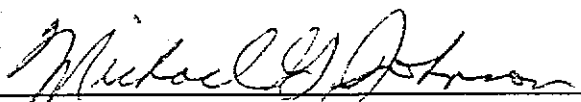


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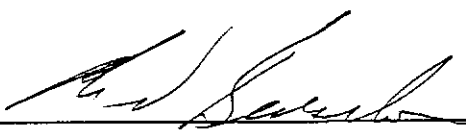
**ISOLATION, PURIFICATION, CHARACTERIZATION, MODE
OF ACTION, AND AMINO ACID SEQUENCE OF
BIFIDOCIN B AND LACTOCOCCIN R**

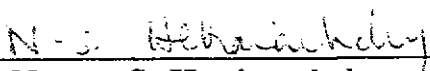
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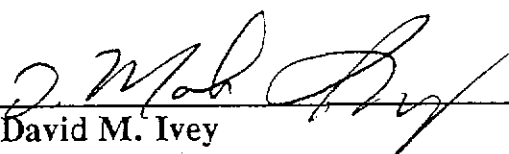
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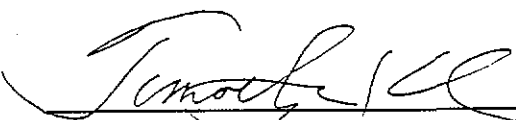

Dr. Timothy A. Kral

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grow at refrigeration temperature. Although the combination of low pH and low temperature could effectively control many foodborne pathogens and food spoilage bacteria in certain foods, some questions remain unanswered in a situation where the pH and the storage temperature of a food are both high enough to permit some growth. In those situations, use of antimicrobial proteins from food grade lactic acid bacteria in these foods could be an effective mean to overcome both microbial spoilage and safety hazards.

The recent trend of food processors to develop foods that are minimally processed may in fact compromise or eliminate previous product safeguards. Minimally processed foods are attractive to the consumer because they are convenient, have a natural, fresh appearance, are viewed as nutritionally correct, and are generally devoid of added preservatives. Consumer bias against chemical preservatives and preference for minimally processed foods reflect consumers' perception of what constitutes a natural and healthy food. To meet the demand and expectation of today's consumer, any preservative or additive under consideration in a product formulation must be carefully evaluated in terms of the consumer's perception of its validity.

Converting sugars into organic acid by lactic acid bacteria (LAB) is a well known fermentation process to improve the flavor and texture of some food products. LAB are also interesting for their ability to inhibit the growth of undesirable bacteria. This inhibitory activity is due to numerous metabolic events, including production

with food spoilage and foodborne illness, such as *Staphylococcus aureus* and *Listeria monocytogenes* while the cells that produce the bacteriocins are insensitive (Tagg *et al.* 1976, Klaenhammer 1993). Their production has been described in bacteria belonging to all main genera of the lactic acid bacteria group, lactococci, lactobacilli, leuconostoc, pediococci and bifidobacteria. Although a number of bacteriocins produced by LAB have been described, today only one bacteriocin, nisin, has found practical application. Following the successful application of the lantibiotic nisin as a biopreservative, the interest in bacteriocins increased considerably, because they might serve as food preservatives able to replace current chemical agents from which potentially carcinogenic compounds can arise. The prospect of using bacteriocins, particularly those from LAB, either directly or indirectly, stimulated enormous research activities and led to an extraordinary increase in knowledge about ribosomally synthesized peptides with antimicrobial activity. The events contributing to the increasing number of applied investigations on bacteriocins are (i) safe and efficacious use of nisin the past 30 years; (ii) FDA approval of nisin as a "generally regarded as safe" (GRAS) substance in certain applications; (iii) consumer resistance to traditional "chemical" preservatives; (iv) realization that bacteriocinogenicity is not a rare occurrence within the lactic acid bacteria; (v) justifiable concerns over the safety of existing food preservatives such as sulfites and nitrites; (vi) feasibility of using bacteriocin production and immunity as selectable genetic markers in starter culture bacteria; (vii) availability of molecular biology tools to transfer, clone, and

