicators	Inhibition with high conc. of bacteriocin from *L. mesenteroides* subsp. *cremoris*
Staphylococcus aureus LMG 3022	-
Bacillus subtilus	-
Staphylococcus aureus	-
Streptococcus thermophilus	-
Klebsiella pneumoniae	-
Salmonella enteritidis	-
E. coli O157:H 7	-
Salmonella enteritidis 38383	-
Salmonella cholerasuis 34088	-
Salmonella paratyphi A 2961	-
Salmonella paratyphi B 33653	-
Salmonella paratyphi B 33656	-
Salmonella paratyphi B 34323	-
Salmonella paratyphi B 38359	-
Salmonella paratyphi B 38470	-
Salmonella paratyphi B 38518	-
Salmonella enteritidis 38055	-
Salmonella enteritidis 33280	-
Salmonella choleraesuis 21	-
Salmonella paratyphi B 4620	-

3.4.2. Comparison of the activity spectrum of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* with some other bacteriocins

Activity spectrum of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was compared with lacticin from *Lactococcus lactis* LMG 2907, lacticin 3147 from *Lactococcus lactis* LMG 2910, *Lactococcus lactis* LMG 2911, *Lactococcus lactis* LMG 2912, lacticin 481 from *Lactococcus lactis* LMG 3113, lactococcin G from *Lactococcus lactis* LMG 2088 and nisin from *Lactococcus lactis* SIK-83 (Table 3.5).

Lactobacillus cremoris RSSK 708, *Lactobacillus delbrueckii* subsp. *lactic* RSSK 498, *Lactococcus lactis* LMG 2909, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923, *Bacillus cereus* LMG 2732, *Pseudomonas fluorescens* LMG 3020 were inhibited by only the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* is similar to other bacteriocins in that they all but lactococcin G inhibited *Listeria innoqua* LMG 2813. It is also similar to nisin with respect to inhibition of *Lactobacillus plantarum* RSSK 675. Other bacteriocins did not inhibit this indicator strain. Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* is also similar to other bacteriocins in terms of not inhibiting *Lactobacillus casei* NRRL 21, NRRL 22, NRRL 23, NRRL 334, *Lactobacillus plantarum* Hacettepe University, *Lactococcus lactis* LMG 2907, *Lactobacillus plantarum* 80B, *Lactobacillus plantarum* RSSK 10.

Table 3.5. Comparison of activity spectrum of the bacteriocin produced by *Leuconostoc mesentereoides* subsp. *cremoris* with other bacteriocins.

Indicator \ producer	Bacteriocin							
	lacticin	**lacticin 3147**	**lacticin 3147**	**lacticin 3147**	**lacticin 481**	**LCN G**	**This study**	**nisin**
	LMG 2907	**LMG 2910**	**LMG 2911**	**LMG 2912**	**LMG 3113**	**LMG 2088**	***L. mesenteroides***	**SIK-83**
Lactobacillus plantarum 73	-	-	-	-	-	+	-	+
Lactobacillus plantarum NRRL 21	-	-	-	-	-	-	-	-
Lactobacillus casei NRRL 22	-	-	-	-	-	-	-	-
Lactobacillus plantarum NRRL 23	-	-	-	-	-	-	-	-
Lactobacillus plantarum Z11 L	-	-	-	-	-	+	-	+
Lactobacillus plantarum HÜ	-	-	-	-	-	-	-	-
Lactobacillus cremoris RSSK 708	-	-	-	-	-	-	+	-
Lactobacillus delbrueckii subsp. *lactic* RSSK 498	-	-	-	-	-	-	+	-
Leuconostoc mesenteroides subsp. *cremoris* (producer)	-	-	-	-	-	-	-	-
Enterococcus faecalis LMG 2602	+	+	+	+	-	-	+	+
Lactobacillus casei NRRL 441	+	+	+	+	-	-	-	+
Lactococcus lactis LMG 2909	-	-	-	-	-	-	+	-
Lactococcus lactis LMG 2908	+	+	+	+	-	-	-	+
Lactobacillus acidophilus ATCC 4356	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides subsp. *cremoris* RSSK 923	-	-	-	-	-	-	+	-
Lactococus lactis LMG 2907	-	-	-	-	-	-	-	-
Listeria innoqua LMG 2813	+	+	+	+	+	-	+	+
Lactococcus lactis LMG 3113	+	+	+	+	-	-	-	+

+, presence of activity; -, absence of activity

Table 3.5. Continue

producer / indicator	Bacteriocin: lacticin	lacticin 3147	lacticin 3147	lacticin 3147	lacticin 481	LCN G	This study	nisin
	LMG 2907	**LMG 2910**	**LMG 2911**	**LMG 2912**	**LMG 3113**	**LMG 2088**	***L. mesenteroides***	**SIK-83**
Lactobacillus plantarum DSM 20246	-	-	-	-	-	-	-	+
Lactococcus lactis LMG 2911	-	-	-	-	-	-	-	+
Lactococcus lactis LMG 2088	+	+	+	+	-	-	-	-
Lactococcus lactis LMG 2910	-	-	-	-	-	-	-	+
Lactococcus lactis IL-1403	-	-	-	-	-	-	-	+
Lactococcus lactis SIK-83	+	+	+	+	-	-	+	-
Lactobacillus plantarum 80B	-	-	-	-	-	-	-	-
Lactobacillus salivarius M7	-	-	-	-	-	-	-	+
Lactobacillus plantarum RSSK 675	-	-	-	-	-	-	+	+
Bacillus cereus LMG 2732	-	-	-	-	-	-	+	-
Lactobacillus sake LMG 2313 (NCDO 2714)	-	-	-	-	-	+	+	+
Pediococcus pentosaceous LMG 2001	-	-	-	-	-	-	-	+
*Lactobacillus plantaru*m RSSK10	-	-	-	-	-	-	-	-
Lactobacillus plantarum LMG 2003	+	+	+	+	+	+	+	+
Pseudomonas fluorescens LMG 3020	-	-	-	-	-	-	+	-
Staphylococcus aureus LMG 3022	+	+	+	+	+	-	-	-
Lactococcus lactis LMG 2912	-	-	-	-	-	-	-	+
Lactobacillus casei NRRL 334	-	-	-	-	-	-	-	-

+, presence of activity; -, absence of activity

3.4.3. Comparison of bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* with other bacteriocins on common indicators.

Activity of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was compared with other bacteriocins in common indicator bacteria in terms of the highest two-fold dilutions (Table 3.6). Table 3.6 compares also the sensitivities of the some indicator bacteria to the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. As shown, the indicator bacteria *Lactococcus lactis* SIK-83 was inhibited by 1/8 dilution of the supernatant of *Leuconostoc mesenteroides* subsp. *cremoris*. The same indicator was inhibited by 1/4 dilution of the other bacteriocins (lacticin 3147 and lacticin 481). When the indicator bacteria was *Lactobacillus sake* LMG 2313 (NCDO 2714), only 1/1 dilution of *Leuconostoc mesenteroides* subsp. *cremoris* supernatant inhibited this indicator organism while 1/16 dilution of lactococcin G was able to inhibit this indicator organism. In other words, NCDO 2714 is 16 times more sensitive to the lactococcin G than to the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. As seen from Table 3.6, Lactococcin G was more bactericidal than the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* against *Lactobacillus plantarum* LMG 2003; Although, 1/2 dilution of the supernatant of the culture of *Leuconostoc mesenteroides* subsp. *cremoris* inhibited *Lactobacillus plantarum* LMG 2003, 1/16 dilution of the lactococcin G was sufficient to inhibit *Lactobacillus plantarum* LMG 2003. Nisin and Lacticin 481 were more effective against *Lactobacillus plantarum* LMG 2003 than the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*. Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was more bactericidal against *Listeria innoqua* LMG 2813 and *Enterococcus faecalis* LMG 2602 than other bacteriocins (Table 3.6). The indicator bacteria which were sensitive to the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* also showed differences with respect to sensitivity to this bacteriocin (Table 3.6). For example, *Lactobacillus sake* NCDO 2714 and *Lactobacillus plantarum* RSSK 675 were inhibited by the 1/1 dilution (undiluted) supernatant of *Leuconostoc mesenteroides* subsp. *cremoris*,

Lactobacillus plantarum LMG 2003 was inhibited by 1/2 dilution of the bacteriocin containing supernatant of *Leuconostoc mesenteroides* subsp. *cremoris*. Other indicator bacteria were inhibited by 1/8 dilution of the supernatant of *Leuconostoc mesenteroides* subsp. *cremoris*. In this view of point, *Lactobacillus sake* NCDO 2714 and *Lactobacillus plantarum* RSSK 675 were the less sensitive indicator microorganisms compared to other sensitive bacteria (Table 3.6). Although *Leuconostoc mesenteroides* subsp. *cremoris* supernatant showed clear inhibition zone on *Lactobacillus plantarum* RSSK 675, this indicator was not as sensitive as other sensitive indicator bacteria.

Table 3.6. Comparison of bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* with that of other bacteriocins on common indicators in a quantitative manner.

	Bacteriocin producers							
Bacteriocin / **Common indicators**	**SIK 83**	**LMG 2907**	**LMG 2912**	***L. mesenteroides***	**LMG 2910**	**LMG 3113**	**LMG 2911**	**LMG 2088**
	Nisin	**Lacticin**	**Lacticin 3147**	**This study**	**Lacticin 3147**	**Lacticin 481**	**Lacticin 3147**	**Lactococcin G**
Lactococcus lactis SIK-83		1/4	1/4	1/8	1/4	1/4	1/4	
Lactococcus lactis LMG 2909		1/2		1/8				
Listeria innoqua LMG 2813	1/4		1/4	1/8	1/4	1/1	1/4	1/2
*Lactobacillus sak*e LMG 2313 (NCDO2714)	1/4	1/2	1/1	1/1	1/1	1/4	1/1	1/16
Enterococcus faecalis LMG 2602	1/2	1/4	1/4	1/8	1/4		1/4	1/1
Lactobacillus cremoris RSSK 708				1/4				
Lactobacillus plantarum RSSK 675				1/1				
Lactobacillus plantarum LMG 2003	1/4	1/2	1/1	1/2	1/2	1/4	1/1	1/16
Pseudomonas fluorescens LMG 3020				1/8				
Lactobacillus delbrueckii subsp. *lactic* RSSK 498				1/8				

***Activities were expressed as the highest dilutions of supernatant showing a complete clear inhibition zone.**

3.5. Sensitivity of the bacteriocin to enzymes

Antagonism by lactic acid bacteria due to metabolic end products such as acid and hydrogen peroxide, can be erroneously attributed to the production of bacteriocin-like compounds. Attributing the inhibitory activity of *Leuconostoc mesenteroides* subsp. *cremoris* to bacteriocin-like substances therefore requires elimination of other inhibitory factors produced by *Leuconostoc mesenteroides* subsp. *cremoris*. Catalase was effective in eliminating the inhibitory activity a 3 % solution of H_2O_2, but did not eliminate the inhibitory activity of the supernatant of *Leuconostoc mesenteroides* subsp. *cremoris.*

The cell-free culture fluid of *Leuconostoc mesenteroides* subsp. *cremoris* was treated by a variety of enzymes shown in Table 3.7 to verify the protein nature of the inhibitor substance. The inhibitory activity of the supernatant was inhibited by all the proteases employed. Activity was not lost after treatment with lysozyme and catalase. These data clearly showed that the antimicrobial substance is of proteinaceous nature, containing cleavage-sites suitable for the mentioned proteases. Characterization of the proteinaceous inhibitor confirmed that the antimicrobial agent produced by *Leuconostoc mesenteroides* subsp. *cremoris* was a bacteriocin according to the criteria outlined by Tagg *et al*. (1976). Surprisingly, this bacteriocin was inhibited by also α-amylase. The α-amylase sensitivity of the bacteriocin is unusual. The results indicate that the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was a glycoprotein which required both the glyco portion and the protein portion of the molecule for activity. There has been a few glycoprotein bacteriocin studied in literature like lactocin prodıced by *Lactobacillus helveticus* LP27 (Upreti and Hinsdill, 1973) and leuconocin S produced by *Leuconostoc paramesenteroides* (Lewus *et al*., 1992). Leuconocin S is an amylase-sensitive bacteriocin produced by *Leuconostoc paramesenteroides* isolated from retail lamb and has a broad spectrum of activity. It was active against *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and some strains of *Clostridium botilunum*. Staphylococcin (Gagliano and Hinsdill, 1970) and thermocin (Fikes *et al*., 1983) are also glycoprotein

bacteriocins. Patek *et al*. (1986) suggested that the bacteriocin-like inhibitor produced by *Corynebacterium glutamicum* is a glycoprotein based on NMR data.

Table 3.7. Effect of enzymes on the activity of bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris.*

Treatment	Activity* (AU/ml)
Catalase	+
Trypsin	-
Chymotrypsin	-
Pepsin	-
Lysozyme	+
Proteinase K	-
Alkali serine protease	-
α-amylase	-

*Determined by the spot-on-the lawn method using *Enterococcus faecalis* LMG 2602 as indicator microorganism

*+,presence of activity; -, absence of activity

3.6. Sensitivity of the bacteriocin to dissociating agents

To determine if the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was present in aggregated form, crude bacteriocin containing culture fluid of *Leuconostoc mesenteroides* subsp. *cremoris* was treated by the dissociating agents, including nonionic and anionic detergents, in various combinations and alone by employing the amounts shown in Table 3.8 as explained in Material and Methods. Bacteriocin

activity was increased by 1% Tween 80, 0.1% SDS, 0.2% β-mercaptoethanol in combination with 0.1% SDS, and 0.1 % Triton X-100 (from 1600 AU/ml to 3200 AU/ml). 6M guanidine HCI, 8 M urea, 1 % SDS, 2 % β-mercaptoethanol in combination with 1 % SDS reduced the bacteriocin activity 50 % (from 1600 AU/ml to 800 AU/ml). 2% β-mercaptoethanol reduced the bacteriocin activity by 75 (from 1600 AU/ml to 400 AU/ml). 5% SDS completely destroyed bacteriocin activity (from 1600 AU/ml to 0 AU/ml) (Table 3.8).

The high concentrations of SDS and β-mercaptoethanol (1%) had a detrimental effect on the bacteriocin activity as shown in Table 3.8. These dissociating was reported to reduce the activities of other bacteriocins also, especially those of class II a bacteriocins, since they reduce the disulfide bonds. The negative effect of β-mercaptoethanol on the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* indicates the presence of at least one disulfide bond in its structure. The characterized bacteriocins of *Leuconostoc* spp. are mostly class IIa bacteriocins (Cystibiotics) that have at least one disulfide bond which is necessary for bactericidal activity, e.g., leucocin A and mesentericin Y105. Disulfide bridge promotes intramolecular rigidity within class IIa bacteriocins, which may affect activity and heat stability under certain conditions.

The results indicated that bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was present in aggregated form since the bacteriocin activity of the crude extract was increased by the reported amount of dissociating agents, which doubled the activity (from 1600 AU/ml to 3200 AU/ml). The increase in bacteriocin activity was confirmed not due to the bactericidal effect of dissociating agents as evidenced by control experiments.

Table 3.8. Effect of dissociating agents on the activity of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*.

Dissociating agent	Bacteriocin activity + Treatment (AU/ml)	Dissociating agent control (AU/ml)
Control	1600	-
0.1 % Tween 80	1600	0
1.0 % Tween 80	3200	0
6M Guanidine HCI	800	0
8M Urea	800	0
0.1 % SDS	3200	0
1.0 % SDS	800	800
5.0 % SDS	0	3200
0.2 % β-mercaptoethanol	1600	0
2.0 % β-mercaptoethanol	400	0
0.2 % β-mercaptoethanol + 0.1 % SDS	3200	0
2.0 % β-mercaptoethanol + 1.0 % SDS	800	0
1.0 % Triton X-100	800	0
0.1 % Triton X-100	3200	0

3.7. Effect of heat treatment and pH on bacteriocin activity

3.7.1. Effect of heat treatment on bacteriocin activity

Effect of heat treatment on the bacteriocin activity was tested by keeping the crude extract (final pH after the fermentation was 4.8) at 45, 50, 60, 70, and 100^0C for various periods (Table 3.9). As seen from Table 3.9, crude bacteriocin was very stable to heat with respect to the all the temperatures and durations because the original

activity of crude extract (1600 AU/ml) was unchanged upon all the heat treatment regimes. The stability of bacteriocin preparations has often been shown to decrease significantly with increased purification. Crude bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was stable at 100^0C for 2 hours as shown in Table 9, with the same bacteriocin titer of 1600 AU/ml.

Heat resistance is a major characteristic of many bacteriocins and bacteriocin-like compounds produced by lactic acid bacteria and can vary dramatically ranging from 60^0C or 100^0C for more than 30 min (e.g. lactocin 27, lactocin S, carnobacteriocins A and B) to autoclaving at 121^0C for 15-20 min (e.g. lactacin B, lactacin F, nisin etc.) (De Vuyst and Vandamme, 1994).

Many of the bacteriocins produced by lactic acid bacteria, particularly the ones of class I and class II, are described as small hydrophobic proteins containing little tertiary structure, which explains their heat stability. Other factors contributing to heat stability of the bacteriocin of LAB are stable cross-linkages, a high glycine content and occurrence of strongly hydrophobic regions. Such heat stability also excludes the possibility of the inhibitory action being due to bacteriophage (De Vuyst and Vandamme, 1994).

Table 3.9. Titration of the bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* upon heat treatment at 45, 50, 60, 70^{0}C and 100^{0}C from 0 min up to 120 min (Activities, AU/ml).

Time (min)	45^{0}C	50^{0}C	60^{0}C	70^{0}C	100^{0}C
0	1600	1600	1600	1600	1600
5	1600	1600	1600	1600	1600
10	1600	1600	1600	1600	1600
20	1600	1600	1600	1600	1600
30	1600	1600	1600	1600	1600
35	1600	1600	1600	1600	1600
40	1600	1600	1600	1600	1600
45	1600	1600	1600	1600	1600
50	1600	1600	1600	1600	1600
55	1600	1600	1600	1600	1600
60	1600	1600	1600	1600	1600
65	1600	1600	1600	1600	1600
70	1600	1600	1600	1600	1600
75	1600	1600	1600	1600	1600
80	1600	1600	1600	1600	1600
85	1600	1600	1600	1600	1600
90	1600	1600	1600	1600	1600
95	1600	1600	1600	1600	1600
100	1600	1600	1600	1600	1600
105	1600	1600	1600	1600	1600
110	1600	1600	1600	1600	1600
115	1600	1600	1600	1600	1600
120	1600	1600	1600	1600	1600

3.7.2. Effect of pH on bacteriocin activity

Supernatant samples taken following incubation and adjusted to pH 2-12 were found to be active on the indicator strain *Enterococcus faecalis* LMG 2602 (Figure 3.3 and Table 3.10.1). Uncultivated sterile MRS broth (pH range 2-12) spotted onto LMG 2602 as control did not show inhibitory activity on *Enterococcus faecalis* LMG 2602 as indicated by the absence of inhibition zone on indicator lawn (Figure 3.4).

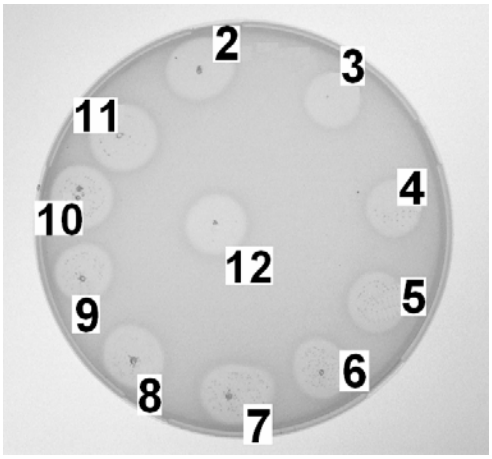


Figure 3.3. Activity of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* at pH 2.0-12 at room temperature after fermentation.

Table 3.10.1. The effect of pH (2-12) on the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* after fermentation (Activities, AU/ml).

pH	Activity
2	1600
3	1600
4	1600
5	1600
6	1600
7	1600
8	1600
9	1600
10	1600
11	1600
12	1600

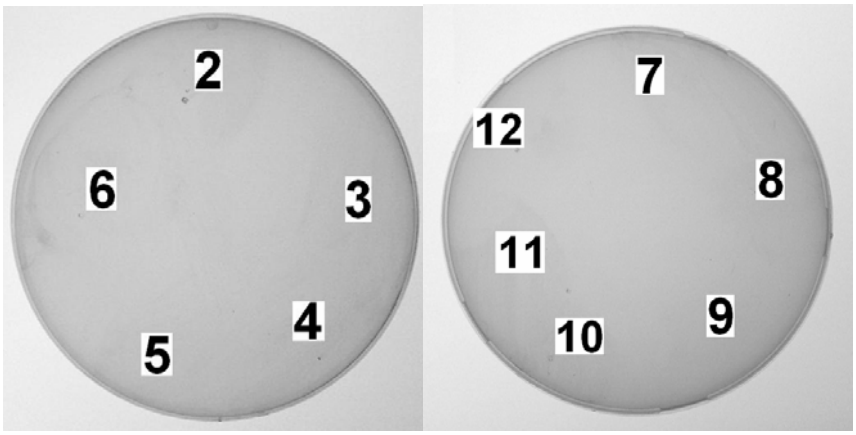


Figure 3.4. Uncultivated sterile MRS broth (pH 2.0-12) spotted on *Enterococcus faecalis* LMG 2602 as control.