CHAPTER II

LITERATURE REVIEW

A. Historical background

Recorded observations of antagonistic interactions between different bacteria probably dates back to the work of Pasteur and Joubert (1877). They noted the inhibitory effect of common bacteria from urine against *Bacillus anthracis*. Many early studies were concerned with controlling diseases such as anthrax and diphtheria by use of nonpathogenic, antagonistic microorganisms. The discovery of penicillin in 1928 by Alexander Fleming gave impulse for the antibiotic revolution. 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*. According to Florey *et al.* (1949), perhaps the first description of bacteriocin-like antagonism between gram-positive bacteria was that of Babes, who in 1885 observed "le staphylococcus empeche surtout le staphylococcus" (staphylococcus especially inhibits staphylococcus) during growth on solid medium. Antagonistic interaction among LAB was first demonstrated by Whitehead as early as 1933. While investigating the reason for failure of some lactic cultures to grow normally in certain lots of milk during cheese manufacture, he isolated a gram-positive *Streptococcus*

C. Classification and nomenclature:

The majority of bacteriocins produced by LAB have been characterized by the initial definition of a proteinaceous inhibitor, estimation of molecular mass, and determination of susceptible strains. LAB bacteriocins have been categorized by their biochemical properties into four major groups by Klaenhammer (1993).

Class I bacteriocins, termed lantibiotics, are composed of small membrane active peptides (<5 kDa) containing the unusual amino acids, lanthionine and dehydrated amino acid residues; e.g. nisin, lactacin 481, carnocin U149, lactocin S. Class II bacteriocins, small non-lanthionine containing membrane active peptides, include bacteriocins that contain a Gly-Gly protease-processing site between the mature peptide and the prepeptide. This class is further subdivided into three groups. Bacteriocins active against listeria and having a consensus sequence at the N-terminal end of -Try-Gly-Asn-Gly-Val-Xaa-Cys- are classified in the subgroup IIa; e.g., pediocin PA-1, pediocin AcH, sakacin A, sakacin P, leucocin A, curvacin A. Subgroup IIb bacteriocins are poration complexes requiring two different peptides for activity; e.g., lactococcin G, lactococcin M, lactacin F. Bacteriocins in subgroup IIc are thiol-activated peptides requiring reduced cysteine residues for activity; e.g., lactococcin B. Class III bacteriocins are large heat-labile proteins (>30 kDa); e.g., helvetecin J, helvetecin V-1829, acidophilus A, lactacin A and B. Class IV includes complex bacteriocins containing carbohydrate or lipid components that are required for their activity; e.g., plantaricin S, leuconocin S, lactocin 27, pediocin SJ-1.

et al. (1983) who showed that 5% of 280 strains surveyed produced proteinaceous inhibitors. On the basis of heat resistance, host range, trypsin sensitivity, and cross reactivity between various bacteriocins/host combinations, Geis *et al.* (1983) recognized eight bacteriocin types (I-VIII) and suggested that each type could be composed of a heterogeneous group of compounds. These categories provide the basis for five distinct types of lactococcal bacteriocins: (i) lactococcin types I, V, VII with heat resistance at pH 9.4 and narrow host range against lactococci; (ii) diplococcins type II, III which are heat sensitive at pH 9.4 and have a narrow host range against lactococci; (iii) lactococcin-poration complexes of type IV which are heat resistant at pH 9.4 and show a wider host range against LAB and clostridia; (iv) lantibiotics types VI, VII which are heat sensitive at pH 9.4 and show inhibition against a wide host range of gram-positive bacteria.

a) Diplococcin

The first proteinaceous inhibitor described in lactococci was diplococcin (Whitehead 1933, Oxford 1944). Diplococcin, produced by *L. lactis* subsp. *cremoris*, is a 5,300 Da peptide that exerts a rapid bactericidal effect against *Lactococcus* strains. Cells treated with diplococcin immediately halt DNA and RNA synthesis and die without cell lysis.