Bacteriocin activity was not affected by all the pH treatments within the first 30 min (Table 3.10.2). The decrease in bacteriocin activity occurred at pH 11 after 45 min, at pH 10 after 60 min and at pH 8 after 4 h; the prolonged exposure to the alkali pH was found to be detrimental to the bacteriocin activity. After three days incubation at $+4^0$C, precipitations were observed in sample with pHs 2.0, 3.0, 11 and 12. After these precipitations were removed by centrifugation, activity of remaining supernatants was tested for bacteriocin activity and activity was found in samples with a pH of 2.0, 3.0, and 11 but not in the sample with pH of 12. However, the activity of this fraction was restored partially when the pH of this fraction (pH 12) was neutralized to pH 6.0.

Table 3.10.2. Residual bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* supernatant (pH 2-12) upon incubation at room temperature for 5, 15, 30, 45, 60 min and 4 h (Activities, AU/ml).

pH	5 min	15 min	30 min	45 min	60 min	4 h
2.0	1600	1600	1600	1600	1600	1600
3.0	1600	1600	1600	1600	1600	1600
4.0	1600	1600	1600	1600	1600	1600
5.0	1600	1600	1600	1600	1600	1600
6.0	1600	1600	1600	1600	1600	1600
7.0	1600	1600	1600	1600	1600	1600
8.0	1600	1600	1600	1600	1600	800
9.0	1600	1600	1600	1600	1600	800
10.0	1600	1600	1600	1600	800	800
11.0	1600	1600	1600	800	800	800
12.0	1600	1600	1600	800	800	400

Bacteriocins differ greatly with respect to their sensitivity to inactivation by changes in pH and temperature. Many of the bacteriocins and bacteriocin-like substances produced by lactic acid bacteria are only stable at acid and neutral pH (De Vuyst and Vandamme, 1994) and are inactivated even at a pH above 8.0 (e.g. nisin, lactostrepcins, pediocin AcH, leucocin A-UAL 187). This can be attributed to the solubility of the bacteriocins of LAB (lactic acid bacteria); the isoelectric points of the bacteriocins produced by LAB are around 8.0-9.0 and the solubility of the bacteriocins decreases with increasing pH.

3.7.3. Combined effect of heat and pH on bacteriocin activity

The combined effect of heat and pH was tested also by exposure of the crude bacteriocin, of which pH was pre-adjusted to 2-12, to different temperature for different durations Table 3.11). The crude extract with a pH value between 2 and 12 was kept for 4 h at room temperature, for 1 h at 100^0C, for 24 h at 4^0C and for 24 h at room temperature. It is seen from Table 3.11 that crude bacteriocin at 4^0C within the pH range of 2 to 12 is rather stable, activity losses occurred at pH 12 only in spite of 24 h incubation at 4^0C. At room temperature incubation for 4 h, the activity losses began to come out at pH 8, activity being dropped to 800 AU/ml from 1600 AU/ml. When the crude bacteriocin incubated at room temperature for 24 h, bacteriocin activity was reduced by 50 % within the pH range of 2 to 10, by 75 % at pH 11 and by 100 % loss at pH 12. When the crude bacteriocin was boiled for 1 h (approximately 100^0C), activity was stable between pH 2 and 7 and dropped at pH above 7.0. Boiling did not affect the stability of the bacteriocin in the pH range of 2.0 to 7.0 (Figure 3.5) but above the pH 7.0, boiling was detrimental to the bacteriocin activity. The bacteriocin activity was reduced by 50% and 75% at pH 8.0 and 9.0, respectively as understood by comparing the first and second columns in Table 3.11 in spite of the clear inhibition zones at pH 8.0 and 9.0 in Figure 3.5 because the detection limit of the bacteriocin is 200 AU/ml. The obvious stability of the bacteriocin at 4^0C for 24 h incubation was also due to the inhibition of the proteases at this temperature.

Table 3.11. Activities of the bacteriocin produced *by Leuconostoc mesenteroides* sıbsp. *cremoris* incubated at different temperatures within the pH range of 2-12 (Activities, AU/ml).

pH	Four hours at room temperature	Boiling for 1 hours	Incubation at 4^0C for 24 hours	Incubation at room temperature for 24 hours
2	1600	1600	1600	800
3	1600	1600	1600	800
4	1600	1600	1600	800
5	1600	1600	1600	800
6	1600	1600	1600	800
7	1600	1600	1600	800
8	800	400	1600	800
9	800	200	1600	800
10	800	0	1600	800
11	800	0	1600	400
12	400	0	400	0

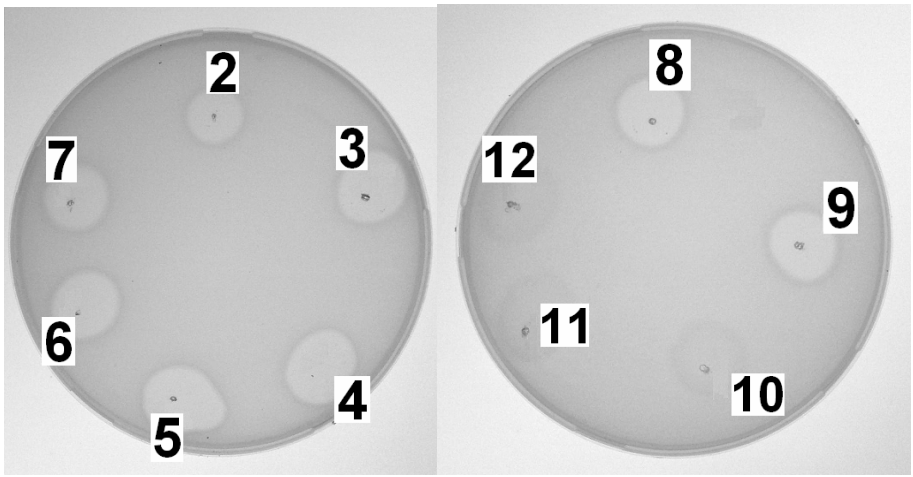


Figure 3.5. The effect of boiling temperature on the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* at pH 2.0-12.

3.7.4. Comparison of combined heat and pH stability of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* with that of other bacteriocins

The combined heat and pH stability of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was compared with that of other bacteriocins through Table 3.12-3.15. The stability was compared within the pH range of 2-12 for various times and temperatures. Table 3.12 illustrates the stability of these bacteriocins at pH 2-12 by keeping the bacteriocins at room temperature for 4 h after fermentation. As shown, bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was more stable compared with others. 100% of the activity of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was retained within the pH range of 2.0-7.0 and then activity was reduced by 50% at pH 8.0. Although, bacteriocin activity was reduced, it was observed up to pH 12 at room temperature for 4 hours. As for nisin, it retained 100% of the bacteriocin activity in between pH 2.0 and 5.0, then its activity was reduced by 50% and 25% at pH 6.0. and pH 8.0, respectively. Nisin activity was completely lost at pH 12 at room temperature for 4 hours. As can be seen from the Table 3.12, bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was also stable when compared to lactococcin G and lacticin 3147 at room temperature for 4 hours.

Table 3.12. Comparison of remaining percentage bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* culture fluid with those of other bacteriocin producers after incubation at pH 2-12 for 4 hours at room temperature.

pH	*L. mesen teroides*	SIK 83 Nisin	2088 LCN G	3113 Lacticin 481	2910 lacticin 3147	2911 Lacticin 3147	2912 lacticin 3147	2907 lacticin
2	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
6	100	50	100	100	100	100	100	100
7	100	50	100	100	50	50	50	50
8	50	25	50	0	25	25	25	25
9	50	25	50	0	0	0	0	0
10	50	25	50	0	0	0	0	0
11	50	25	12,5	0	0	0	0	0
12	25	0	0	0	0	0	0	0

Stability to combined pH and boiling stress (for 1 hour) was also tested for the above bacteriocins. Lacticin activity was completely destroyed after the pH 6 and lacticin 481 was lost completely after pH 5.0. Nisin and lactococcin G was also completely destroyed after pH 6 and 7, respectively, while bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* was completely destroyed after the pH 9 (Table 3.13). Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was stable to boiling for 1 h as nisin was. Although, nisin activity was reduced at around neutral pH, bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* was retained completely at neutral pH values. The solubility, stability and biological activity of nisin are highly dependent on the pH of the solution. These drop suddenly as the pH is increased. In neutral and alkaline conditions nisin is almost insoluble (Liu and Hansen, 1990). The stability of nisin solutions to heat and storage depends not only on pH, but also on

several other factors such as chemical composition of the solution, the protective effect of proteins, the temperature etc. Nisin can be reversibly adsorbed by some proteins. This adsorption accounts for the protective effect when nisin solutions are exposed to heat (Hall, 1966).

Table 3.13. Comparison of remaining percentage bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* culture fluid with those of other bacteriocin producers after boiling for 60 min at pH 2-12.

pH	*L. mesenteroides.*	Sık 83 Nisin	2088 LCN G	3113 Lacticin 481	2910 lacticin 3147	2911 Lacticin 3147	2912 lacticin 3147	2907 lacticin
2	100	100	100	25	25	25	25	25
3	100	100	100	25	25	25	25	25
4	100	100	25	25	25	25	25	25
5	100	50	25	25	25	25	25	25
6	100	25	25	25	25	25	25	25
7	100	0	25	0	0	0	0	0
8	25	0	0	0	0	0	0	0
9	12,5	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0

The similar pH stability test was performed by keeping the bacteriocins at room temperature and at 4^0C for 24 h (Table 3.14 and 3.15). When looked at the tables, it is shown that bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* is more stable than other bacteriocins involved with respect to pH and heat stability. As shown in Table 3.15, all the bacteriocins retained their activities without any loss at

refrigerator temperature (+4^{0}C). The inactivation of proteases in crude bacteriocin fluid may be an explanation for these observations.

Piard *et al*. (1990) reported that biological activity of lacticin 481 was unchanged after 1 h at 100^{0}C at pH 4.5 or 7.0. However, 50% activity was lost after 20 min at 115^{0}C and pH 2.0. Lacticin 481 therefore was reported to be more stable than nisin at neutral pH, and the opposite is observed at acid pH (Hurst, 1981).

Table 3.14. Comparison of remaining percentage bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* culture fluid with those of other bacteriocin producers after incubation at room temperature for 24 hours at pH 2-12.

pH	*L. mesenteroides*	Sık 83 Nisin	2088 LCN G	3113 Lacticin 481	2910 lacticin 3147	2911 Lacticin 3147	2912 lacticin 3147	2907 lacticin
2	100	100	100	50	50	50	50	50
3	100	100	100	50	50	50	50	50
4	100	100	100	50	50	50	50	50
5	100	100	100	50	50	50	50	50
6	100	50	100	50	50	50	50	50
7	100	50	100	50	50	50	50	50
8	100	25	25	0	50	50	50	50
9	100	25	25	0	25	25	25	25
10	100	0	12.5	0	0	0	0	0
11	25	0	6.25	0	0	0	0	0
12	0	0	0	0	0	0	0	0

Table 3.15. Comparison of remaining percentage bacteriocin activity of *Leuconostoc mesenteroides* subsp. *cremoris* culture fluid with those of other bacteriocin producers after incubation at 4^0C for 24 hours at pH 2-12.

pH	*L. mesenteroides*	*Sık 83 Nisin*	*2088 LCN G*	*3113 Lacticin 481*	*2910 lacticin 3147*	*2911 Lacticin 3147*	*2912 lacticin 3147*	*2907 lacticin*
2	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100
7	100	50	100	100	100	100	100	100
8	100	50	100	50	50	50	50	50
9	100	50	100	50	50	50	50	50
10	100	50	12.5	0	25	25	25	25
11	100	25	12.5	0	0	0	0	0
12	25	0	0	0	0	0	0	0

3.8. Effect of organic solvents on the bacteriocin activity

Crude bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was tested for the stability against the organic solvents shown in Table 3.16 as explained in Material and Methods. 10 % (v/v) and 25 % (v/v) concentrations of the organic solvents were mixed with bacteriocin and incubated. After the evaporation of the organic solvent, the remaining bacteriocin titers were found as in Table 3.16. Of these organic solvents di-ethylether reduced the bacteriocin activity by 50 % (from 1600 AU/ml to 800 AU/ml). Bacteriocin activity was not altered by the treatment with all other organic solvents and remained 1600 AU/ml. Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*

was found to be stable methanol, hexane, toluene, petroleum ether, ethanol, acetonitrile, isoamylalcohol, 2-propanol, formaldehyde, acetone, 1-butanol and chloroform in specified concentrations. Being stable to organic solvents shows its hydrophobic character and important in the isolation-extraction of the bacteriocin with organic solvents from the liquid culture supernatant. Moreover, elution of the bacteriocin from Amberlite XAD-16, Phenyl Sepharose and Reverse Phase columns requires high concentrations of some organic solvents like 2-propanol, ethanol, methanol, and acetonitrile.

Table 3.16. Determination of the stability of the bacteriocin produced by *Leuconostoc mesenteroides* subsp*. cremoris* against organic solvents with 10 % and 25 % concentrations (Activities, AU/ml).

Organic solvent	**% 10 conc. (v/v)**	**% 25 conc. (v/v)**
Methanol	1600	1600
Hexane	1600	1600
Toluene	1600	1600
Petroleum ether	1600	1600
Ethanol	1600	1600
Acetonitrile	1600	1600
Isoamylalcohol	1600	1600
2-propanol	1600	1600
Formaldehyde	1600	1600
Acetone	1600	1600
Di-ethylether	800	800
1-butanol	1600	1600
Chloroform	1600	1600

3.9. Extraction of the bacteriocin with organic solvents

Methods of extracting bacteriocins are based on their affinity to organic solvents, their variation in solubility in concentrated salt solutions at a given pH value. The presence of hydrophobic regions in bacteriocin molecules is essential for their activity against sensitive bacteria. Inactivation of microorganisms with bacteriocins depends on the hydrophobic interaction between cells and bacteriocin molecules (Abee *et al*., 1991). The amphiphilic properties of bacteriocins were made use of separating bacteriocins at the interface of immiscible liquids in this study. Chloroform, chloroform-methanol (2:1, v/v), methanol, n-propanol, chloroform-isopropanol (2:1,v/v), hexane, isoamylalcohol, di-ethylether, petroleum ether, toluene, 1-butanol were tested for the extraction of the bacteriocin.

It was observed that chloroform, chloroform-methanol and chloroform-isopropanol extracted the bacteriocin very efficiently (Table 3.17). Di-ethylether and petroleum ether did not remove the bacteriocin from aqueous phase to organic phase sufficiently. 1-butanol did not result in the removal of the bacteriocin from aqueous phase to organic phase. Hexane, toluene, isoamylalcohol, methanol and n-propanol extracted approximately 80 % of the bacteriocin to organic phase. Chloroform containing mixtures resulted in precipitates at the solvent-aqueous interface with strong bacteriocin activity.

Table 3.17. Influence of organic solvents in the extraction of bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*.

Organic solvent	Percentage of the bacteriocin in both phases after extraction	
	Organic phase	**Aqueous phase**
Chloroform	100	0
Chloroform-methanol	100	0
Chloroform-isopropanol	100	0
Methanol	80	20
Hexane	80	20
Petroleum ether	30	70
Di-ethylether	40	60
n-propanol	70	30
Toluene	80	20

3.9.1. Extraction of the bacteriocin with chloroform and chloroform-methanol from 1 liter culture supernatant of *Leuconostoc mesenteroides* subsp. *cremoris*

The majority of bacteriocin activity was found at the interface between the solvent and the culture supernatant. Also, some activity was found in the sediments collected from the sides and bottom of the centrifuge tube. Bacteriocin activity was not found in the solvent phases. Precipitate at the interface between the chloroform and culture supernatant contained most of the the bacteriocin activity in the mixture. When 1 liter of supernatant was used, bacteriocin activities from the top (culture fluid), interface and bottom (chloroform) layers of the mixture were 2 x 10^5, 3 x 10^6 and 0 AU, respectively. A comparison of ammonium sulphate precipitation (80% saturation), chloroform extraction and pH mediated cell adsorption-desorption for the extraction of

the bacteriocin from culture supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* is shown in Table 3.18.

Table 3.18. Extraction of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* using chloroform extraction, ammonium sulphate precipitation and pH mediated cell adsorption-desorption methods.

Method	Total AU recovered fom 1 liter supernatant	Specific activity (AU/µg protein)
No extraction	1.6×10^6	0.5
Chloroform extraction	3×10^6	9.7
80%Ammonium sulphate precipitation	16×10^6	3.2
pH mediated cell adsorption-desorption	0.5×10^6 or 1.6×10^6	40

* Total activity recovered from 1 liter culture supernatant by cell mediated adsorption-desorption varies with the amount of cell; if 1 liter supernatant is treated with the cells resulted from 1 liter broth, only 15-20 % of the total bacteriocin recovered. The larger the amount of cells, the more bacteriocin was attained.

Ammonium sulfate precipitation recovered more bacteriocin from the supernatant than the other two methods. Specific activity of the bacteriocin preparation was highest for the pH mediated cell adsorption-desorption method and lowest for the ammonium sulphate precipitation. Total activity was higher in the fractions obtained by ammonium sulphate precipitation and chloroform extraction than in the culture supernatant. This indicates that ammonium sulphate precipitation and chloroform extraction dispersed bacteriocin aggregates. Burianek and Yousef (2000) applied

chloroform extraction to recover lacidin, pediocin, nisin, bacillin, and subtilin. They reported that recovery of bacteriocins from culture fluid by chloroform was greater for pediocin, nisin and subtilin than lacidin. Chloroform effectively recovered pediocin, nisin and bacillin from culture fluid.

Bacteriocins are amphiphilic peptides with high affinity towards lipid membranes. The interface between chloroform and aqueous media creates ideal environments for the concentration of amphiphilic substances, such as bacteriocins. Sufficient mixing of culture supernatant with chloroform allows for the migration of bacteriocins from the aqueous medium to the interfacial layer (Burianek and Yousef, 2000). Chloroform has a polarity index (PI) of 4.1; PI values of hexane and dimethyl sulphoxide are 0.1 and 7.2, respectively. Solubility of chloroform in water is only 0.815 %. Therefore, chloroform is a solvent with intermediate polarity and is immiscible with water. These properties make chloroform most suitable for bacteriocin concentration from culture fluid (Burianek and Yousef, 2000).

3.10. Recovery and partial purification of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* by diatomite calcium silicate

3.10.1. Adsorption of the bacteriocin onto Micro-Cel.

Culture supernatant of *Leuconostoc mesenteroides* subsp.*cremoris* (1600 AU/ml, pH 4.8) was adsorbed with Micro-Cel (1 %, wt/vol) for 60 min at room temperature. All the bacteriocin found in the culture supernatant was adsorbed onto Micro-Cel, with no activity detected in the supernatant after the separation of Micro-Cel by centrifugation. 100 % activity was recovered in the Micro-Cel. The amount of bacteriocin adsorbed per gram of Micro-Cel was 160 000 AU.

3.10.2. Desorption of the bacteriocin from Micro Cel E with acidic and alkaline pH conditions

The Micro-Cel adsorbed bacteriocin was dissolved in distilled water and the pH of the mixture was tested between 2.0 and 12 to eluate the adsorbed bacteriocin. After the centrifugation, the pH of the resulting supernatant was adjusted to 5.0 and assayed for the bacteriocin activity. No bacteriocin was desorbed between pH 2.0 and 12 (Table 3.19).

Table 3.19. Desorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* from Micro-Cel at room temperature for 60 min with acidic and alkaline pH conditions.

pH	Activity of the bacteriocin on *Enterococcus faecalis* LMG 2602 (AU/ml, %)	
	Desorbed into eluent	Remaining in Micro-Cel
2	0	1600 100 %
3	0	1600 100 %
4	0	1600 100 %
5	0	1600 100 %
6	0	1600 100 %
7	0	1600 100 %
8	0	1600 100 %
9	0	1600 100 %
10	0	1600 100 %
11	0	1600 100 %
12	0	1600 100 %

In addition, the same experiments in these pH ranges were performed by adding NaCI such that the final concentration of NaCI would be 0.1 M. (Table 3.20) but none of these conditions desorbed the bacteriocin of *Leuconostoc mesenteroides* subsp.*cremoris* from Micro-Cel. It is interesting that when the pH of the eluate was re-adjusted to 5.0 from 2.0, a large amount of protein aggregation was observed suddenly but this fraction did not show bacteriocin activity.

Table 3.20. Desorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* from Micro-Cel at room temperature for 60 min with pH 2 to 12 by adding 0.1 M NaCI.

pH	Activity of the bacteriocin on *Enterococcus faecalis* LMG 2602 (AU/ml, %)	
	Desorbed into eluent	Remaining in Micro-Cel
2	0	1600 100 %
3	0	1600 100 %
4	0	1600 100 %
5	0	1600 100 %
6	0	1600 100 %
7	0	1600 100 %
8	0	1600 100 %
9	0	1600 100 %
10	0	1600 100 %
11	0	1600 100 %
12	0	1600 100 %

3.10.3. Desorption of the bacteriocin from Micro Cel E with organic solvents, salts and urea

Desorption of the bacteriocin from Micro-Cel was also tested by organic solvents (Chloroform, methanol, hexane, toluene, ethanol, acetonitrile and isoamyalcohol), 0.2 M phosphate buffer, 0.1 M NaCI in 0.2 M phosphate buffer, 1 % urea, 1 % disodium EDTA at room temperature for 1 hour. These agents were tested whether to produce a zone of inhibition on the lawn of the indicator bacterium *Enterococcus faecalis* LMG 2602. Consequently, none of these agents produced a zone of inhibition on the indicator lawn or resulted in desorption of the bacteriocin from Micro-Cel. 100 % of the bacteriocin was left on the adsorbent (Table 3.21).