by and primarily act on gram-positive bacteria. The term lantibiotics was introduced as an abbreviation for lanthionine-containing peptides and refers to the most prominent features of this group, which are the strong antimicrobial activity and the intramolecular rings formed by the thioether amino acids lanthionine (Lan) and 3-methyllanthionine (MeLan). In addition to these unusual amino acids, 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) also occur commonly in lantibiotics. These unusual amino acids are synthesized by posttranslational side-chain modifications of ribosomally produced precursor peptides (Klaenhammer 1993, de Vos and Kulpers 1995, Sahl *et al.* 1995). They can be broadly divided into two groups on the basis of their structures: type-A lantibiotics are elongated, amphiphilic peptides, while type-B lantibiotics are compact and globular. In the last decade there has been a marked increase in research interest in these peptides due both to the novel biosynthetic mechanisms by which they are produced, as well as to their potential applications (Sahl *et al.* 1995).

Among the bacteriocins produced by lactic acid bacteria, members of the antibiotic class are unique because of their efficacy and the established safety record of nisin. The prototype lantibiotic nisin enjoys recognition as the first LAB bacteriocin that has realized commercial applications in food processing and fermentation. It is the first bacteriocin granted GRAS (generally recognized as safe) status in the USA. Nisin is a 3,488 Da hydrophobic peptide consisting of 34 amino acid residues, five thioether crossbridges, and three dehydrated residues formed from

involves the addition of the sulfhydryl group of a neighboring Cys residue to the double bond of either Dha or Dhb, forming a covalent ring structure and yielding Lan or MeLan, respectively (Schnell *et al.* 1988, Jung 1991). This reaction is stereospecific in that, while the prepeptide sequence contains only L-serine and L-threonine residues, both Lan and MeLan are found in the meso-configuration (i.e. DL) with the Ser/Thr-derived 'half' in the D-configuration and the Cys-derived 'half' present as a L-isomer. Furthermore, an additional characteristic of the type-A lantibiotics is that the Cys-derived 'half' of Lan and MeLan is located on the C-terminal side of the diamino acid, while the Ser- or Thr-derived 'half' is located toward the N-terminus (Jung 1991, Sahl *et al.* 1995).

The reason for the presence of lanthionine in these particular peptides, in preference to the more commonly encountered (disulfide-linked) cystine residues, remains unclear. While these dehydroamino acids can remain stable within the peptide chain, their presence has been shown to cause failure of N-terminal amino acids sequencing by Edman degradation (Gross and Morrell 1971, Kellner *et al.* 1988, Jung 1991), posing difficulties in determinations of peptide primary structures. These studies have shown that when the dehydroamino acid becomes N-terminally located (such as occurs after cleavage of the preceding amino acid during sequencing), spontaneous oxidative deamination occurs, resulting in formation of a sequence-blocking pyruvyl group. Thus, during the structure determination of nisin, mild acid hydrolysis of the Dha33-Lys34 peptidyl bond results in the formation

interaction whereupon pores would form and/or reactions with sulfhydryl groups could occur. Perhaps the covalent interactions eliminate the need for a specific membrane receptor and serve as binding groups to orient nisin molecules into some active conformation needed for membrane insertion or stability. Operation of one or both mechanisms may explain why nisin is capable of exerting its antimicrobial action toward so many different types of bacteria.