Table 3.21. Desorption of the bacteriocin from the Micro-Cel at room temperature for 60 min with organic solvents (Chloroform, methanol, hexane, toluene, ethanol, acetonitrile and isoamyalcohol), 0.2 M phosphate buffer, 0.1 M NaCI in 0.2 M phosphate buffer, 1 % urea, 1 % disodium EDTA.

Organic solvent	**Activity of the bacteriocin on *Enterococcus faecalis* LMG 2602 (AU/ml, %)**	
	Desorbed into eluent	**Remaining in Micro-Cel**
Chloroform	0	1600 100 %
Methanol	0	1600 100 %
Isoamylalcohol	0	1600 100 %
Hexane	0	1600 100 %
Toluene	0	1600 100 %
Acetonitril	0	1600 100 %
Ethanol	0	1600 100 %
0.2 M phosphate buffer	0	1600 100 %
0.1 M NaCI in 0.2 M phosphate buffer	0	1600 100 %
1 % urea	0	1600 100 %
1 % disodium EDTA		

3.10.4. Desorption of the bacteriocin from Micro Cel E with surfactants

The Micro-Cel adsorbed bacteriocin was desorbed for 60 min at room temperature with 1 % (wt/vol) of various surfactant solutions (Tween 80, Triton X-100, SDS) in distilled water in their natural pH. None of these surfactant controls, at the specified controls and pHs, resulted in inhibition zone on the indicator lawn apart from 1.0 % SDS. Also illustrated in Table 3.8 previously while mentioning about the effects of dissociating agents on the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*, 1.0 % SDS has proven to be producing inhibition zone on the indicator bacteria. However, this situation is not so important because excess SDS could be cold-precipitated.

Triton X-100 and Tween 80 did not desorb the bacteriocin at all. However, SDS in 0.1-1.0 % (wt/vol) desorbed 100 % of the bacteriocin adsorbed onto the Micro-Cel (Table 3.22). The effect of SDS concentration on desorption of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was investigated by extracting Micro-Cel E adsorbed bacteriocin with a volume equal to that of the adsorption culture supernatant, using various concentrations (0.1-1.0 %, wt/vol) of SDS for 60 min at room temperature.

Coventry *et al*. (1996) reported that adsorption of bacteriocins onto Micro-Cel most probably involved both electrostatic and hydrophobic interactions. The electrostatic interactions would be facilitated by the negatively charged diatomite particles over a wide range of pH (2.0 to 11) and by the positively charged bacteriocin molecules at a pH below their isoelectric points. The electrostatic interactions between the Micro-Cel particles and the bacteriocin molecules were decreased at a pH higher than the isoelectric point of the bacteriocin (Coventry *et al*., 1996).

Table 3.22. Desorption of the bacteriocin from Micro-Cel at room temperature for 60 min with surfactants (Triton X-100, Tween 80 and SDS).

Detergent	Activity of the bacteriocin on *Enterococcus faecalis* LMG 2602 (AU/ml, %)	
	Desorbed into eluent	**Remaining in Micro-Cel**
1 % Triton X-100	0	1600 100 %
1 % Tween 80	0	1600 100 %
1 % SDS (SDS removed by cold precipitation after desorption)	1600 100 %	0 0 %

The hydrophobic interactions between the Micro-Cel surface and the bacteriocin molecules were influenced by the use of an appropriate surfactant. Micro-Cel adsorbed bacteriocin was desorbed by SDS effectively but not by Tween 80 and Triton X-100 (Table 3.22). Results obtained from these studies suggest that the hydrophobic interactions between Micro-Cel particles and the bacteriocin molecules are important than the electrostatic interactions and these hydrophobic interactions were overcome more effectively by the use of SDS.

Micro-Cel is inexpensive for use as an adsorbing agent and is suitable for addition as a food-grade anticaking agent to foods for direct human consumption.

The preferred desorption agent, SDS, has been utilized as a food-grade emulsifier at up to 0.1%. Also, SDS was removed from unconcentrated bacteriocin preparations by cold-precipitation. Acetone precipitation of the bacteriocin was also tested to get rid of SDS because of the fact that SDS dissolves in acetone.

3.11. Mode of action of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* and adsorption of the bacteriocin onto indicator cells

3.11.1. Adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto various gram-positive and gram-negative bacteria

The bactericidal action of the bacteriocins of lactic acid bacteria is caused by functional destabilization of the cytoplasmic membrane. Bacteriocin adsorption is a critical step in initiation of lethal interactions between these peptides and sensitive cells. Following adsorption of bacteriocin molecules onto cell surface, entrance of the molecules through the cell wall and finally contact with the cytoplasmic membrane occur. Bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* was examined for adsorption to both sensitive and insensitive indicator organisms. Bacteriocin was adsorbed on both sensitive and insensitive strains of all gram-positive bacteria tested regardless of whether they are sensitive or insensitive to the bacteriocin. Cells of gram-negative bacteria did not adsorb the bacteriocin (Table 3.23). Adsorption of bacteriocin resulted in cell death in the sensitive, but not in the resistant strains. This could be due to the presence of non-specific receptors or binding sites in resistant strains. The sensitive strains probably have both non-specific and specific (lethal) receptors. In sensitive strains the adsorption and lethal action of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was very rapid. Cell death was observed as soon as the bacteriocin was added to the cells of *Enterococcus faecalis* LMG 2602 (Figure 3.7), *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 (Figure 3.9), *Lactococcus lactis* SIK-83 (Figure 3.11) and *Listeria innoqua* LMG 2813 (Figure 3.14, 3.15 and 3.17). Bacteriocin molecules were probably prevented from entering into the normal gram-negative bacterial cells by the lipopolysaccharide barrier. It has been well established that in gram-negative bacteria lipopolysaccharide molecules act as a barrier against the entrance of many molecules including hydrophobic molecules such as sodium lauryl sulfate and deoxycholate, and provide resistance. In sublethally injured cells, the barrier functions of the lipopolysaccharide molecules are temporarily impaired due to conformational change that allows the hydrophobic molecules to pass

through the cell wall (De Vuyst and Vandamme, 1994). Nonlethal binding has been reported for a number of gram-positive bacteriocins, including lactocin 27 produced by *Lactobacillus helveticus* (Barefoot and Klaenhammer, 1983) and pediocin AcH produced by *Pediococcus acidilactici* H (Bhunia *et al*., 1991). Studies with pediocin AcH showed that bacteriocin molecules were adsorbed onto both sensitive and insensitive strains but not to gram-negative bacteria (Bhunia *et al*., 1991). It was shown that lipoteichoic acid molecules in gram-positive bacteria are the receptors of pediocin AcH. Due to their lack of cell-wall lipoteichoic acid, gram-negative bacteria could not adsorb pediocin AcH. The adsorption of bacteriocin onto cell wall of gram-positive bacteria does not lead to passage of the molecules through the cell wall and contact with the cytoplasmic membrane. Passage of the bacteriocin molecules and contact with the cytoplasmic membrane occur only in the cells of sensitive gram-positive bacteria. Ray (1992) suggested that in the gram-positive sensitive cells only, binding of pediocin AcH probably bring about a conformational change in the three-dimensional configuration of the cell wall that cause the loss of its barrier functions. This change allows the pediocin AcH molecules to pass through the cell wall, come in contact with the cytoplasmic membrane and give rise to functional destabilization by their hydrophobic action. In a resistant gram-positive cell either this change in the cell wall does not occur or the immunity proteins interfere with the action of bacteriocin on the cytoplasmic membrane.

Table 3.23. Adsorption of the bacteriocin to resistant and sensitive strains.

Resistant indicators	Adsorption (%)	Sensitive indicators	Adsorption (%)
Lactobacillus plantarum LP73	100	*Lactobacillus cremoris* RSSK 708	100
Lactobacillus casei NRRL 21	100	*Lactobacillus delbrueckii* subsp. *lactic* RSSK 498	100
Lactobacillus casei NRRL 22	100	*Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923	100
Lactobacillus casei NRRL 23	100	*Lacto*coccus *lactis* LMG 2909	100
Lactobacillus plantarum Z11 L	100	*Enterococcus faecalis* LMG 2602	100
Lactobacillus plantarum HÜ	100	*Lactococcus lactis* SIK-83	100
Lactobacillus casei NRRL 441	100	*Listeria innoqua* LMG 2813	100
Lactococcus lactis LMG 2908	100	*Staphylococcus aureus* LMG 3022	50
Lactobacillus acidophilus ATCC 4356	100	*Lactobacillus plantarum* RSSK 675	100
Lactobacillus plantarum 80B	100	*Bacillus cereus* LMG 2732	100
Lactobacillus salivarius M7	100	*Lactobacillus sake* LMG 2313	100
Lactobacillus plantarum DSM 20246	100	*Pediococcus pentosaceous* LMG 2001	100
Lactococcus lactis LMG 2911	100	*Lactobacillus plantaru*m RSSK10	100
Lactococcus lactis LMG 2088	100	*Lactobacillus plantarum* LMG 2003	100
Lactococcus lactis LMG 2910	100	*Pseudomonas fluorescens* LMG 3020	0
Lactococus lactis LMG 2907	100		
Lactococcus lactis LMG 3113	100		
Lactococcus lactis LMG 2912	100		
Lactobacillus casei NRRL 334	100		
Lactococcus lactis IL-1403	100		
E.coli LMG 3083	0		
Salmonella enterica typhimurium LMG 3085	0		

3.11.2. Measurement of lysis and loss of viability of sensitive cells by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris*

Effect of the bacteriocin on the lysis of *Enterococcus faecalis* LMG 2602 was illustrated in Figure 3.6. As shown, the O.D. of bacteriocin treated culture of *Enterococcus faecalis* LMG 2602 did not change for the first 20 min and increased slightly up to 60 min although it lost its viability by % 99.99 (from $4x10^9$ to 10^3 cfu/ml) within the first minutes (Figure 3.7); some delay between the decrease in cell viability and cell lysis of *Enterococcus faecalis* LMG 2602 has been observed. Some structural integrity may be retained by the affected cells, and this may account for the slower decrease in OD. After 1 h, the OD of the bacteriocin treated culture decreased rapidly up to 120 min and then the turbidity of the culture approached to original sterile medium M17 while the OD of the control increased highly. These results indicated that cells of this indicator organism were lysed in contrast to control which not treated with the bacteriocin.

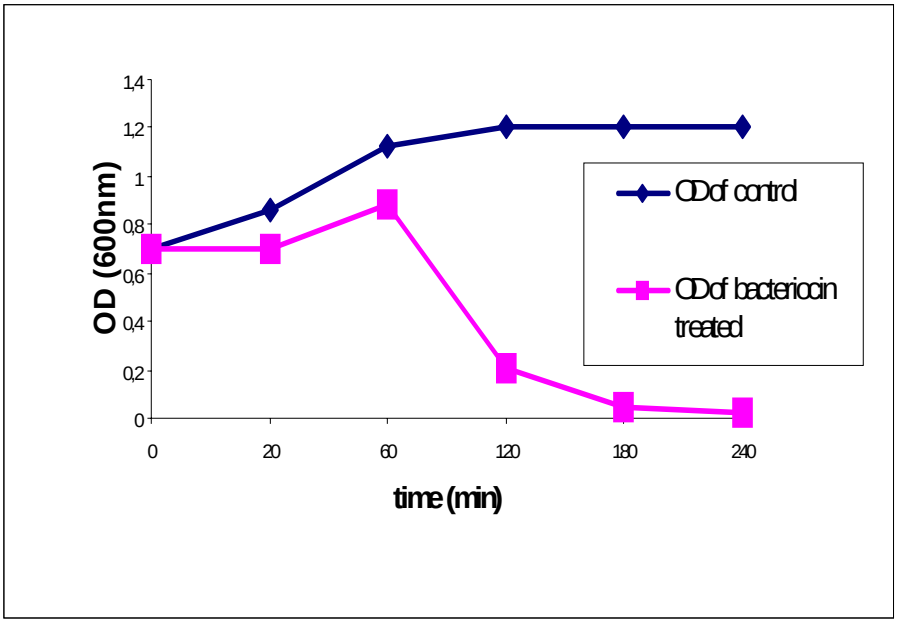


Figure 3.6 Measurement of lysis of *Enterococcus faecalis* LMG 2602 in M17 broth containing 300 AU/ml bacteriocin.

Figure 3.7 shows the viability loss of *Enterococcus faecalis* LMG 2602. Indicator strain *Enterococcus faecalis* LMG 2602 with the bacteriocin lost its viability by % 99.99 (from $4x10^9$ to 10^3 cfu/ml) within the first minutes; the reduction in the viable colony count was 8 log within the first 20 min and did not change along the rest of the incubation. At the end of the experiment, the viable colony count dropped from $2x10^{14}$ cfu/ml to 800 cfu/ml, with a 12 log difference (Figure 3.7).

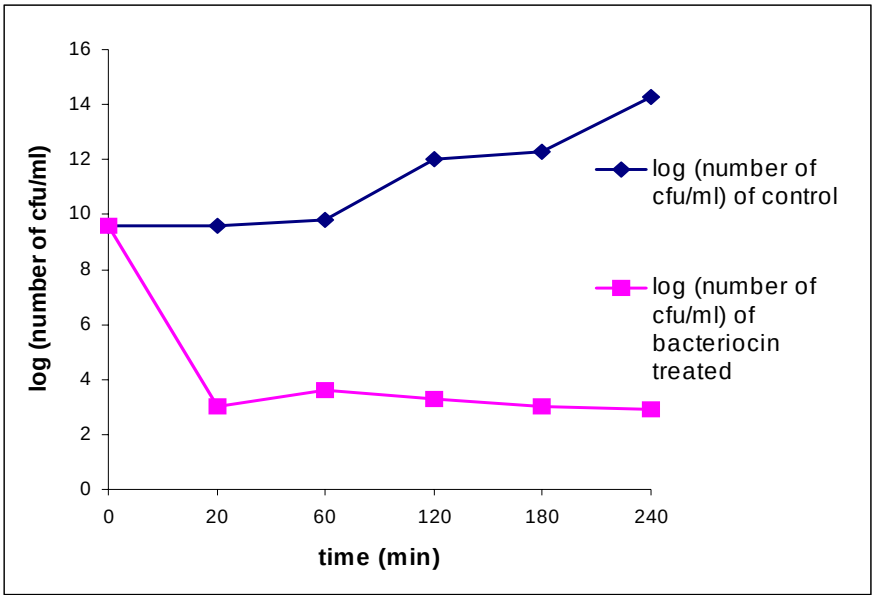


Figure 3.7 Effect of 300 AU/ml bacteriocin treatment on the viable count of *Enterococcus faecalis* LMG 2602 in M17 broth.

Some delay between the decrease in cell viability and cell lysis has been observed in the mechanism of action of other bacteriocins also. Morgan *et al*. (1995) also reported a slower decrease in OD of *Lactococcus lactis* subsp. *lactis* MG 1614 in the presence of 800 AU/ml bacteriocin of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* DPC938, which produces lactococcins A, B, and M, in spite of the fact that > 99.98% of cells are nonviable within 10 min. Ten hours after addition, only 57% of the total amount of the activity of an intracellular marker was released, indicating that bacteriolysis follows loss of viability.

Martinez *et al*. (2000) showed that the bacteriocin produced by *Lactococcus lactis* IFPL105 is bactericidal against several *Lactococcus* and *Lactobacillus*. Interestingly, the two *Lactobacillus* strains showing the highest lytic response to the

bacteriocin, *L. plantarum* IFPL935 and *L. rhamnosus* JLC1211, showed the least loss of viability, whereas *L. casei* IFPL731 and JCL1237 exhibited a loss of viability similar to that of the *Lactococcus* strains. In all strains studied, reduction of cell viability and OD were not simultaneous, suggesting that it involves two steps. First, viability is lost due to insertion of the bacteriocin into the membrane of the sensitive cell and depletion of cellular energy. Second, a gross imbalance between cell wall buildup and degradation caused in *L. lactis* AcmA leads to the observed cell lysis.

Bacteriocin treated *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 started to lyse after some time by the addition of the bacteriocin as shown in Figure 3.8. O.D. of the bacteriocin treated culture declined rapidly after the first 20 min (Figure 3.8). The decrease in OD of the bacteriocin treated culture continued throughout the incubation. In contrast, OD of the control increased as long as incubation continued.

The viability loss of *Leuconostoc mesenteroides* subsp. *cremoris* was a 5 log reduction within the first 20 min; indicator microorganism lost its viability by 99% within the first 20 min; viable colony count dropped rapidly from $8x10^8$ to 10^4. At the end of the incubation period, the viable colony count of bacteriocin treated culture was 400 cfu/ml while that of the control was $4x10^9$ cfu/ml with a 7 log reduction (Figure 3.9).

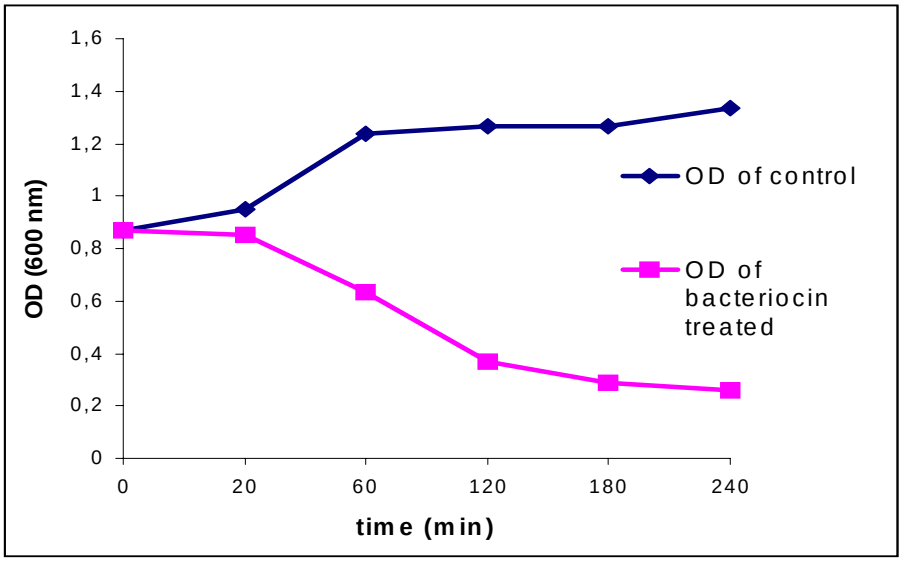


Figure 3.8 Measurement of lysis of *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 in MRS broth containing 300 AU/ml bacteriocin.

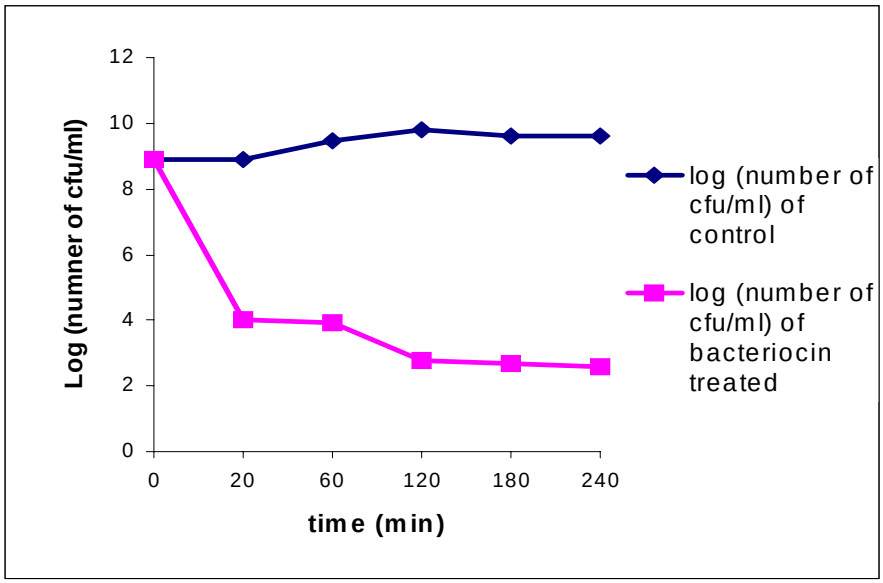


Figure 3.9 Effect of 300 AU/ml bacteriocin treatment on the viable count of *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 in MRS broth.

Optical density (OD) of bacteriocin treated *Lactococcus lactis* SIK-83 did not decrease in contrast to other indicator strains. OD of the bacteriocin treated culture increased very slowly when compared to OD of the control culture (Figure 3.10). This situation was observed for loss of viability also (Figure 3.11). The prevention in further increase in OD with a loss of cell viability was reported also by Bhunia *et al*. (1991). Bhunia *et al*. (1991) demonstrated that treatment of growing sensitive cells of *Lactobacillus plantarum* NCDO 955 with pediocin AcH prevented any further increase in OD and resulted in a loss of cell viability but did not decrease the OD of the culture. However, under similar conditions, the OD of pediocin AcH-treated *Leuconostoc mesenteroides* Ly cells decreased significantly suggesting that cell lysis had occurred. Transmission electron microscopy of the two strains after treatment further confirmed that lysis occurred only in *Leuconostoc mesenteroides* Ly. In a separate study, three of seven sensitive strains of *Listeria monocytogenes* treated with pediocin AcH showed lysis; in the other four strains, there was viability loss but the OD remained unchanged (Motlagh *et al*., 1991).

O'Sullivan *et al*. (2002) reported that lacticin 481 causes elevated enzyme release without the associated and expected decline in optical density of the target strain is a novel phenomenon for bacteriocin-induced lysis. To investigate whether lacticin 481 has a bacteriolytic effect on other lactococcal strains, a purified preparation of lacticin 481 was added in increasing concentrations to an exponentially growing *Lactococcus lactis* MG1614 culture. An 15-fold increase in the level of the intracellular enzyme LDH was observed upon addition of 8 AU/ml of the purified bacteriocin while the culture maintained an active but reduced rate of growth. Since it is hard to envisage how such enzyme-releasing cells could remain viable, it was assumed that such a phenomenon could be explained by the gradual death and lysis/permeabilization of some cells in the culture, while simultaneously other cells continue to grow and multiply.

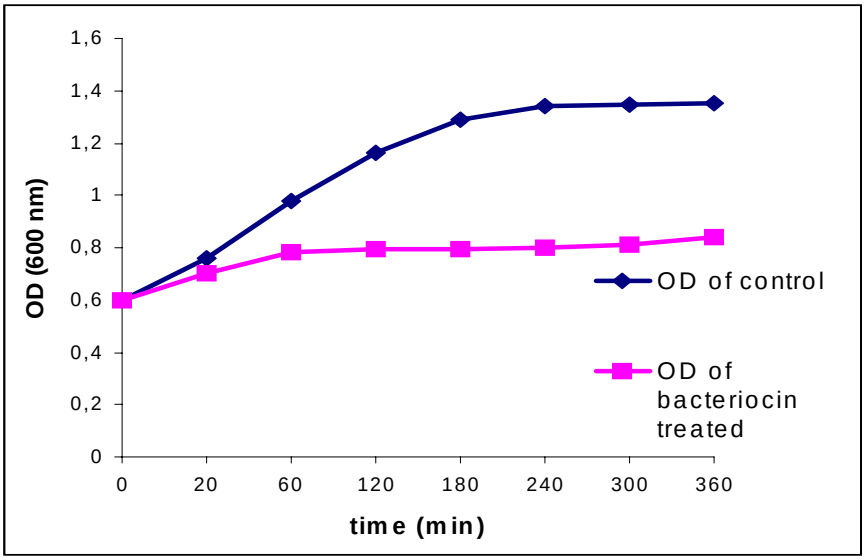


Figure 3.10 Measurement of lysis of *Lactococcus lactis* SIK-83 in M17 broth containing 300 AU/ml bacteriocin**.**

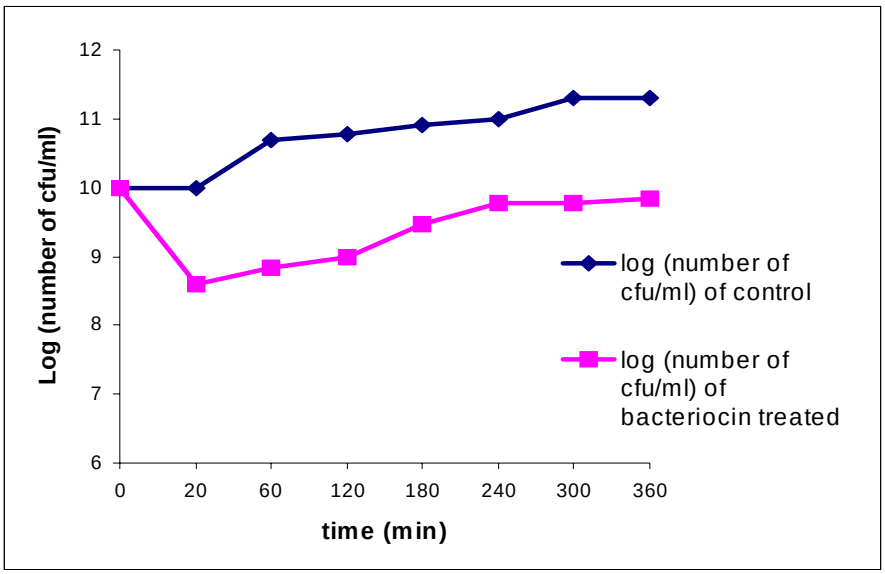


Figure 3.11 Effect of 300 AU/ml bacteriocin treatment on the viable count of *Lactococcus lactis* SIK-83 in M17 broth.

Measurement of lysis of *Listeria innoqua* LMG 2813 was performed in M17 broth (Figure 3.12) with 300 AU/ml of final bacteriocin concentration. OD of the bacteriocin treated culture in both medium decreased rapidly and continued up to 240 min. However, it was observed that the decline in OD in M17 broth appeared substantial at all the points where data taken. The same behaviour of the bacteriocin treated *Listeria innoqua* LMG 2813 in M17 broth was reflected also in loss of viability (Figure 3.13).

The viability loss of *Listeria innoqua* LMG 2813 in M17 broth containing 300 AU/ml of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was a 8 log reduction within the first 20 min and 11 log reduction after 60 min (Figure 3.13). Then, viable cell number started to increase slightly, possibly because of resistant surviving cells or may be due to inactivation of the bacteriocin due to proteases of the indicator bacteria. It has been observed that some bacteria are capable of developing phenotypes of resistance to bacteriocins. For example, nisin-resistant mutants can be generated from sensitive strains by exposure to sublethal concentrations of the bacteriocin. The decrease in the viability of cells in control indicates that cells entered death phase.

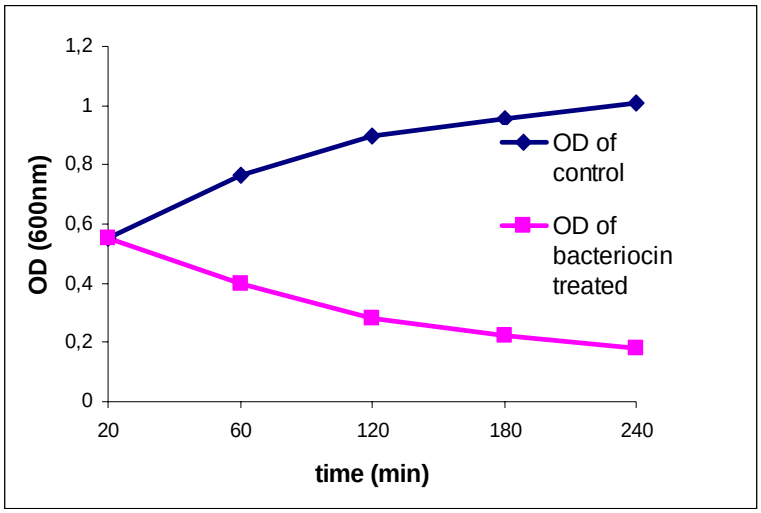


Figure 3.12 Measurement of lysis of *Listeria innoqua* LMG 2813 in M17 broth containing 300 AU/ml bacteriocin.

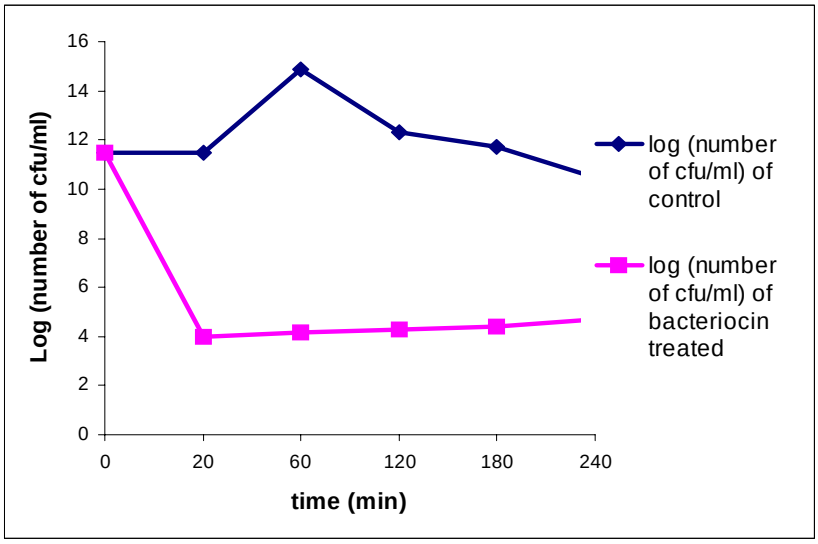


Figure 3.13 Effect of 300 AU/ml bacteriocin treatment on the viable count of *Listeria innoqua* LMG 2813 in M17 broth.

When the lysis measurement of *Listeria innoqua* LMG 2813 was performed with 900 AU/ml of final bacteriocin concentration, the decline of OD was similar to that performed with 300 AU/ml of bacteriocin concentration (Figure 3.14). However, the effect of this 900 AU/ml of final bacteriocin concentration was appreciated when consideration is given to loss of viability (Figure 3.15).

The effect of the bacteriocin produced from *Leuconostoc mesenteroides* subsp. *cremoris* on the viable count of *Listeria innoqua* LMG 2813 was also tested by increasing the final concentration of the bacteriocin from 300 AU/ml to 900 AU/ml (Figure 3.15). As shown, the reduction in viable colony count has changed from 8 log (Figure 3.13) to 10 log (Figure 3.15) for the first 20 min. Viable colony count dropped rapidly from $6{,}5x10^{11}$ to $2{,}2x10^{2}$ within the first 20 min. However, with the increased concentration of the bacteriocin, the viable colony count for *Listeria innoqua* LMG 2813 at the end of experiment (240 min) was $3x10^{2}$ for 900 AU/ml in contrast to $5x10^{4}$ for 300 AU/ml. Increased concentration of the bacteriocin resulted in a decrease in resistant cell population.

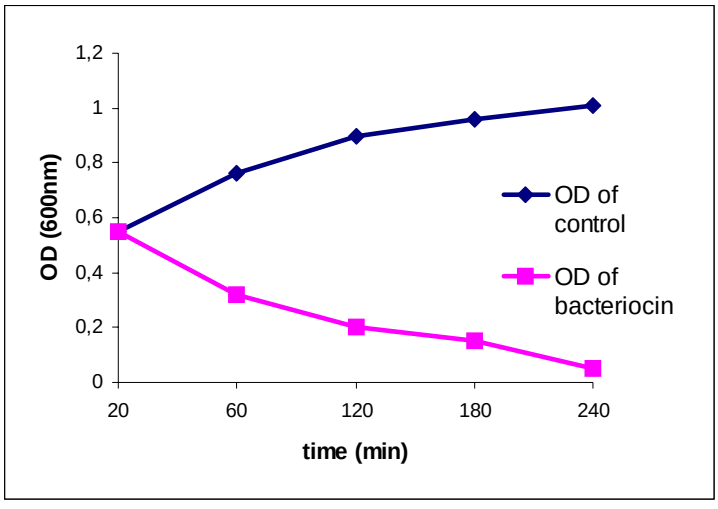


Figure 3.14 Measurement of lysis of *Listeria innoqua* LMG 2813 in M17 broth containing 900 AU/ml bacteriocin.

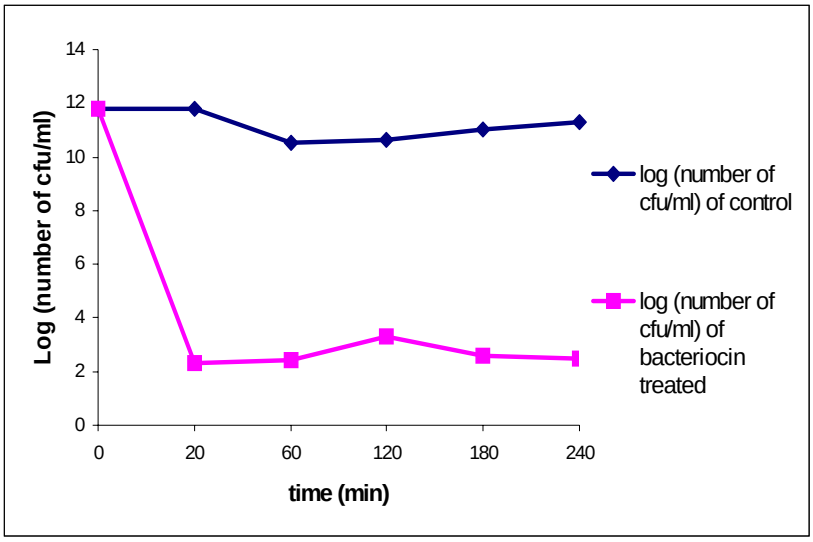


Figure 3.15 Effect of 900 AU/ml bacteriocin treatment on the viable count of *Listeria innoqua* LMG 2813 in M17 broth.

Bacteriolytic effect of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was tested on other sensitive bacteria namely, *Lactobacillus delbrueckii* subsp.*lactic* RSSK 498, *Lactobacillus cremoris* RSSK 708, *Bacillus cereıs* LMG 2732, *Lactococcus lactis* LMG 2909 and *Pseudomonas fluorescens* LMG 3020 with the final bacteriocin concantration of 300 AU/ml. The optic densities of *Lactobacillus delbrueckii* RSSK 498 and *Lactobacillus cremoris* RSSK 708 decreased rapidly after 60 min (Figure 3.16 and Figure 3.17, respectively). OD values of bacteriocin treated *Bacillus cereıs* LMG 2732 (Figure 3.18), *Lactococcus lactis* LMG 2909 (Figure 3.19) and *Pseudomonas fluorescens* LMG 3020 (Figure 3.20) did not decrease while the culture maintained an active but reduced rate of growth. The prevention in further increase in OD with a loss of cell viability was reported also by Bhunia *et al*. (1991) as stated previously when the bacteriolytic effect of the bacteriocin on *Lactococcus lactis* SIK-83 was examined (Figure 3.10).

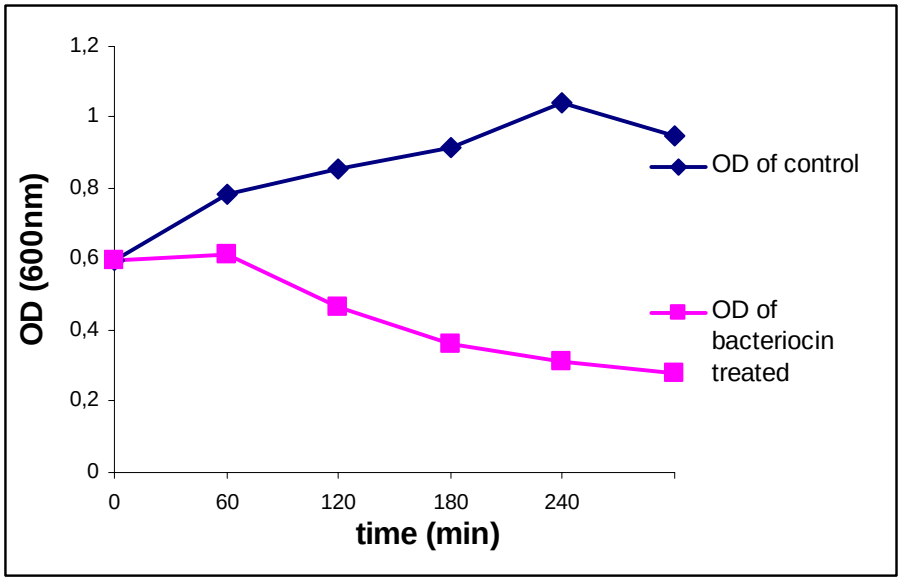


Figure 3.16 Measurement of lysis of *Lactobacillus delbrueckii* subsp.*lactic* RSSK 498 in MRS broth containing 300 AU/ml bacteriocin.

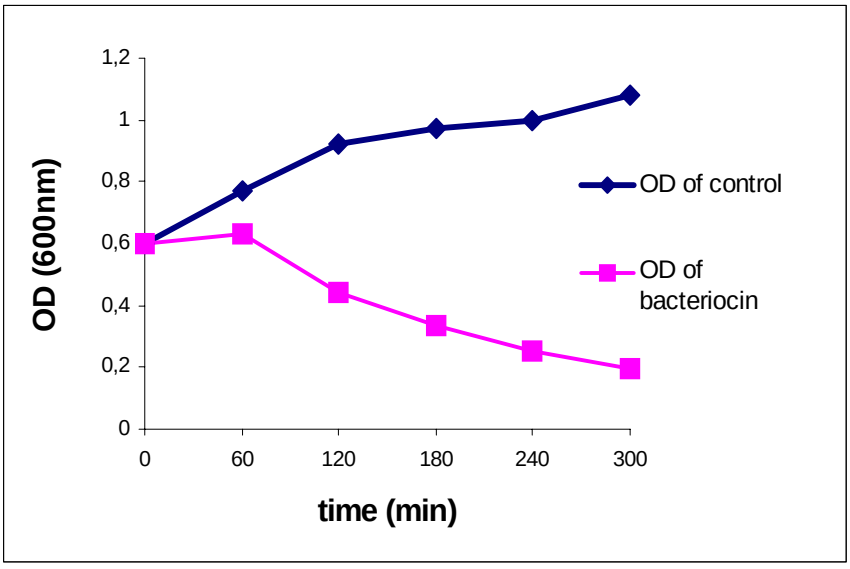


Figure 3.17 Measurement of lysis of *Lactobacillus cremoris* RSSK 708 in MRS broth containing 300 AU/ml bacteriocin.

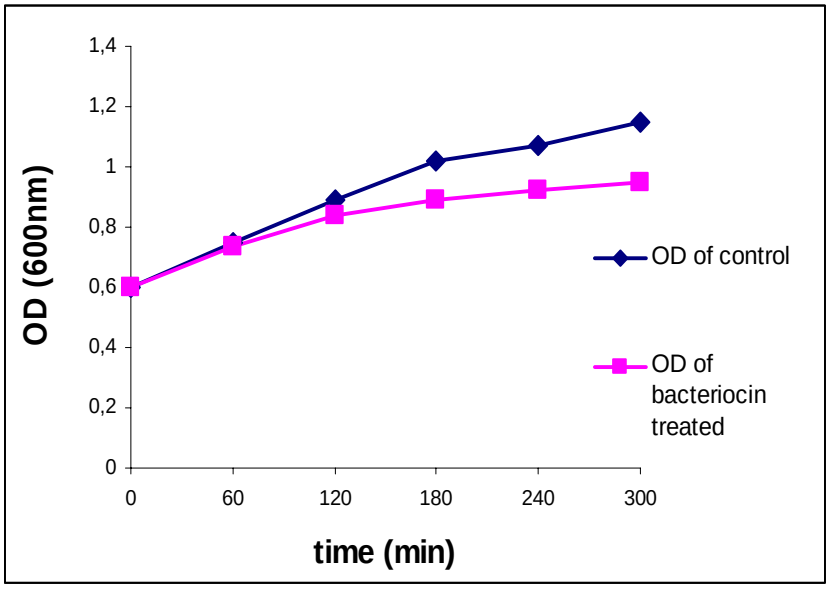


Figure 3.18 Measurement of lysis of *Bacillus cereus* LMG 2732 in M17 broth containing 300 AU/ml bacteriocin.

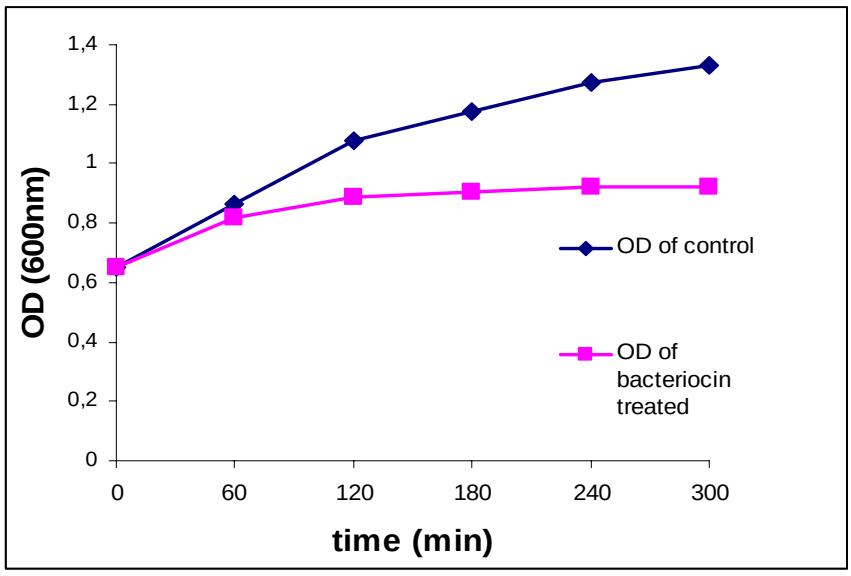


Figure 3.19 Measurement of lysis of *Lactococcus lactis* LMG 2909 in M17 broth containing 300 AU/ml bacteriocin.