2. *Lactobacillus* bacteriocins

The lactobacilli are most often cited for production of bacteriocins. Bacteriocins produced by *Lactobacillus* are generally active against closely related types which occupy similar ecological niches. An expanded host range has been noted recently for a number *Lactobacillus* bacteriocins which kill *E. faecalis*, *L. monocytogenes*, *C. botulinum*, *C. tyrobutyricum*, *S. aureus* (Klaenhammer 1993). Bacterial antagonism by lactobacilli was first demonstrated by Metchinkoff (1908). Since then many bacteriocins, more than 25, from *Lactobacillus* spp. have been isolated and characterized. Some of the reported lactobacilli bacteriocins are lactocidin from *Lb. acidophilus* (Vincent *et al.* 1959), acidolin from *Lb. acidophilus* 2181 (Hamden and Mikolajcik 1974), lactocin 27 from *Lb. helveticus* LP 27. (Upriti and Hindsill 1975), lactacin B from *Lb. acidophilus* (Barefoot and Klaenhammer 1983, 1984), bulgarican from *Lb. bulgaricus* (Reddy *et al.* 1983), helveticin J from *Lb. helveticus* 481 (Joerger and Klaenhammer 1986), lactacin F from *Lb. acidophilus*

by LAB. Production of inhibitory substances by *Pediococcus* species was first reported by Fleming *et al.* (1975). They found that *P. cerevisiae* FBB-61 inhibited the growth of *Lactobacillus plantarum* and other *Pediococcus* species. Later, Graham and McKay (1985) isolated a bacteriocin-like substance from *P. cerevisiae* FBB-63 and suggested that production of this bacteriocin was plasmid linked. In the same year, Daeshel and Klaenhammer (1985) reported pediocin A from *P. pentasaceus* FBB-61 and L 7230. Bhunia *et al.* (1987, 1988) isolated pediocin AcH produced by H. In 1987 Gonzales and Kunka reported pediocin PA-1 from *P. acidilactici* PAC 1.0. Hoover *et al.* (1988) also reported that several strains of *P. acidilactici* produced bacteriocins that inhibited growth of other bacteria. Reports of isolation of pediococcus bacteriocin from *Pediococcus* species include pediocin JD from *P. acidilactici* (Berry *et al.* 1989), pediocin SJ-1 from *P. acidilactici* (Schved *et al.* 1993), pediocin L50 from *P. acidilactici* (Cintas *et al.* 1995), pediocin PO2 from *P. acidilactici* (Coventry *et al.* 1995).

4. *Leuconostoc* bacteriocins

The first evidence for bacteriocin production in *Leuconostoc* was a report by Harding and Shaw (1990) that *Lc. gelidum* produced a heat-stable protein that inhibited *L. monocytogenes* and closely related LAB. However, the bacteriocin leuconocin S lost 50% of its activity after heating at only 60°C for 30 min (Lewus *et al.* 1992). Recent reports have now identified a number of bacteriocin producing

6. *Propionibacterium* bacteriocins

The propionibacteria are industrially significant due to their important contributions to the flavor, texture and eye formation in Swiss cheese. Two bacteriocins have been detected and characterized from this genus, jensenin G (Grinstead and Barefoot 1992) and propionicin PLG-1 (Lyons and Glatz 1991).

7. *Enterococcus* bacteriocins

Bacteriocin production by *Enterococcus faecium* has been known for many years (Kramer 1975). Notable examples include enterocin E1A, a plasmid-encoded bacteriocin (Kramer *et al.* 1983), and the hemolysin/bacteriocin activities associated with conjugative plasmids related to pADI (Ike *et al.* 1992, Gilmore 1991). Enterococci commonly produce bacteriocins active against *Listeria* and closely related bacteria. Examples for bacteriocins produced by *Enterococci* species are enterocin 226 NWC from *E. faecalis* (Villani *et al.* 1993), enterocin CRL35 from *E. faecium* CRL35 (Farias *et al.* 1996), enterocin 900 from *E. faecium* BFE900 (Franz *et al.* 1996), enterocin 4 from *E. faecalis* INIA 4 (Joosten *et al.* 1996), enterocin A from *E. faecium* (Aymerich *et al.* 1996), enterocin P from *E. faecium* P13 (Luis *et al.* 1997), enterocin B from *E. faecium* T136 (Casaus *et al.* 1997).