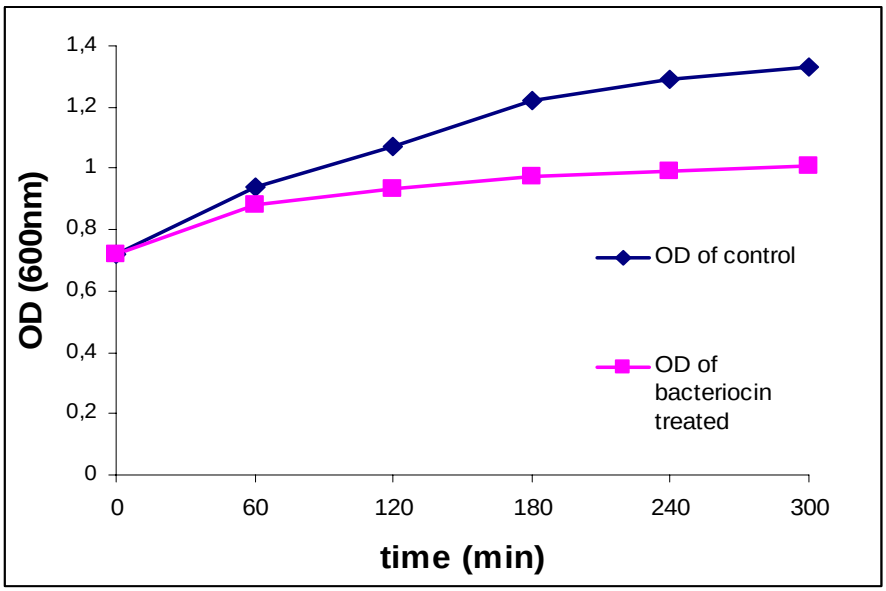


Figure 3.20 Measurement of lysis of *Pseudomonas fluorescens* LMG 3020 in M17 broth containing 300 AU/ml bacteriocin.

To summarize, the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* is bactericidal with a concomitant bacteriolysis for the indicator bacteria of *Enterococcus faecalis* LMG 2602, *Leuconostoc mesenteroides* subsp. *cremoris* RSSK 923 and *Listeria innoqua* LMG 2813, *Lactobacillus delbrueckii* subsp. *lactic* RSSK 498, *Lactobacillus cremoris* RSSK 708. However, the bacteriocin treated cultures of *Lactococcus lactis* SIK-83, *Bacillus cereus* LMG 2732, *Lactococcus lactis* LMG 2909 and *Pseudomonas fluorescens* LMG 3020 maintained an active but reduced rate of growth. Interestingly, it was found that there was no relationship between the extent of the sensitivities and bacteriolytic effect; Although *Lactococcus lactis* SIK-83, *Lactococcus lactis* LMG 2909 and *Pseudomonas fluorescens* LMG 3020 were among the most sensitive indicator bacteria in terms of

two-fold diutions (Table 3.6), they were not lysed by the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* in contrast to moderately sensitive *Lactobacillus cremoris* RSSK 708, which was lysed by the same bacteriocin.

Depending on the strain of sensitive bacteria, cell death and membrane damage following bacteriocin treatment may or may not be associated with cell lysis. This variation among sensitive strains could be due to differences in the degree of damage caused by bacteriocin in the membrane of a particular strain or to some inherent traits, such as an autolytic system, in the strain. Bacteriocin probably does not directly cause lysis. It results in functional destabilization of the cytoplasmic membrane in all sensitive gram-positive cells leading to cell death. Following cell death, the autolytic systems carried by some lytic strains probably became activated and lysed the cells.

Pucci *et al*. (1988) reported that treatment of growing sensitive cells of *Listeria monocytogenes* LM 01 and *Pediococcus pentosaceus* FBB63 with pediocin PA-1 resulted in cell lysis as observed by the reduction of optical density (OD). The two strains differed in lysis rate and in the minimum concentrations of pediocin PA-1 that were capable of inducing lysis, with the more sensitive cells lysing at a higher rate.

3.11.3. Temperature effect on the adsorption of the bacteriocin onto *Enterococcus faecalis* LMG 2602

As shown in Table 3.24, temperatures did not affect adsorption of the bacteriocin to the cells of *Enterococcus faecalis* LMG 2602. The amount of bacteriocin binding was the same as the control (room temperature).

Table 3.24. Effect of temperature on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602 cells.

Temperature	Adsorption (%)
0	100
20	100
30	100
40	100
50	100
60	100
70	100
80	100

3.11.4. Effect of salts on the adsorption of the bacteriocin onto *Enterococcus faecalis* LMG 2602

Effect of salts on the adsorption of the bacteriocin was shown in Table 3.25. As can be seen from the Table 3.25, phosphate buffer, NaCI, KCI, KI treated samples did not adsorb the bacteriocin. EDTA and sodium acetate had no inhibitory effect on the adsorption of the bacteriocin onto *Enterococcus faecalis* LMG 2602 cells. Adsorption of the bacteriocin to the cell surface of *Enterococcus faecalis* LMG 2602 was reduced by salts including phosphate buffer, NaCI, KCI. The anions in these salts are related with this adsorption inhibition because of the competition for the cell wall associated non-specific receptors since the binding of the bacteriocins to cell wall associated non-specific receptors are mediated by ionic interaction. The fact that bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* was adsorbed to gram-positive cell surface but not to gram-negative cell surface regardless of

their sensitivity pointed out the role of the teichoic acid and lipoteichoic acid for the initial interaction with bacteriocin. As indicated by Bhunia *et al*. (1991) lipoteichoic acid molecules are the probable non-specific receptors. In sensitive cells only, when these non-specific receptors have been saturated, bacteriocin molecules bind to specific or lethal receptors and cause cellular changes related with cell death. CI^- and PO_4^{3-} can compete with bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* for binding to these receptors.

Table 3.25. Effect of different salts on adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602.

Treatment	Adsorption (%)
Phosphate buffer (100 mmol/l)	0
NaCI (100 mmol/l)	0
KCI (100 mmol/l)	0
KI (100 mmol/l)	25
$MgCI_2$ (100 mmol/l)	50
Tris-HCI (100 mmol/l)	50
Sodium acetate (100 mmol/l)	100
EDTA (100 mmol /l)	100
$NaHCO_3$ (100 mmol/l)	50

3.11.5. Effect of organic solvents on adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602 cells

Treatment of cells with ethanol, chloroform, methanol, butanol, isopropanol, acetone and hexane did not affect adsorption of the bacteriocin from produced by *Leuconostoc mesenteroides* subsp. *cremoris* onto the cells of *Enterococcus faecalis* LMG 2602 as shown in Table 3.26.

Table 3.26. Effect of organic solvents on adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* to *Enterococcus faecalis* LMG 2602 cells.

Treatment	% adsorption
ethanol	100
chloroform	100
methanol	100
butanol	100
isopropanol	100
acetone	100
hexane	100

3.11.6. Effect of detergents on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602

As given in Table 3.27, treatment of the cells of *Enterococcus faecalis* LMG 2602 with detergents (1 % SDS, 1 % Triton X-100, 1 % Tween 80 and 4 M guanidine–HCI) had no effect on the adsorption of the bacteriocin.

Table 3.27. Effect of detergents on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* to *Enterococcus faecalis* LMG 2602.

Treatment	% adsorption
1 % SDS	100
1 % Triton X-100	100
1 %Tween 80	100
4 M Gn-HCI	100

3.11.7. Effect of enzymes on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602

Adsorption of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* on to the cells of *Enterococcus faecalis* LMG 2602 was not influenced by the treatment of the cells with enzymes (proteinase K, pepsin, trypsin, α-chymotrypsin, lysozyme and alpha-amylase). Adsorption remained 100 % as shown in Table 3.28.

Table 3.28. Effect of enzymes on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* to *Enterococcus faecalis* LMG 2602.

Treatment	% adsorption
proteinase K	100
pepsin	100
trypsin	100
α-chymotrpsine	100
lysozyme	100
α-amylase	100

3.11.8. Effect of pH on the adsorption of the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602

The adsorption of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* onto the cells of *Enterococcus faecalis* LMG 2602 was maximum at pH 6.0 (Figure 3.21). Adsorption was reduced below the pH 4.5 and above the pH 7.0-7.5 as shown in Figure 3.21. No adsorption occurred at pH 2.0, 3.0, 8.0, 9.0.

It was reported that adsorption of the bacteriocins from lactic acid bacteria onto cell surface of gram positive bacteria including the producer organisms is maximum around pH 6.0 and minimum around pH 2.0.

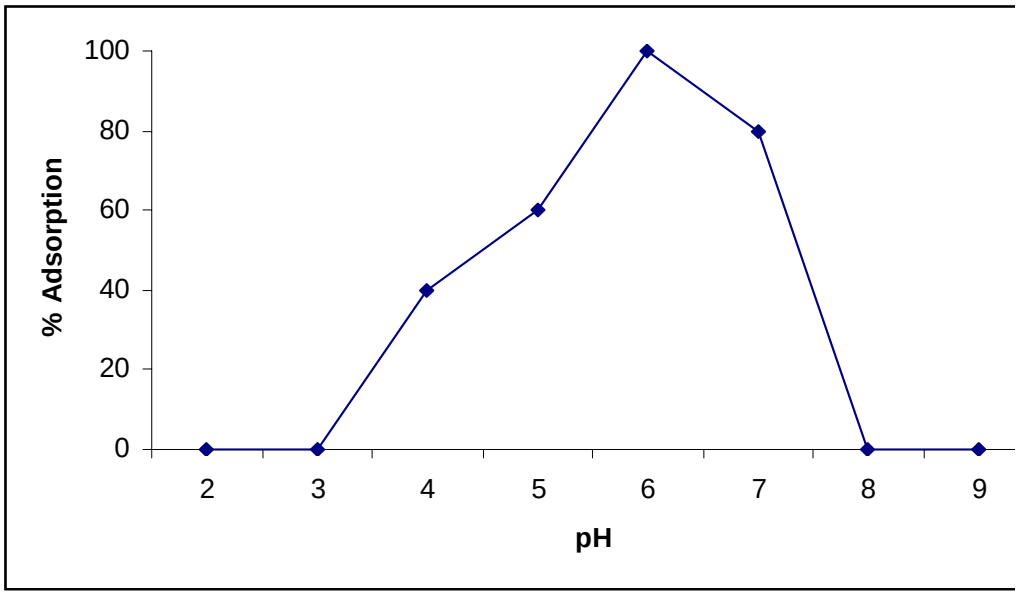


Figure 3.21 Effect of pH on the adsorption of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* onto *Enterococcus faecalis* LMG 2602 cells.

3.12. Purification of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*

Many bacteriocins produced by LAB are polypeptides ranging from 3.0 kDa to 6.0 kDa in molecular weights. These polypeptides have a surplus of positively charged amino acid residues and posses hydrophobic properties. Therefore, purification protocols involve ion-exchange chromatography and hydrophobic interaction chromatography. The purification protocol for the bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* is summarized in Table 3.29.

A 62-fold purification of the bacteriocin (62 fold increase in the specific activity) and 100% recovery of the activity were achieved after pH mediated cell surface adsorption-desorption method (Table 3.29, fraction I). Specific activity increased 138 fold and recovery reduced to 56% after bacteriocin binding to Amberlite XAD-16 and eluting it with 70% 2-propanol, pH 2.0 (Table 3.29, fraction II). The specific activity of the bacteriocin increased 259 fold with a recovery of 59% after binding to cation-exchange medium Macro-Prep High S (Table 3.29, fraction III). After binding to Phenyl-Sepharose CL-4B and eluting it with 70% ethanol, the specific activity increased 345 fold and the recovery decreased to 9.3% (Table 3.29, fraction IV). The increase in the specific activity of the bacteriocin after binding to Phenyl-Sepharose CL-4B could be due to activation of the bacteriocin in a more hydrophobic environment.

Table 3.29. Purification protocol of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris.*

Purification stage	Vol (ml)	Bac.act (AU/ml)	Total Bac (AU/ml)	Protein (mg/ml)	Bac sp activity (AU/mg)	Activity recovered	Fold purifica-tion
Culture supernatant	1000	1600	$1.6X10^6$	4.8	334	100	1
pH 2 Extract	250	6400	$1.6X10^6$	0.31	20645	100	62
Amberlite XAD-16	100	12000	$1.2X10^6$	0.26	46154	56	138
MacroPrep High S	100	9500	$0.95X10^6$	0.11	86364	59	259
Phenyl Sepharose CL-4B	65	2307	$0.15X10^6$	0.02	115350	9.3	345

*Protein concentration was determined by Lowry method

Upon chromatography on Phenyl-Sepharose CL-4B column, the bacteriocin eluted as a symmetrical peak of activity at around 70% ethanol and co-migrated with a major absorbance peak (Figure 3.22 and 3.23). Fractions on the both sides of the

peak showed activity when pooled and concentrated. Activity staining of the fraction purified by the protocol outlined in Table 3.29 was illustrated in Figure 3.24. The single band in lane 4 showed antimicrobial activity against *Enterococcus faecalis* LMG 2602 in activity staining gel (Figure 24 a). As shown, the molecular weight of the band showing activity was smaller than 6 kDa. Micro Cel mediated adsorption-desorption fraction and pH mediated cell adsorption-desorption fraction also resulted in inhibition zone in activity staining gel. However, Micro Cel mediated adsorption-desorption fraction and pH mediated cell adsorption-desorption fraction resulted in several bands, with the latter resulted in better purity. These contaminating proteins were observed also when the pH mediated cell adsorption-desorption fraction was chromatographed by size-exclusion chromatography on Sephadex G-50 column (Figure 3.25). Activities of other purified fractions against the indicator organism by the agar-well diffusion assay were given in Figure 3.26. Figure 3.25 shows the size-exclusion chromatography of the pH mediated cell adsorption-desorption fraction. One major peak showing antimicrobial activity was obtained after size-exclusion chromatography. The major band showed antimicrobial activity against the indicator organism *Enterococcus faecalis* LMG 2602.

Adsorption of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* to hydrophobic resins Amberlite XAD-16 and Phenyl Sepharose CL-4B and elution with 70% 2-propanol (pH 2.0) and 70% ethanol, respectively indicates the very strong hydrophobic nature of this bacteriocin. Strong hydrophobicity is a characteristic property of the class II peptide bacteriocins. This strong hydrophobic character is one of the main reasons why bacteriocin purification is tedious and cumbersome. Adsorption of the bacteriocin to cation-exchange resins Macro Prep High S and Carboxy methyl cellulose indicated its cationic nature.

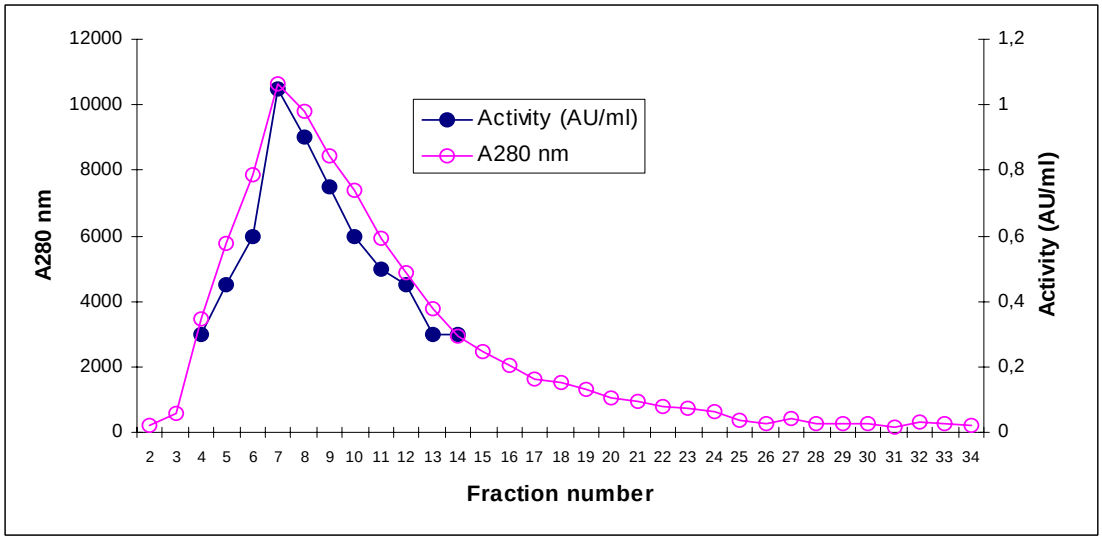


Figure 3.22 Hydrophobic interaction chromatography on Phenyl Sepharose CL 4B column (fraction IV).

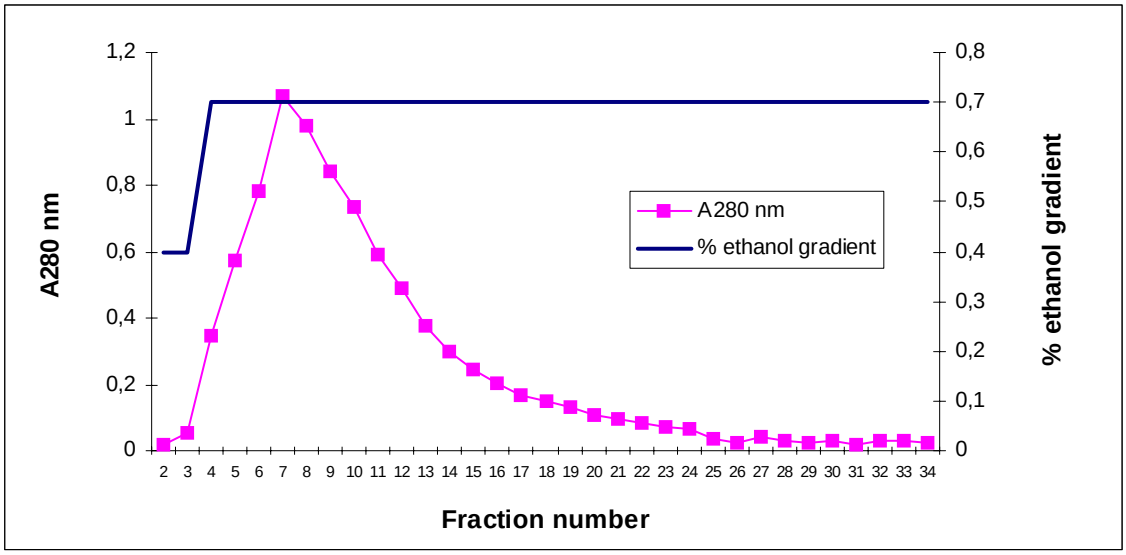


Figure 3.23. Gradient elution profile from Phenyl Sepharose CL-4B column.

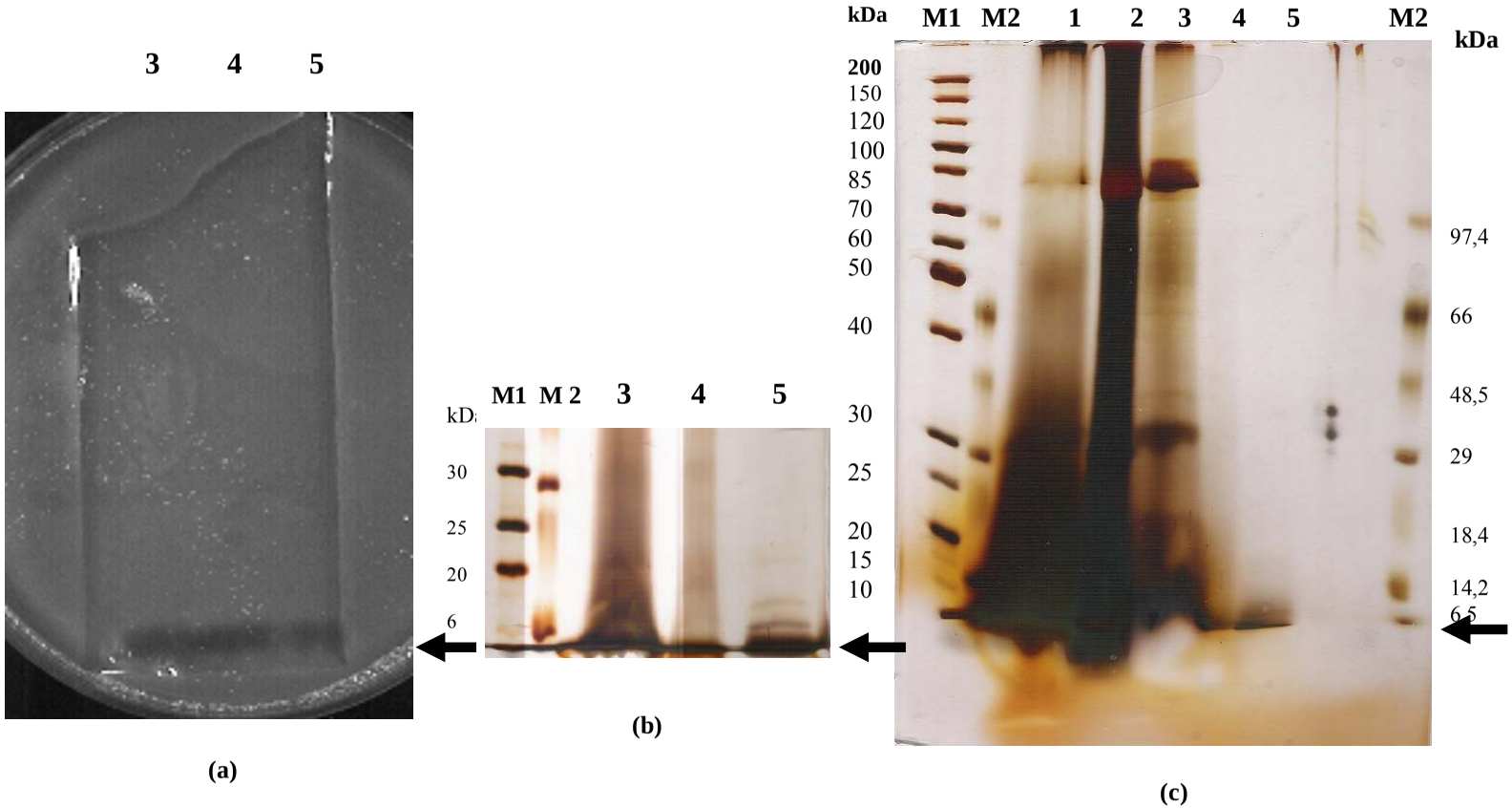


Figure 3.24. SDS PAGE of various bacteriocin preparations obtained by different protocols. Lane 1, pellicle from crude extract; Lane 2, chloroform-methanol extracted pellicle; Lane 3, Micro Cel adsorbed fraction; Lane 4, bacteriocin purified by the protocol outlined in Table 3.29; Lane 5, pH mediated cell adsorption-desorption fraction.; M1, SDS-PAGE marker (Fermentas); M2, SDS-PAGE marker (Sigma). (a) activity staining with *Enterococcus faecalis* as indicator; (b) SDS-PAGE gel; (c) SDS-PAGE gel.

Pellicle formation from ammonium sulphate precipitation (40 %), and chloroform-methanol extraction (2:1, v/v) of this pellicle was tested for purification. Researchers have been familiar with pellicle formation when performing ammonium sulphate precipitation of cell free supernatant containing bacteriocin of lactic acid bacteria. When crude cell free culture supernatant made up to 35-40% ammonium sulphate saturation, a surface pellicle containing the bacteriocin was recovered. Therefore, a simple purification protocol of the bacteriocin from *Leuconostoc mesenteroides* subsp. *cremoris* was performed by ammonium sulphate precipitation of culture supernatant brought to 40% saturation and methanol-chloroform (1:2, vol/vol) extraction. However, no distinct banding pattern could be observed with the bacteriocin containing pellicle and its methonol-chloroform extract. The floating pellicle was eluted as a smearing band in SDS-PAGE (Figure 3.24, Lane 1). This smearing band indicated the formation of large bacteriocin aggregates due to hydrophobic interaction of the bacteriocin with fatty acids of Tween 80, a non ionic detergent present in MRS broth. A simple one-step methanol-chloroform extraction was performed to remove fatty acid contamination from pellicle fraction. However, the smearing band was observed again (Figure 3.24 c, lane 2).

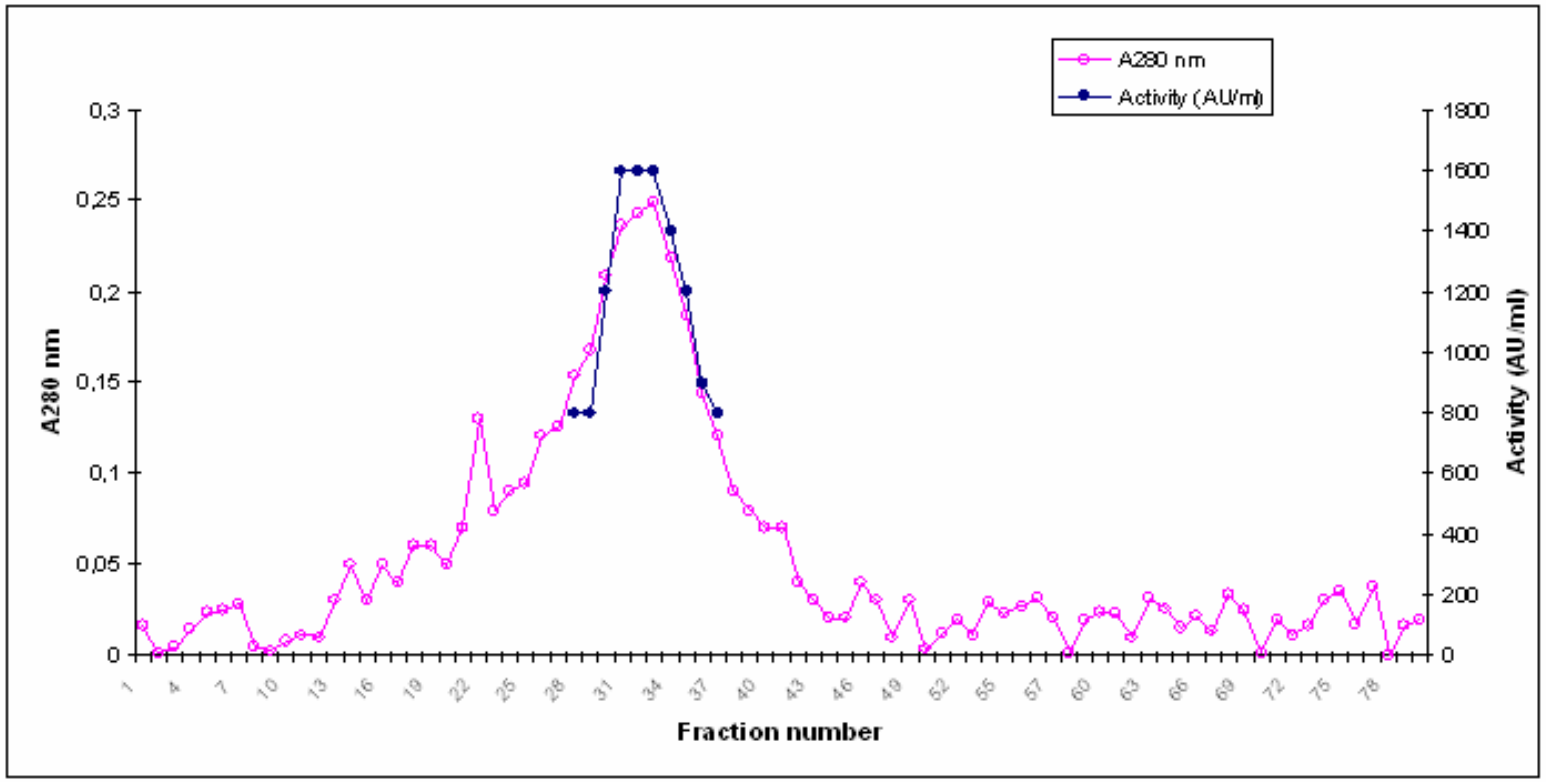


Figure 3.25. Size exclusion chromatography of the pH mediated cell surface adsorption-desorption fraction on Sephadex G-50 column.

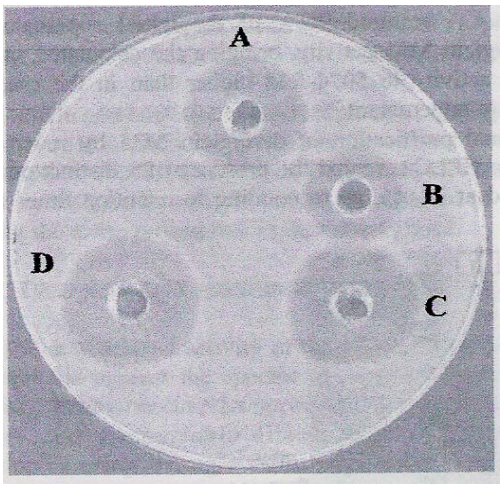


Figure 3.26. Antimicrobial activities of the purified fractions against the indicator microorganism *Enterococcus faecalis* LMG 2602 by agar-well diffusion method. **(A)** pH-mediated cell adsorption-desorption fraction, (fraction I) **(B)** Size-exclusion G-50 fraction (major peak shown in Figure 3.25) of the pH mediated cell adsorption-desorption fraction, **(C)** Amberlite XAD-16 extract of the pH mediated cell adsorption-desorption fraction (fraction II), **(D)** Phenyl-Sepharose fraction shown in Figure 3.22 (fraction IV).

CHAPTER 4

CONCLUSION

In recent years bacteriocins produced by laxtic acid bacteria have attracted great attention due to their application in food processing and preservation to control undesirable organisms such as *Listeria monocytogenes* and *Clostridium* spp. A potential means of preserving fermented foods from outgrowth of listeriae is through the use of bacteriocin-producing starter cultures. Only strains of *Lactococcus lactis* and and *Pediococcus*, producer strains of nisin and pediocin PA-1, respectively have been used up to now. Nisin is the most widely used bacteriocin and it has been granted the status of GRAS (generally recognized as safe) in the United States for food. However, nisin has several deficiencies: instability at neutral to alkaline pHs. Decay in its antimicrobial activity when incorporated into complex foods, low solubility over the physiological pH range, and a spectrum of activity restricted to gram-positive bacteria. Hence, it would be desirable to find other antimicrobial compounds which could be successfully exploited as food preservatives (Mendoza *et al*., 1999).

Sensitivity of the inhibitory agent produced by *Leuconostoc mesenteroides* subsp. *cremoris* to proteolytic enzymes confirmed that it was proteinaceous in nature. Also, it was sensitive to α-amylase. The α-amylase sensitivity of the bacteriocin is unusual. The results indicate that the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* was a glycoprotein which required both the glyco portion and the protein portion of the molecule for activity. Its synthesis and

secretion by the producer organism starts in the early exponential phase and continues up to the end of the exponential phase. Bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* is unique due to its physico-chemical properties. It had quite high activity between pH 2.0 and 12 at room temperature after fermentation and protected its activity after boiling for 2 hours between pH 2.0 and 9.0. Crude supernatant of *Leuconostoc mesenteroides* subsp. *cremoris* which was boiled between pH 2.0 and 6.0 showed bacteriocin activity even after 12 months. This means that bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* has a potential for use in both high-acid and low-acid canned foods. By adding bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* to low-acid foods, it should be possible to reduce the extent of heat-processing required, thus resulting in improved flavour, increased nutritional value and an overall more economical process. Such applications may be particularly beneficial for products such as canned milk puddings where heat penetration is often a problem.

Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* showed a bactericidal mode of action with a concomitant cell lysis. There was a gradual growth of some indicator bacteria following an initial decrease or suppression of the growth depending on bacteriocin concentration. This can be attributed to the presence of resistant variant cells capable of growing in the presence of bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris.*

Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was very stable to heating at a broader pH range relative to some other available bacteriocins of lactic acid bacteria, e.g., lacticin 3147, lacticin 481, lactococcin G and nisin. It was stable to organic solvents and showed inhibitory activity against closely related LAB species like *Leuconostoc mesenteroides* and some distantly related gram-positive bacteria such as *Listeria innoqua*, *Listeria monocytogenes*, *Bacillus cereus*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Lactobacillus cremoris*, *Lactobacillus fructivorans*, *Lactobacillus delbrueckii*, *Lactococcus lactis* and gram-

negative bacterium *Pseudomonas fluorescens*. Most of the bacteriocin-insensitive strains and species were inhibited by the high concentration of the bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris*. It had an inhibition spectrum that is broader than lacticin 3147, lacticin 481, lactococcin G and that is comparable to nisin. It showed a strong anti-listerial effect and seems to be different from other bacteriocins based on comparison of activity spectrum although classification based on the activity spectrum is not the sole criterion.

It was adsorbed to producer strain *Leuconostoc mesenteroides* subsp. *cremoris* at pH 6.0 and desorbed at pH 2.0 quite specifically. This specific interaction of the bacteriocin had a great advantage in the purification protocol. Bacteriocin was also adsorbed to other gram positive sensitive and insensitive strains. Bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was very hydrophobic; it was adsorbed to hydrophobic matrix Amberlite XAD-16 and Phenyl Sepharose CL-4B. It could be extracted by some organic solvents due to its hydrophobic nature. Strong hydrophobicity is a characteristic property of the class II peptide bacteriocins. This strong hydrophobic character is one of the main reasons why bacteriocin purification is tedious and cumbersome.

It is also cationic; it was adsorbed to Macro Prep High S and carboxy methyl cellulose strongly. Taking these properties of the bacteriocin into consideration, bacteriocin was purified to homogeneity by pH mediated cell adsorption-desorption method, solid phase extraction with Amberlite XAD-16, ion-exchange chromatography on Macro Prep High S column and final hydrophobic interaction chromatography on Phenyl Sepharose CL-4B column. The purification resulted in an electrophoretically pure protein with a molecular weight of approximately 6 kDa as judged by SDS-PAGE gel electrophoresis.

It was adsorbed to Micro Cel E (a food grade calcium silicate) and because of the specificity of this interaction, Micro Cel E was employed in a separate chapter for

the purpose of purification. The activity loss brought about by treatment of the bacteriocin with 2% beta-mercaptoethanol points out that bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* has at least one disulphide bond comprised from two cysteine residues, which is also a common characteristics of class IIa bacteriocins of lactic acid bacteria.

An important disadvantage while using bacteriocins in is the potential reduction of their long term effectiveness due to pH changes, particularly in fermented foods. In this respect bacteriocin of *Leuconostoc mesenteroides* subsp. *cremoris* was not affected by pH; activity levels were still high at acidic and basic pH values. Bacteriocin maintained enough bactericidal activity over a wide pH range. This bacteriocin is therefore more interesting than other tested bacteriocins such as nisin, lactococcin G, lacticin 481, lacticin 3147, which are active only at relatively low pH values.

Taking all these biochemical characteristics (heat stability, hydrophobicity and its cationic character) into consideration, bacteriocin produced by *Leuconostoc mesenteroides* subsp. *cremoris* appears to belong to the class IIa bacteriocins of lactic acid bacteria. Elucidation of the amino acid sequence and the genetic determinants of the bacteriocin can provide insight into the structure and function relationships and mode of action.

Due to its quite stability under the indicated conditions, this bacteriocin is worth studying for its potential as natural food preservative.

REFERENCES

Adams, M. R. and Hall, C. J. 1988. Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. Int. J. Food Microbiol. 23: 287-292.

Allgaier, H., Jung, G., Werner, R. G., Schneider, U., Zahner, H. 1986. Epidermin: sequencing of a heterodetic tetracyclic 21-peptide amide antibiotic. Eur. J. Biochem. 60: 9-22.

Aly, R., Shinefield, H., and Maibach, H. 1982. Bacterial interference among *Staphylococcus aureus* strains, p.13-23. In R. Ally and H. Shinefield (ed.), Bacterial interference. CRC press, Boca Raton, Fla.

Anderssen, E. L., Bao Diep, D., Nes, I. F., Eijsink, V. G. H., Nissen-Meyer, J. 1998. Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricin EF and JK, and the induction factor plantaricin A. Appl. Environ. Microbiol. 64: 2269-2272.

Axelsson, L.and Holck, A. 1995. The genes involved in production of and immunity to sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Bacteriol. 177: 2125-2137.

Banerjee, S., Hansen, J. N. 1988. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J. Biol. Chem. 263: 9508-9514.

Bárcena, B. J. M., Siñeriz, F., González de Llano D., Rodriguez, A., Suárez, J. E. 1998. Chemostat production of plantaricin C by *Lactobacillus plantarum* L 441. Appl. Environ. Microbiol. 64: 3512-3514.

Barefoot, S. F. and Klaenhammer, T. R. 1983. Detection of Activity of Lactacin B, a Bacteriocin Produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45: 1808-1815.

Barefoot, S. F., Chen, Y.-R., Hughes, T. A., Badine, A. B., Shearer, M. Y., Hughes, M. D. 1994. Identification and purification of a protein that induces production of the *Lactobacillus acidophilus* bacteriocin lactacin B. Appl. Environ. Microbiol. 60: 3522-3528.

Benz, R., Jung, G., Sahl, H.-G. 1991. Mechanism of channel formation by lantibiotics in black lipid membranes, p. 359-372. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, The Netherlands.

Berridge, N. J., Newton, G. G. F. and Abraham, E. P. 1952. Purification and nature of the antibiotic nisin. Biochem. J. 52: 529-535.

Bhunia, A.K., Johnson, M. C. and Ray, B. 1987. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Indus. Microbiol. 2: 319-322.

Bhunia, A.K., Johnson, M. C. and Ray, B. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. J. Appl. Bacteriol. 65: 261-268.

Bhunia, A.K., Johnson, M. C. and Ray, B. 1991. Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. J. Appl. Bacteriol. 70: 25-33.

Bierbaum, G. and Sahl, H.-G. 1991. Induction of autolysis of *Staphylococcus simulans* 22 by Pep5 and nisin and influence of the cationic peptides on the activity of the autolytic enzymes, p. 386-396. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, The Netherlands.

Bierbaum, G., Sahl, H.-G. 1985. Induction of autolysis of staphylococci by the basic peptide antibiotic Pep5 and nisin and their influence on the activity of autolytic enzymes. Arch. Microbiol. 141: 249-254.

Bierbaum, G., Sahl, H.-G. 1987. Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. J. Bacteriol. 169: 5452-5458.

Biswas, S. R., Ray, P., Johnson, M. C., Ray, B. 1991. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. Appl. Environ. Microbiol. 57: 1265-1267.

Broekaert, W. F., Terras, F. R., Cammue, B. P. A., Osborn, R. W. 1995. Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant. Physiol. 108: 1353-1358.

Brötz, H., Josten, M., Wiedemann, I., Schneider, U., Götz, F., Bierbaum, G. 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. 30: 317-327.

Brurberg, M. B., Nes, I. F. and Eijsink, V. G. H. 1997. Pheromone-induced production of antimicrobial peptides in *Lactobacillus*. Mol Microbiol. 26: 347-360.

Burianek, L. L. and Yousef, A. E. 2000. Solvent extraction of bacteriocins from liquid cultures. Lett. Appl. Microbiol. 31: 193-197.

Callewaert, R., Holo, H., Devreese, B., Van Beeumen, J., Nes, I. F., De Vuyst, L. 1999. Characterization and production of amylovorin L471, a bacteriocin purified from *Lactobacillus amylovorus* DCE 471 by a novel three-step method. Microbiology. 145: 2559-2568.

Casaus, P., Nilsen, T., Cintas, L. M., Nes, I. F., Hernández, P. E. and Helge, H. 1997. Enterocin B, anew bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiol. 143: 2287-2294.

Chatterjee, S., Chatterjee, D. K., Jani, R. H., Blumbach, J., Ganguli, B. N., Klesel, N., Limbert, M., Seibert, G. 1992. Mersacidin, a new antibiotic from Bacillus. In vitro and in vivo antibacterial activity. J. Antibiot. (Tokyo) 45: 839-845.

Cheeseman, G. C. and Berridge, N. J. 1957. An improved method of preparing nisin. Biochem. J. 65: 603-608.

Chikindas, M. L., Garcia-Garcerá, M. J., Driessen, A. J. M., Ledeboer, A. M., Nissen-Meyer, J., Nes, I. F., Abee, T., Konings, W. N. and Venema, G. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC 1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. Appl. Environ. Microbiol. 59: 3577-3584.

Christensen, D. P. and Hutkins R. W. 1992. Collapse of the proton motive force in *Listeria monocytogenes* caused by a bacteriocin produced by *Pediococcus acidilactici*. Appl. Environ. Microbiol. 58: 3312-3315.

Cintas, L. M., Casaus, P., Håverstein, L. S., Hernández, P. E. and Nes, I. F. 1997. Biochemical and genetic chracterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. 63: 4321-4330.

Cintas, L. M., Casaus, P., Holo, H., Hernandez, P. E., Nes, I. F., Håverstein, L. S. 1998. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. 180: 1988-1994.

Contreras, B. G. L., De Vuyst, L., Devreese, B., Busangova, K., Raymaeckers, J., Bosman, F., Sablon, E. and Vandamme, E. J. 1997. Isolation, Purification, and Amino Acid Sequence of Lactobin A, One of the Two Bacteriocins Produced by *Lactobacillus Amylovorus* LMG P-13139. 63: 13-20.

Copeland, R. A.1994. Electrophoretic and Chromatographic Methods for Assessing Protein Purity. In: Methods for protein analysis, a practical guide to laboratory protocols, pp. 59-98, Chapman and Hall, New York.

Coventry, M. J., Gordon, J. B., Alexander, M., Hickey, M. W. and Wan, J. 1996. A food-grade process for isolation and partial purification of bacteriocins of lactic acid bacteria that uses diatomite calcium silicate. Appl. Environ. Microbiol. 62: 1764-1769.

Daba, H., Lacroix, C., Huang, J., Simard, R. 1993. Influence of growth conditions on production and activity of mesenterocin 5 by a strain of *Leuconostoc mesenteroides*. Appl. Microbiol. Biotechnol. 39: 166-173.

Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43: 91-94.

Daeschel, M. A., Jung, D. S. and Watson, B. T. 1991. Controlling wine malolactic fermentation with nisin and nisin-resistant strains of *Leuconostoc oenos*. Appl. Environ. Microbiol. 57: 601-603.

Dalet, K., Cenatiempo, Y., Cossart, P., Héchard, Y. 2001. A sigma (54)-dependent PTS permease of the mannose family is responsible for sensitivty of *Listeria monocytogenes* to mesentericin Y105. Microbiol. 147: 3263-3269.

Davidson, P. M. and Parish, M. E. 1989. Methods for testing the efficacy of food antimicrobials. Food. Technol. 43: 148-155.