8. *Bifidobacteria* bacteriocins

Bifidobacteria are one of the predominant bacteria in the normal flora of the

E. Detection and assay of bacteriocin activity

Methods to detect and assay bacteriocin activity from LAB have been borrowed from prior studies with gram-negative bacteriocins, primarily the colicins (Mayr-Harting *et al.* 1972). Direct or deferred antagonism (i.e., either simultaneous growth of bacteriocin-producing and indicator cells or inhibition of indicator lawns by performed bacteriocin, respectively) are methods frequently used to detect bacteriocin activity. These methods may involve (1) spot-on-lawn test: spot serial dilutions of neutralized and filtered sterilized culture supernatants on indicator lawns (Mayr-Harting *et al.* 1972, Tagg *et al.* 1976); (2) agar well diffusion test: add neutralized and filtered sterilized culture supernatant into wells bored into an indicator lawn (Tagg and McGiven 1971); (3) Sandwich overlay: plate bacterial dilution, cover with sterile medium, incubate until colonies are visible, and overlay with an indicator lawn (Mayr-Harting *et al.* 1972); (4) Flip plate method: grow colonies on agar, "flip" agar onto inside of petri plate cover, and overlay with indicator organism (Kekessy and Piguet 1970); and (5) Lutri-plates (special two-sided agar plates): grow colonies on one side, and overlay other side with indicator lawn (Lutri-Plate, Inc., Starksville, Mississippi).

Although various methods have been devised to measure bacteriocin activity, the most common method used is the 'critical dilution method' or 'agar spot method' (Mayr-Harting *et al.* 1972). Briefly, culture supernatant (bacteriocin) and dilutions thereof are spotted on an indicator lawn and activity is quantitated subjectively in

exclusion, can simultaneously concentrate bacteriocins and eliminate undesirable proteins. Precipitation of bacteriocins from culture supernatants has been accomplished by salt fractionation (e.g., ammonium sulfate), acid precipitation (Hastings *et al.* 1991), or organic solvent precipitation (Upreti and Hinsdill 1973). These procedures are well suited for an initial step in the purification process because they accommodate large volumes of starting materials. Other methods that have proven to simultaneously concentrate and purify bacteriocins include freeze-drying, dialysis and ultrafiltration.

Yang *et al.* (1992) and Daba *et al.* (1994) have reported a simple method of the purification of pediocin involving adsorption of the bacteriocin onto producer cells at pH 5.5 followed by extraction at pH 2.0. de Vast and Vandamme (1994) provide a brief review of industrial approaches to the recovery of nisin, including adsorption of nisin onto silica-type adsorbents (Baranova *et al.* 1987, 1984 and 1983). Daeschel *et al.* (1992) and Bower *et al.* (1995) have adsorbed purified nisin in phosphate buffer solution to hydrophilic and hydrophobic silicon surfaces as an approach to using nisin as a surface-active in situ sanitizer. Coventry *et al.* (1996) partially purified nisin, pediocin PO2, brevicin 286, and piscicolin 126 by adsorption onto Micro-Cel (a food grade diatomite calcium silicate anticaking agent) and subsequently desorption. Seventy five percent of the bacteriocin activity was desorbed by using 1% sodium dodecyl sulfate (SDS). Higher levels (up to 100%) of desorption were achieved by repeated elution or by an increase in surfactant

purification protocol led to a 107,506-fold increase in the specific activity and 11.4% recovery of lacticin 481. Lactocin S from *Lb. sake* L45 was purified to homogeneity by ion exchange, hydrophobic interaction, reverse-phase chromatography, and gel filtration and purification resulted in approximately a 40,000 increase in the specific activity and 3% yield of lactocin S (Mortvedt *et al.* 1991). Bavaricin MN from *Lb. sake* was purified by ammonium sulfate precipitation, anion exchange and cation exchange chromatography with an 135-fold increase in purity and a 10.7% recovery (Kaiser and Montville 1996).