De Klerk, H. C. 1967. Bacteriocinogeny in *Lactobacillus fermenti*. Nature. 214: 609.

De Vos, W. M. 1987. Gene cloning and expression in lactic streptococci. FEMS Microbiol. Rev. 46: 281-295.

De Vuyst, L., Vandamme, E. J. 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentation. J. Gen. Microbiol. 138: 571-578.

De Vuyst, L., Vandamme, E. J. 1994. Nisin, lantibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications. In: De Vuyst L., Vandamme E.J.(eds), Bacteriocins of lactic acid bacteria. Blackie, London, pp. 151-221.

Dicks, L. M. T., van Jaarsveld, D. E. and van Vuuren, H. J. J. (1992) Caseicin LHS, a broad spectrum bacteriocin produced by *Lactobacillus casei*. Abstr. Book 7th Biennial Congrss of the South African Soc. Microbiol., p.214.

Diep, D .B., Håverstein, L. S., Nes, I. F. 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J. Bacteriol. 178: 4472-4483.

Dodd, H. M., Horn, N. and Gasson, M. J. 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. 136: 555-566.

Diep, D. B., Axelsson, L., Grefsli, C. and Nes, I. F. 2000. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. Microbiology. 146: 2155-2160.

Eijsink, V. G. H., Brurberg, Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H. and Nes, I. F. 2002. Production of class II bacteriocins by lactic acid bacteria: an

example of biological warfare and communication. Antonie van Leewenhoek. 81: 639-654.

Eijsink, V. G. H., Brurberg, M. B., Middelhoven, P. H., Nes, I. F. 1996.Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. J. Bacteriol. 178: 2232-2237.

Eijsink, V. G. H., Skeie, M., Middelhoven, P. H., Brurberg, M. B. and Nes, I. F. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64: 3275-3281.

Ennahar, S., Sashihara, T., Sonomoto, K., Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. FEMS Microbiol. Rev. 24: 85-106.

Elegado, F. B., Kim, W. J., Kwon, D. Y. 1997. Rapid purification, and antimicrobial spectrum of the bacteriocin, Pediocin AcM, from *Pediococcus acidilactici* M. Int. J. Food microbial. 37: 1-11.

Eugenio, P., Brienza, C., Moles, M., Ricciardi, A. 1995. A comparison of methods fort he measurement of bacteriocin activity. J. Microbiol. Methods. 22: 95-108.

Faith, M. J., Skvirsky, R., Gilson, L., Mahanty, H. K. and Kolter, R. 1992. The secretion of colicin V, p.331-348. In R. James, C. Ladunski and F. Pattus (ed.), Bacteriocins, microcins, and lantibiotics. Springer-Verlag, New York.

Fikes, J. D., Crabbtree, B. L. and Barridge, B. D. 1983. Studies on the mode of action of a bacteriocin produced by *Bacillus stearothermophilus*. J. Microbiol. 29: 1576-1582.

Franz, C. M. A. P., Van Belkum, M. J., Worobo, R. W., Vederas, J. C. and Stiles, M. E. 2000. Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: the immunity gene confers cross-protection to enterocin B. Microbiology. 146: 621-631.

Fregeau Gallagher, N. L., Sailer, M., Niemezura, W. P., Nakashima, T. T., Stiles, M. E. & Vederas, J. C. 1997. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. Biochemistry. 36: 15062-15072.

Freund, S., Jung, G., Gutbrod, O., Folkers, G. and Gibbons, W. A. 1991. The three-dimensional structure of gallidermin determined by NMR-based molecular graphics, p. 91-102. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, The Netherlands.

Freund, S., Jung, G., Gutbrod, O., Folkers, G. and Gibbons, W. A., Allgaier, H. and werner, R. 1991. The solution structure of the lantibiotic gallidermin. Biopolymers 31: 803-811.

Froseth, B. R. and McKay, L. L. 1988. Cloning of nisin resistance determinant and replication origin of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* DRC3. Appl. Environ. Microbiol. 57: 804-811.

Gagliano, V. J. and Hinsdill, R. D. 1970. Characterization of a *Staphylococcus aureus* bacteriocin. J. Bacteriol. 104: 117-125.

Gao, F. H., Abee, T. L. and Konings, W. N. 1991. Mechanism of action of the peptide antibiotic nisin in liposome and cytochrome c oxidase containing proteolyposomes. Appl. Environ. Microbiol. 57: 2164-2170.

Garneau, S., Martin, N. I., Vederas, J. C. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie. 84: 577-592.

Gasson, M. J. 1984. Transfer of sucrose fermenting ability, nisin resistance and nisin production into *Streptococcus lactis* 712. FEMS Microbiol. Lett. 21: 7-10.

Gasson, M. J. 1993. Progress and potential in the biotechnology of lactic acid bacteria. FEMS Microbiol. Rev. 87: 43-60.

Geis, A., Singh, J. and Teuber, M. 1983. Potential of lactic Streptococci to produce bacteriocin. Appl. Environ. Microbiol. 45: 202-211.

Gerritse, K., Posno, M., Schnellekens, M. M., Boersma, W. J. A. and Claassen, E. 1990. Oral administration of TNP-Lactobacillus conjugates in mice: a model for evaluation of mucosal and systemic immune responses and memory formation elicited by transformed lactobacilli. Res. Microbiol. 141: 955-962.

Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol Rev. 87: 175-188.

Giraffa, G., Neviani, E. and Veneroni, A. 1990. Use of conductance to detect bacteriocin activity. J. Food Prot. 53: 772-776.

Hall, R. H. 1966. Nisin and food preservation. Proc. Biochem. 1: 461-464.

Hansen, J. N., Chung, Y. J., Liu, W. and Sten, M. T. 1991. Biosynthesis and mechanism of action of nisin and subtilin, p. 287-302. In G. Jung and H.-G Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, The Netherlands.

Harding, C. and Sham, B. G. 1990. Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. J. Appl. Bacteriol. 69: 648-658.

Hastings, J. W. and Stiles, M. E. 1991. Antibiosis of *Leuconostoc gelidum* isolated from meat. J. Appl. Bacteriol. 70: 127-134.

Håvarstein, L. S., Diep, D. B. and Nes, I. F. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. 16: 229-240.

Héchard, Y., Derijard, B., Letellier, F. and Cenatiempo, Y. 1992. Characterization and purification of mesentericin Y105, an anti-Listeria bacteriocin from *Leuconostoc mesenteroides*. J. Gen. Microbiol. 138: 2725-2731.

Héchard, Y., Dherbomez, M., Cenatiempo, Y. and Letellier, F. 1990. Antagonism of lactic acid bacteria from goats' milk against pathogenic strains assessed by the sandwich method. Lett. Appl. Microbiol. 11: 185-188.

Héchard, Y., Pelletier, C., Cenetiempo, Y., Frere, J. 2001. Analysis of sigma (54)-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII(Man) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiol. 147: 1575-1580.

Héchard, Y., Sahl, H. G. 2002. Mode of action of modified and unmodified bacteriocins from gram-positive bacteria. Biochimie. 84: 545-557.

Henning, S., Metz, R. and Hammes, W. P. 1986. New aspects for the application of nisin to foods based on its mode of action. Int. J. Food Microbiol. 3: 135-141.

Hetz, C., Bono, M. R., Barros, L. F., Lagos, R. 2002. Microcin E492, a channel-forming bacteriocin from *Klebsiella pneumoniae*, induces apoptosis in some human cell lines. Proc. Natl. Acad. Sci. USA. Mar 5; 99(5): 2696-2701.

Hill, R. P. and Farkas-Himsley, H. 1991. Further studies of the action of a partially purified bacteriocin against a murine fibrosarcoma. Cancer Res. Mar 1; 51(5): 1359-1365.

Holck, A., Axelsson, L., Birkeland, S.-E., Aukrust, T. and Blom, H. 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb 706. J. Gen. Microbiol. 138: 2715-2720.

Horn, N., Swindell, S., Dodd, H. M. and Gasson, M. J. 1991. Nisin biosynthesis genes are encoded by a novel conjugative transposon. Mol. Gen. Genet. 228: 129-135.

Hurst, A. 1978. Nisin: its preservative effect and function in the growth cycle of the producer organism. Soc. Appl. Bacteriol. Symp. Ser. 7: 297-314.

Hurst, A. 1981. Nisin. Adv. Appl. Microbiol. 27: 85-123.

Hühne, K., Axelsson, L., Holck, A. and Kröckel, L. 1996. Analysis of the sakacin P gene cluster from *Lactobacillus sake* Lb674 and its expression in sakacin-negative *L. Sake* strains. Microbiol. 142: 1437-1448.

Jack, R. W., Carne, A., Metzger, J., Stefanovic, S., Sahl, H.-G., Sung, G. and Tagg, J. R. 1994. Elucidation of the structure of SA-FF22, a lanthionine-containing antibacterial peptide produced by *Streptococcus pyogenes* strain FF22. Eur. J. Biochem. 220: 455-462.

Jack, R. W., Tagg, J. R., Ray, B. 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. 59: 171-200.

Jamuna, M., Jeevaratnam, K. 2004. Isolation and partial purification of bacteriocins from Pediococcus species. 65: 433-439.

Jung, G. 1991. Lantibiotics-ribosomally synthesized biologially active polypeptides containing sulfide bridges and α-β-didehydroamino acids. Angew. Chemie. 30: 1051-1068.

Kalchayanad, N., Hanilin, M. B. and Ray, B. 1992. Sublethal injury make gram-negative and resistant gram-positive bacteria sensitive to the bacteriocins pediocin AcH and nisin. Lett. Appl. Microbiol. 15: 239-243.

Kaletta, C., Entian, K. D., Jung, G. 1991. Prepeptide sequence of cinnamycin (Ro 09-0198): the first structural gene of a duramycin-type lantibiotic. Eur. J. Biochem. 199: 411-415.

Kékessy, D. A. and Piguet, J. D. 1970. New method for detection bacteriocin production. Appl. Microbiol. 20: 282-283.

Kim, W. S., Hall, R. J., Dunn, N. W. 1998. The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 48: 449-453.

Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. Biochimie. 70: 337-349.

Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12: 39-85.

Klaenhammer, T. R. and Sanozky, R. B. 1985. Conjugal transfer from *Streptococcus lactis* ME2 of plasmids encoding phage resistance, nisin resistance and lactose-fermenting ability: Evidence for a high-frequency conjugative plasmid responsible for abortive infection of virulent bacteriophage. J. Gen. Microbiol. 131: 1531-1541.

Kleerebezem, M., Quadri, L. E. N., Kuipers, O. P., Vos, W. M. De .1997. Quorum sensing by peptide pheromones and two-component signal transduction systems in gram-positive bacteria. Mol. Microbiol. 24: 895-904.

Kleerebezem, M., and Quadri, L. E. N. 2001. Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behaviour. Peptides. 22: 1579-1596.

Kleinkauf, H. and von Döhren, H. 1990. Nonribozomal biosynthesis of peptide antibiotics. Eur. J. Biochem. 192: 1-15.

Kordel, M., Schüller, F. and Sahl, H.-G. 1989. Interaction of the pore forming peptide antibiotics Pep5, nisin and subtilin with non-energized liposomes. FEBS Lett. 224: 99-102.

Kuipers, O. P., Beerthuizen, M. M., Ruyter, P. G. G. A. De, Lucsink, E. J., Vos, W. M. De. 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. 270: 27299-27304.

Larsen, A. G. and Nørrung, B. 1993. Inhibition of *Listeria monocytogenes* by bavaricin A, bacteriocin produced by *Lactobacillus bavaricus* MI401. Lett. Appl. Microbiol. 17: 132-134.

Lehrer, R. I., Lichtenstein, A. K., Ganz, T. 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. 11: 105-128.

Lewus, C. B., Kaiser, A. and Montville, T. J. 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl. Environ. Microbiol. 57: 1683-1688.

Lewus, C. B., Sun, S. and Montville , T. J. 1992. Production of an amylase-sensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain. Appl. Environ. Microbiol. 5: 143-149.

Li, C., Bai, j., Li, W., Cai, Z. and Ouyang, F. 2001. Optimization of conditions for bacteriocin extraction in PEG (Salt Aqueous Two-Phase Systems Using Statistical Experimental Designs) Biotechnol. Prog. 17: 366-368.

Limbert, M., Isert, D., Klesel, N., Markus, A., Seibert, G., Chatterjee, S., Chatterjee, D. K., Jani, R. H. and Ganguli, B. N. 1991. Chemotherapeutic properties of mersacidin in vitro and in vivo, p. 448-456. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. Escom Publishers, Leiden, the Netherlands.

Liu, W. And Hansen, J. N. 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. Appl. Environ. Microbiol. 56: 2551-2558.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Maftah, A., Renault, D., Vignoles, C., Hechard, Y., Bressollier, P., Ratinaud, M. H., Cenatiempo, Y. and Julion, R. 1993. Membrane permeabilization of *Listeria monocytogenes* and mitochondria by bacteriocin mesentericin Y105. J. Bacteriol. 175: 3232-3235.

Martinez-Cuesta, M. C., Kok, J., Herranz, E., Peláez, C., Teresa, R. and Buist, G. 2000. Requirement of autolytic activity for bacteriocin-induced lysis. Appl. Environ. Microbiol. 66: 3174-3179.

Martinez, M. I., Rodriguez, E., Medina, M., Hernandez, P. E. and Rodriguez J. M. 1998. Detection of specific bacteriocin-producing lactic acid bacteria by colony hybridization. J. Appl. Microbiol. 84: 1099-1103.

Matsusaki, H., Endo, N., Sonomoto, K., De Vuyst, L. 1998. Modelling the growth and bacteriocin production by *Lactococcus lactis amylovorus* DCE 471 in batch cultivation. J. Appl. Bacteriol. 84: 159:168.

Mattila, T. 1987. A modified Kelsey-Sykes method for testing disinfectants with 2,3,5-triphenyltetrazolium chloride reduction as an indicator of bacteriol growth. J. Appl. Bacteriol. 62: 551-554.

Mattila, T. and Sandholm, M. 1989. Antibacterial effect of the glucose oxidase-glucose system on food poisoning organisms. Int. J. Food Microbiol. 8: 165-174.

Mayr-Harting, A., Hedges, A. J. and Berkeley, R. C. W. 1972. Methods for studying bacteriocins. Methods Microbiol. 7A, 315-422.

Mazzotta, A. S. and Montville, T. J., 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10^0C and at 30^0C. J. Appl. Bacteriol. 82: 32-38.

McKay, L. L. and Baldwin, K. A. 1984. Conjugative 50-megadalton plasmid in *Streptococcus lactis* subsp. *diacetylactis* DRC3 is associated with resistance to nisin and bacteriophage. Appl. Environ. Microbiol. 47: 68-74.

Meghrous, J., Huot, E., Quittelier, M., Petitdemange, H. 1992. Regulation of nisin biosynthesis by continuous cultures and by resting cells of *Lactococcus lactis* subsp. *lactis*. Res. Microbiol. 143: 879-890.

Mendoza, F., Maqueda, M., Gálvez, A., Martinez-Bueno, M. and Valdivia, E. 1999. Antilisterial activity of peptide AS-48 and study of changes induced in the cell envelope properties of an AS-48-adapted strain of *Listeria monocytogenes*. Appl. Environ. Microbiol. 65: 618-625.

Métivier, A., Boyaval, P., Duffes, F., Dousset, X., Compoint, J.-P. and Marion, D. 2000. Triton X-114 phase partitioning for the isolation of a pediocin-like bacteriocin from *Carnobacterium divergens*. Lett. Appl. Microbiol. 30: 42-46.

Ming, X. and Daeschel, M. A. 1995. Correlation of cellular phospholipid content with nisin resistance of *Listeria monocytogenes* Scott A. J. Food. Prot. 58: 416-420.

Moll, G. N., Konings, W. N., Driessen, A. J. M. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie van Leewenhoek. 76: 185-198.

Moll, G. N., Roberts, G. C. K., Konings, W. N., Driessen, A. J. M. 1996. Mechanism of lantibiotic-induced pore formation. Antonie van Leewenhoek. 69: 185-191.

Morgan, S., Ross, R. P. and Hill, C. 1995. Bacteriolytic activity caused by the presence of a novel Lactococcal plasmid encoding Lactococcins A, B, and M. Appl. Env. Microbiol. 61: 2995-3001.

Møretrø, T. 2000. Optimization of production of the bacteriocin sakacin P by *Lactobacillus sakei*; cultural conditions and genetics. Academic Thesis, Agricultural University of Norway.

Mørtvedt, C. I., and Nes, I. F. 1990. Plasmid-associated bacteriocin production by *Lactobacillus sake* strain. J. Gen. Microbiol. 136: 1601-1608.

Motlagh, A, Johnson, M. C. and Ray, B. 1991. Viability loss of foodborne pathogens by starter culture metabolites. J. Food. Prot. 54: 873-878.

Muriana, P. M. and Klaenhammer, T. R. 1991. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57: 114-121.

Nes, I. F. and Holo, H. 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopolymers. 55: 50-61.

Nes, I. F., Bao Dien, D., Håverstein, L. S., Eijsink, V., Holo, H. 1996. Biosynthesis of bacteriocins of lactic acid bacteria. Antonie van Leeuwenhoek. 70: 113-128.

Nes, I. F., Tagg, J. R. 1996. Novel lantibiotics and their pre-peptides. Antonie van Leeuwenhoek. 69: 89-97.

Nes, I. F., Diep, D. B., Håvarstein, L. S., Brurberg, M. B., Eijsink, V. and Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leewenhoek. Int. Gen. Mol. Microbiol. 70: 113-128.

Nikaido, H.and Vaara, M. 1987. Outer membrane, p.7-22. In F. C. Neidhardt (ed), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology Washington, D. C.

Nilsen, T., Nes, I. F., Holo, H. 1998. An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. J. Bacteriol. 180:1848-1854.

Nissen-Meyer, J., Håvarstein, S., Holo, H., Sletten, K. and Nes, I. F. 1993. Association of the lactococcin A immunity factor with the cell membrane: purification and characterization of the immunity factor. J. Gen. Microbiol. 139: 1503-1509.

Nissen-Meyer, J., Holo, H., Håvarstein, S., Sletten, K. and Nes, I. F. 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. J. Bacteriol. 174: 5686-5692.

Noerlis, Y. and Ray, B. 1993. Factors influencing immunity and resistance of *Pediococcus acidilactici* to the bacteriocin, pediocin AcH. Lett. Appl. Microbiol. 18: 138-143.

Ogden, K., Waites, M. J. and Hammond, J. R. M. 1988. Nisin and Brewing. J. Inst. Brewing. 94: 233-238.

Okereke, A. and Montville, T. J. 1991. Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria ar refrigeration and abuse temperatures. Appl. Environ. Microbiol. 57: 3423-3428.

Oscáriz, J. C., Pisabarro, A. G. 2001. Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. Int. Microbiol. 4: 13-19.

O' Sullivan, L., Morgan, S. M., Ross, R. P. and Hill, C. 2002. Elevated enzyme release from Lactococcal starter cultures on exposure to the lantibiotic Lacticin 481, produced by *Lactococcus lactis* DPC5552. J. Dairy Sci. 85: 2130-2140.

Papathanasopoulos, M. A., Dykes, G. A., Revol-Junelles, A. M., Delfour, A., von holy, A. and Hastings, J. W. 1998. Sequence and structural relationships of leucocins A-, B- and C-TA33a from *Leuconostoc mesenteroides* TA33a. Microbiol. 144: 1343-1348.

Parente, E., Hill, G. 1992. A composition of factors affecting the production of two bacteriocins from lactic acid bacteria. J. Appl. Bacteriol. 73: 290-298.

Parente E., Ricciardi, A. 1994. Effect of nitrogen and carbohydrate sources on the lactic acid and bacteriocin production by *Enterococcus faecium* DPC 1146. Agro-Industry. Hi-Tech. 58: 35-39.

Parente, E., Ricciardi A., Addario, G. 1994. Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. Appl. Microbiol. Biotechnol. 41: 388-394.

Patek, M., Hochmannova, J., Nesvera, J. and Stransky, J. 1986. Glutamicin CBII, a bacteriocin-like substance produced by *Corynebacterium glutamicin*. Antonie Leewenhoek. 52: 129-140.

Piard, J.-C. and Desmazeaud, M. 1991. Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. Lait. 71: 525-541.

Piard, J.-C. and Desmazeaud, M. 1992. Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. Lait 72: 113-142.

Proctor, A. and Palaniappan, S. 1990. Adsorption of soy oil free fatty acids by rice hull ash. J. Am. Oil. Chem. Soc. 67: 15-17.

Proctor, A., Adhikari, C. and Blyholder, G. D. 1995. Mode of oleic acid adsorption on rice hull ash cristobalite. J. Am. Oil. Chem. Soc. 72: 331-335.

Pucci, M. J., Vedamuthu, E. R., Kunka, B. S. and Vanderbergh, P. A. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. Appl. Environ. Microbiol. 54: 2349-2353.

Pugsley, A. P. 1984. The ins and outs of colicins. I. Production and translocation across membranes. Microbiol. Sci. 1: 203-205.

Quadri, L. E. N., Kleerebezem, M., Kuipers, O. P., Vos, W. M. De, Roy, K. L., Vederas, J. C., Stiles, M. E. 1997. Characterization of a locus from *Carnobacterium*

piscicola LV17B involved in bacteriocin production and immunity: evidence for global inducer-mediated transcriptional regulation. J. Bacteriol. 19: 6163-6171.