G. Antimicrobial spectrum

LAB bacteriocins are inhibitory to specific strains of gram-positive bacteria, strains from within the species or genus, or strains from different genera. Under normal conditions gram-negative bacteria are resistant to these bacteriocins. An interesting discovery is that gram-negative bacteria can be sensitized to nisin by exposure to chelating agents, sublethal heat, and to freezing (Blackburn *et al.* 1989, Stevens *et al.* 1991, Stevens *et al.* 1992). It is recognized that gram-negative cell walls are far less permeable than gram-positive cells to various antimicrobial agents such as anionic detergents, antibiotics, lysozyme, and to various bacteriocins including nisin. The gram-negative bacteria can be rendered sensitive by conversion to spheroplasts. Treatment of the gram-negative bacteria with EDTA and other chelating agents makes them sensitive to agents they normally resist. The exact mode

are amphiphilic cationic peptides showing alpha-helix, β -sheet, or in the case of lantibiotics, screwlike secondary structures (Montville and Winkowski 1997). Kaiser (1987) noted that secondary structures play a significant role in the biological activity of membrane-active peptides in which amphiphilic alpha-helices or β -sheets form two faces, one hydrophilic and one hydrophobic. Based on the amphiphilic characteristics of bacteriocins, there are at least two different mechanisms that may explain their membrane-permeabilization action (Fig 2.1) (Montville and Winkowski 1997). Bacteriocins may act as a multiple-step poration complex in which bacteriocin monomers bind, insert, and oligomerize in the cytoplasmic membrane to form a pore with the hydrophilic residues facing inward and the hydrophobic ones facing outwards (barrel-stave mechanism) (Nissen-Mayer *et al.* 1992, Kok *et al.* 1993, Ojcius and Young 1991). Pore formation in the cytoplasmic membrane is a common mechanism of action of those LAB bacteriocins for which the mode of action has been determined. Nisin and a number of the Class II peptides (lactococcin A, B, G, lacticin F, pediocin JD, pediocin AcH) have been shown to be membrane active peptides that destroy integrity via formation of membrane channels. Loss of membrane integrity generally leads to ion leakage, loss of proton motive force (PMF), and cell death. This increased flux of compounds across the membrane rapidly dissipates chemical and electrical gradients across the membrane. The PMF, an electrochemical gradient which serves as the major driving force for many vital energy dependent processes, is dissipated within minutes of bacteriocin addition

(Bruno *et al.* 1992, Bruno and Montville 1993). The chemical gradient of H^+ of the PMF is dissipated faster than the electrical gradient, probably as a result of rapid influx of protons into the cytoplasm. Bacteriocin-treated cells have decreased intracellular ATP levels. The loss of ATP can be caused by ATP efflux or ATP hydrolysis (Abee *et al.* 1994, Winkowski *et al.* 1994). Ultimately, these changes in permeability render the cell unable to protect its cytoplasm from the environment. This leads to cell inhibition and possibly death. Alternatively, bacteriocins may disrupt the membrane integrity by a detergent-like membrane solubilization action. A generalized solubilization mechanism would result in an all-or-none lysis of the cells, with a sudden collapse of the bioenergetic parameters and no saturation kinetics (Montville and Winkowski 1997). The lantibiotic bacteriocins act on energized membranes and do not require receptor proteins to exert their action (Gao *et al.* 1991, Kordel and Sahl 1986). Alternatively, most non-lantibiotic bacteriocins act on energized membranes but may require a membrane receptor protein (Chikindas *et al.* 1993, van Belkum *et al.* 1991, Venema *et al.* 1993). The activity of some bacteriocins, including lactacin F and lactococcin M and G, occurs through action of two peptides. These two-component bacteriocins are postulated to form a pore in the cytoplasmic membrane (Klaenhammer 1993, Kok *et al.* 1993).