Quadri, L. E. N., Sailer, M., Terebiznik, M. R., Roy, K. L., Vederas, J. C. and Stiles, M. E. 1995. Characterization of the protein conferring immunity to the antimicrobial peptide carnobacteriocin B2 and expression of carnobacteriocins B2 and BM1. J. Bacteriol. 177: 1144-1151.

Quiao, M., Koponen, O., Usabiaga, M., Immonen, T. 1996. Regulation of the nisin operons in *Lactococcus lactis* N8. J. Appl. Bacteriol. 80: 626-634.

Radler, F. 1990a. Possible use of nisin in wine-making I. Action of nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Am. J. Enology and Viticulture. 41: 1-6.

Radler, F. 1990b. Possible use of nisin in wine-making II. Experiments to control lactic acid bacteria in the production of wine. Am. J. Enology and Viticulture. 41: 7-11.

Ramnath, M., Beukes, M., Tamura, K., Hastings, J. W. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. 66: 3098-3101.

Rasch, M and Knøchel, S. 1998. Variations in tolerance of *Listeria monocytogenes* to nisin, pediocin PA-1 and bavaricin A. Lett. Appl. Microbiol. 27: 275-278.

Rauch, P. J. G. and De Vos, W. M. 1992. Characterization of the novel nisin-sucrose conjugative transposon Tn5276 and its insertion in *Lactococcus lactis*. J. Bacteriol. 174: 1280-1287.

Ray, B., Motlagh, A. and Johnson, M. C. 1993. Processing of prepediocin in *Pediococcus acidilactici*. FEMS Microbiol. Rev. 12: 119.

Reid, F. L., Ahmed, K. A., Waites, W. M. and Stewart, G. S. A. B. 1990. The rapid detection of antimicrobials using bioluminescent lactic acid bacteria. FEMS Microbiol. Rev. 87: 88.

Reis, M., Eschbach-Bludau, M., Iglesias-Wind, M. I., Kupke, T., Sahl, H. G. 1994. Producer immunity towards the lantibiotic Pep5: identification of the immunity gene pepI and localization and functional analysis of its gene product. Appl. Environ. Microbiol. 60: 2876-2883.

Revol-Junelles, A.-M., Lefebvre, G. 1996. Purification and N-terminal amino acid sequence of Dextranicin 24, a bacteriocin of *Leuconostoc* sp. Current Microbiol. 33: 136-137.

Ryan, M. P., Flynn, J., Hill, C., Ross, R. P., Meaney, W. J. 1999. The natural food grade inhibitor, Lacticin 3147, reduced the incidence of mastitis after experimental challenge with *Streptococcus dysgalactiae* in nonlactating dairy cows. J. Dairy Sci. 82: 2108-2114.

Ryan, M. P., Meaney, W. J., Ross, R. P., Hill, C. 1998. Evaluation of Lacticin 3147 and a teat seal containing this bacteriocin for inhibition of mastitis pathogens. Appl. Environ. Microbiol. 64: 2287-2290.

Sahl, H.-G. 1985. Influence of staphylococcin-like peptide Pep5 on membrane potential of bacterial cells and cytoplasmic membrane vesicles. J. Bacteriol. 162: 833-836.

Schnell, N., Entian, K.-D., Schneider, U., Gotz, F., Zähner, H., Kellner, R. and Jung, G. 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature. 333: 276-278.

Schüller, F., Benz, R. and Sahl, H.-G. 1989. The peptide antibiotic subtilin acts by formation of voltage-dependent multi-state pores in bacterial and artificial membranes. Eur. J. Biochem. 182: 181-186.

Skyttä, E. and Sandholm, T. M. 1991. A quantitative method for assessing bacteriocins and other food antimicrobials by automated turbidometry. J. Microbiol. Methods. 14: 77-88.

Spelhaug, S. R. and Harlander, S. K. 1989. Inhibition of food-borne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceous*. J. Food Prot. 52: 856-862.

Stevens, K. A., Sheldon, B. W., Klapes, N. A. and Klaenhammer, T. R. 1991. Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria. Appl. Environ. Microbiol. 57: 3613-3615.

Stiles, M. E. and Hastings, J. W. 1991. Bacteriocin production by lactic acid bacteria: potential for use in meat preservation. Trends Food Sci. Technol. 2: 247-251.

Stoffels, G., Nissen-Meyer, J., Gudmundsdottir, A., Sletten, K., Holo, H. and Nes, I. F. 1992. Purification and characterization of a new bacteriocin isolated from a *Carnobacterium* sp. Appl. Environ. Microbiol. 58: 1417-1422.

Stoffels, G., Sahl, H. G. and Gudmundsdottir, Á. 1993. Carnocin UI49, a potential biopreservative produced by *Carnobacterium piscicola*: large scale purification and activity against various Gram-positive bacteria includind *Listeria* sp. Int. J. Food Microbiol. 20: 199-210.

Stoffels, G., Gudmundsdottir, A. and Abee, T. 1994. Membrane-associated proteins encoded by the nisin cluster may function as a receptor for the lantibiotic carnocin U149. Microbiol. 140: 1443-1450.

Suárez, A. M., Azcona, J. L., Rodriguez, J. M., Sanz, B., Hernández, P. 1997. One-step purification of nisin A by immunoaffinity chromatography. Appl. Environ. Microbiol. 63: 4990-4992.

Tagg, J. R. and McGiven, A. R. 1971. Assay system for bacteriocins. Appl. Microbiol. 21: 943.

Tagg, J. R., Dajani, A. S. and Wannamaker, L.W. 1976. Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. 40: 722-756.

Tantrakulsiri, J., Jeyashoke, N. and Krisanangkura, K. 1997. Utilization of rice hull ash as a support material for immobilization of *Candida cylindracea* lipase. J. Am. Oil. Chem. 74: 173-175.

Tichaczek, P. S., Nissen-Meyer, J., Nes, I. F., Vogel, R. F. and Hammes, W. P. 1992. Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH 1174 and sakacin P from *L. Sake* LTH 673. System. Appl. Microbiol. 15: 460-468.

Tsai, H. J. and Sandine, W. E. 1987. Conjugal transfer of nisin plasmid genes from *Streptococcus lactis* 7962 to *Leuconostoc dextranicum* 181. Appl. Environ. Microbiol. 53: 352-357.

Ungermann, V., Goeke, K., Fiedler, H.-P.and Zähner, H. 1991. Optimization of fermentation of gallidermin and epidermin, p. 410-421. In G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics, Escom Publishers, Leiden, The Netherlands.

Upreti, G. C. and Hinsdill, R. D. 1973. Isolation and characterization of a bacteriocin from a homofermentative Lactobacillus. Antimicrob. Agents Chemother. 4: 487-494.

Upreti, G. C. and Hinsdill, R. D. 1975. Production and mode of action of lactocin 27: bacteriocin from a homofermentative Lactobacillus. Antimicrob. Agents. Chmother. 7: 139-145.

Van Belkum, M. J., Kok, J., Venema, G., Holo, H., Nes, I. F., Konings W. N. and Abee, T. 1991. The bacteriocin lactococcin A specifically increases the permeability of lactococcal cytoplasme membranes in a voltage-independent, protein-mediated manner. J. Bacteriol. 173: 7934-7941.

Van Belkum, M. J., Kok, J.,Venema, G. 1992. Cloning, sequencing, and expression in *Esherichia coli* of *lcnB*, a third bacteriocin determinant from the lactococcal bacteriocin plasmid p9B4-6. Appl. Environ. Microbiol. 58: 572-577.

Van Belkum, M. J.,Hayema, B. J., Jeeninga, R. E., Kok, J., Venema, G. 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. 57: 492-498.

van de Ven, F. J. M., van den Hooven, H. W., Konings, R. N. H. and Hilbers, C. W. 1991. NMR studies of lantibiotics, the structure of nisin in aqueous solution. Eur. J. Biochem. 202: 1181-1188.

Van de Ven, F. J. M., van den Hooven, H. W., Konings, R. N. H. and Hilbers, C. W. 1991. The spatial structure of nisin in aqueous solution. In: Jung G & Sahl H-G (Eds) Nisin and novel lantibiotics (pp 35-44). Escom publishers, Leiden, the Netherlands.

Van den Hooven (1995) Structure elucidation of the lantibiotic nisin in aqueous solution and in membrane-like environments. Ph.D. Thesis, University of Nijmegen, The Netherlands.

Venema, K., Abee, T., Haandrikman, A. J., Leenhauts, K. J., Kok, J., Konings, W. N., and Venema, G. 1993. Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. Appl. Environ. Microbiol. 59: 1041-1048.

Venema, K., Chikindas, M. L., Seegers, J. F. M. L., Haandrikman, A. J., Leenhouts, K. J., Venema, G., Kok, J. 1997. Rapid and efficient purification method for small, hydrophobic, cationic bacteriocins: purification of lactococcin B and pediocin PA-1. Appl. Environ. Microbiol. 63: 305-309.

Venema, K., Venema, G. and Kok, J. 1995. Lactococcal bacteriocins: mode of action and immunity. Trends Microbiol. 8: 299-304.

Von Tersch, M. A. and Carlton, B. C. 1983. Bacteriocin from *Bacillus megaterium* ATCC19213: compararive studies with megacin A-216. J. Bacteriol. 155: 866-871.

Wachsman, M. B., Castilla, V., de Ruiz Holgado, A. P., de Torres, R. A., Sesma, F., Coto, C. E. 2003. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. Antiviral Research. 58: 17-24.

Von Wright, A., Wessels, S., Tynkkynen, S. and Saarela, M. 1990. Isolation of a replication region of a large lactococcal plasmid and use in cloning of a nisin resistance determinant. Appl. Environ. Microbiol. 56: 2029-2035.

Wang, Y., Henz, M. E., Fregeau Gallagher, N., Chai, S., Gibbs, A. C., Yan, L. Z., Stiles, M. E., Wishart, D. S. & Vederas, J. C. 1999. Solution structure of carnobacteriocin B2 and implications fro structure-activity relationships among type IIa bacteriocins from lactic acid bacteria. Biochemistry: 38: 15438-15447.

Watanabe, T. and Saito, H. 1980. Cytotoxicity of pyocin S2 to tumor and normal cells and its interaction with cell surfaces. Biochim. Biophys. Acta . Nov 17; 633 (1): 77-86.

Whitford, M. F., McPherson, M. A., Forster, R. J. and Teather, R. M. 2001. Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. And isolation and characterization of Bovicin 255. Appl. Environ. Microbiol. 67: 569-574.

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. 276: 1772-1779.

Woodruff, W. A., Novak, J., Caufield, P. W. 1998. Sequence analysis of mutA and mutM genes involved in the biosynthesis of the lantibiotic mutacin II in *Streptococcus mutans*. Gene 206: 37-43.

Wu, C.-W., Yin, L.-J. and Jiang, S.-T. 2004. Purification and characterization of bacteriocin from *Pediococcus pentosaceus* ACCEL. J. Agric. Food Chem. 52: 1146-1151.

Yan, L. Z., Gibbs, A. C., Stiles, M. E., Wishar, D. S., Vederas, J. C. 2000. Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. J. Med. Chem. 43: 4579-4581.

Yang, R., Johnson, M. C., and Ray, B. 1992. Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. 58: 3355-3359.

Yang, R., Ray, B. 1994. Factors influencing production of bacteriocins by lactic acid bacteria. Food. Microbiol. 11: 281-291.

Zajdel, J. K., Ceglowski, P., Dobranski, W. T. 1985. Mechanism of action of lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris* 202. Appl. Environ. Microbiol. 49: 969-974.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active froms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA 84: 5449-5453.

APPENDIX A

COMPOSITION OF REAGENTS OF LOWRY METHOD

Reagent A

2 g $C_4H_4KNaO_6.4H_2O$ and 100 g Na_2CO_3 are dissolved in 500 ml and 1N NaOH and diluted with distilled water to 1 L. The solution is stored at room temperature.

Reagent B

2 g C4H4KNaO$_6$.4H$_2$O and 1 g $CuSO_4.5H_2O$ are dissolved in 90 ml distilled water. 10 ml of 1 N NaOH is added to this solution. The solution is stored at room temperature.

Reagent C

1 parts of volume Folin-Ciocalteu reagent is diluted with 15 parts of volume of distilled water.

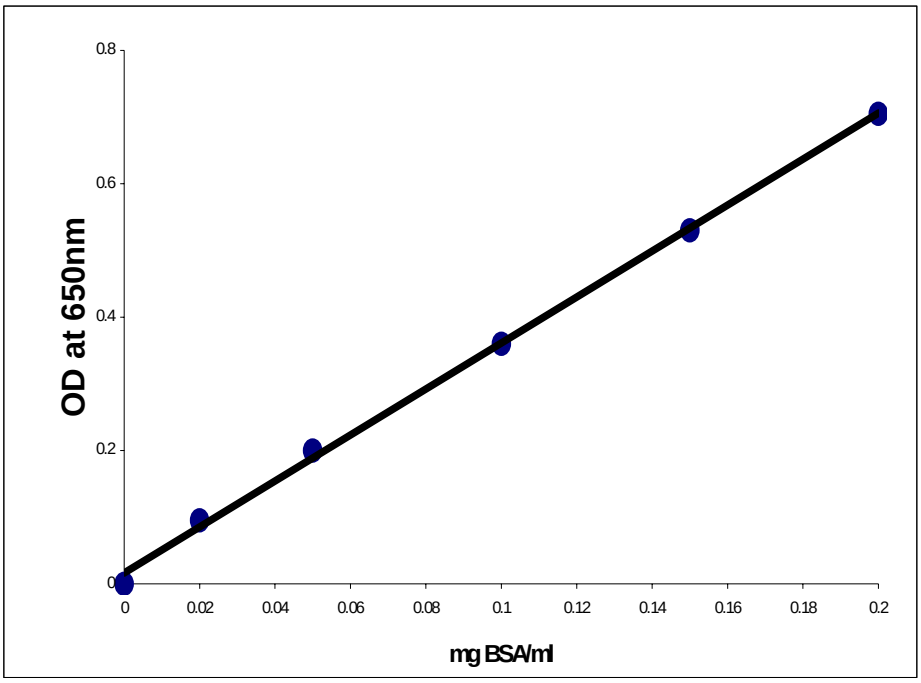
APPENDIX B

PROCEDURE OF LOWRY METHOD

1) 0.9 ml of reagent A is added to tubes containing 0.1 ml samples and mixed on a vortex.
2) Tubes are heated at 50 ^{0}C for 10 minutes.
3) Tubes are cooled at room temperature.
4) 0.1 ml of reagent B is added and mixed by means of a vortex.
5) Tubes are leaved at room temperature for 10 minutes.
6) 3 ml of reagent C is added and mixed on a vortex immediately.
7) Tubes are heated at 50 C for another 10 minutes.
8) Tubes are cooled at room temperature.
9) Optical density is read in a spectrophotometer at 650 nm.

APPENDIX C

STANDARD CURVE FOR LOWRY METHOD



APPENDIX D

PREPARATION OF REAGENTS OF SDS-PAGE

1. 10% Ammonium persulfate

Dissolve 10 mg ammonium persulfate in 100 ml water. Prepare fresh daily.

2. N, N'-tetramethylene-ethylenediamine (TEMED)

Use TEMED from bottle, use only pure, distilled TEMED. Store cool, dry, protect from light.

3. 30% Acrylamide/0.8% Bisacrylamide

Mix 30.0 g acrylamide and 0.8 g N, N'-methylene-bisacrylamide in a total volume of 100 ml distilled water. Filter the solution through a 0.45-μm filter and store at 4^0C in the dark. Discard after 30 days, since acrylamide gradually hydrolyzes to acrylic acid and ammonia.

4. 4X Tris-CI/SDS, pH 6.8 (0.5 M Tris CI containing 0.4% SDS, also called 4X Upper Tris)

Dissolve 6.05 g Tris base and 4 ml of 10% SDS in 40 ml distilled water. Adjust to pH 6.8 with 1 N HCI. Add distilled water to 100 ml toal volume. Filter the solution through a 0.45 μm filter and store at 4^0C.

5. Tris-CI/SDS, pH 8.8 (1.5 M Tris-CI containing 0.4% SDS, also called 4X lower Tris

Dissolve 18.17 g Tris base and 4 ml of 10% SDS in 40 ml distilled water. Adjust to pH 8.8 with 1 N HCI. Add distilled water to 100 ml total volume. Filter the solution through a 0.45-μm filter and store at 4^0C.

6. 5X SDS/Electrophoresis Buffer

Dissolve 15.1 g Tris base, 72.0 g glycine, and 5.0 g SDS in about 800 ml of distilled water. After the solutes are dissolved, bring the volume to 1.0 liter. Filter the solution through a 0.45 μm filter and store at 4^0C. To prepare 1X SDS/electrophoresis buffer, dilute one volume of the above solution with four volumes of distilled water.

7. 2X SDS/Sample Buffer

Mix 30 ml of 10% SDS, 10 ml glycerol, 5.0 ml 2-mercaptoethanol, 12.5 ml of 4X Tris-CI/SDS, pH 6.8, and 5-10 mg bromphenol blue. Bring the volume to 100 ml with distille water. Divide into 1.0 ml aliquots, and store at -20^0C.

APPENDIX E

SILVER NITRATE STAINING

1) Silver Nitrate Solution

Add 3.5 ml concentrated ammonium hydroxide (30%) tp 42 ml 0f 0.36% NaOH and bring the volume to 200 ml with distilled water. Mix with a magnetic stirrer and slowly add 8 ml of 19.4% (1.6 g/8ml) silver nitrate

2) Silver Staining Procedure

a) Place the polyacrylamide gel in a plastic box on an orbital shaker and add fixing solution. Incubate for 30 min.
b) Decant the fixing solution, and fix the gel in destaining solution for at least 60 min.
c) Decant the destain, and cover the gel with 10% glutaraldehyde. Agitate for 30 min.
d) Wash the gel four times with water, for at least 30 min each wash.
e) Stain the gel with silver nitrate solution for 15 min with vigorous shaking.
f) Transfer the gel to another plastic box and wash five times with water (exactly 1 min for each wash)
g) Prepare the developer by diluting 25 ml developing solution with 500 ml water. Transfer the gel to another plastic box, add developer, and shake vigorously until the bands appear as intense as desired. If the developer turns brown, change to fresh developer.

h) Transfer to Kodak Rapid Fix for 5 min.

i) Wash the gel exhaustively in water to remove Rapid Fix.

3) Destaining Solution

50% (v/v) methanol, 10% (v/v) acetic acid and 50% distilled water were mixed.

4) Fixing Solution

50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) distilled water were mixed.

APPENDIX F

COMPOSITION OF CULTURE MEDIA

MRS Broth

Composition	g/l
Peptone	10.0
Meat extract	8.0
Yeast extract	4.0
D(+) Glucose	20.0
K_2HPO_4	2.0
Tween 80	1.0 ml
di-ammonium hydrogen citrate	2.0
Sodium acetate	5.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.H_2O$	0.04

Add 52.2 g to 1 liter of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121^0C for 15 minutes.

M17 Broth

Composition	g/l
Balanced peptone or pancreatic digest of casein	5.0
Soy peptone	5.0
Beef extract	5.0
Yeast extract	2.5
Meat extract	5.0
D(+) Lactose	5.0
Ascorbic acid	0.5
Na-β-glycerophosphate	19.0
Magnesium sulphate	0.25

pH 7.2

Suspend 37.25 g powder in 950 ml purified water. Mix well and distribute into final containers. Sterilize by autoclaving at 121^0C for 15 minutes.

Tryptone Soy Broth

Composition	g/l
Tryptone	15.0
Soy peptone	5.0
NaCI	15.0

APPENDIX G

LIST OF CHEMICALS AND THEIR SUPPLIERS

Chemical	Supplier
MRS broth	Merck
MRS Agar	Merck
Tryptone Soy Broth	LAB M
Agar	DIFCO
Sephadex G-25	Sigma
Sephadex G-50	Sigma
Macro Prep High S	Bio Rad
Phenyl Sepharose CL 4B	Sigma
Amberlite XAD-16	Rohm and Haas
Micro Cel E	Lompoc
2-Propanol	Merck
Methanol	Merck
Chloroform	Merck
Silver nitrate	Sigma
N,N'-Methylene-bis-Acrylamide	Sigma
SDS (Sodium dodecyl sulfate)	Sigma
Tris(hydroxymethyl) aminomethane	Sigma
2-Mercaptoethanol	Sigma
TEMED(N,N,N',N'-Tetramethylethylenediamine)	Sigma
Ammonium Persulfate	Sigma
Bromphenol Blue	Sigma
Ammonium Sulphate	Merck
NaCI	Merck
Proteinase K	Sigma
Pepsine	Sigma
Trypsine	Sigma
Chymotrypsine	Sigma
α-amylase	Sigma
Catalase	Sigma
Lysozyme	Sigma
0.22-micron pore size membrane filter	Sartorius

CURRICULUM VITAE

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EDUCATION

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WORK EXPERIENCE

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PUBLICATIONS

Z.B. Ögel, K. Yarangümeli, H. Dündar, I. Ifrij, 2001, Submerged cultivation of *Scytalidium thermophilum* on complex lignocellulosic biomass for endoglucanase production, *Enzyme and Microbial Technology.* Vol 28 (7-8), pp 689-695.