2. Mechanism of action against spores

Most information about the mechanisms of bacteriocin action against bacterial

diplococcin (Davey and Richardson 1981) lost 75% of its activity after heating at 100°C for 1 min, whereas partially purified bacteriocin preparations were resistant to heat at low pH. Most bacteriocins are active at wide pH range, more in acidic pH. At pH 2.5 in dilute HCl (0.02 N) solutions the activity of nisin remains stable for a long time, especially under refrigeration. This could be due to higher solubility at acidic pH. The activity of pediocin AcH was retained after exposure to pHs ranging from 2.5 to 9 at 25°C for 24 h or at 93°C for 15 min, but was lost at pH 10. At pH 12 even a 2 h exposure at 25°C resulted in the complete loss of activity (Bhunia *et al.* 1988). At high pH, the molecules probably undergo irreversible changes either in their configuration or in the amino acid sequence that is necessary for their antimicrobial activity. Most bacteriocins are stable over several months during frozen and refrigeration storage and after drying. Purified dry nisin powder retains its potency indefinitely under refrigeration storage. However, in food systems, even at refrigeration temperature, the activity is gradually lost (Ray 1992).

K. Solubility

Nisin is insoluble in nonpolar solvents. It is soluble in aqueous media, and the solubility is pH dependent. Hurst (1981) reported that at pH 2.5 the solubility of nisin is only 12% and that this is reduced to only 4% at pH 5.0 and almost zero at neutral and alkaline pH. Pediocin AcH preparations stored either as liquid, concentrate (5 to 10 times) or in freeze-dried form retained the activity without any

further open the way to directed in vitro modification of their antibacterial spectra (Jack *et al.* 1995).

1. Location of bacteriocin genes

The genes encoding bacteriocin production and immunity are located on plasmids, on the chromosome, or both. To show that bacteriocin production is plasmid mediated, (i) phenotypic evidence (e.g. spontaneous loss of the Bac⁺ phenotype), (ii) physical evidence (e.g., the isolation of a plasmid from the Bac⁺ strain which is missing in Bac⁻ strains) and (iii) genetic confirmation (e.g., the introduction of the plasmid isolated from the Bac⁺ strains into a Bac⁻ strain reestablishes the Bac⁺ phenotype) must be provided (Montville and Winkowski 1997).

The production of lactococcins (Kok *et al.* 1993, Rince *et al.* 1994), diplococcin (Davey 1984), pediocin A (Daeshel and Klaenhammer 1985), pediocin PA-1 (Gonzales and Kunka 1987), pediocin AcH (Ray *et al.* 1989), carnobacteriocins A and B (Ahn and Stiles 1992), pediocin SJ-1 (Schved *et al.* 1993), and sakacin A (Axelson *et al.* 1995) were determined to be plasmid mediated. Plasmid-curing studies and comparison of the plasmid profiles of both bacteriocin-producing and -non-producing strains have provided further evidence for a plasmid location for a number of these bacteriocins. The plasmids isolated vary greatly in size, ranging from 6.0 kb for the pediocin SJ-1-associated plasmid (Schved *et al.*

1991, Rauch and de Vos 1992).

2. Genetic organization of bacteriocins

The DNA sequence or amino acid composition of most bacteriocins is unknown. However, a general picture of the genetic organization of bacteriocin genes is emerging. The structural genes for many bacteriocins appear to be located in an operon-like structure (Klaenhammer 1993). The organization of a generic operon is shown in Fig 2.2 (Jack et al. 1995, Montville and Winkowski 1997). The operon is organized so that it clusters the genes containing the structural information together with an array of genes involved in immunity, maturation, processing, and export of the bacteriocin molecules as well as genes encoding products involved in the regulation of bacteriocin biosynthesis. Not all operons contain all of the genes depicted, nor is the organization of the genes in each operon identical, but they share many similarities (Montville and Winkowski 1997).

The structural gene usually codes for a prepeptide which is the precursor of the mature bacteriocin preceded by an N-terminal extension or "leader sequence". The secondary structure of this N-terminal extension is predicted to be an alpha-helix that is cleaved during the maturation or export process. Class II bacteriocins encode a prepeptide containing a consensus sequence Gly²-Gly¹ at the active site and present strong homology in their hydrophobicity profile. The role of the N-terminal extension is still unknown. It may be required for recognition by the maturation or

export machinery. In this regard, most bacteriocin leader sequences do not exhibit characteristics of sec-dependent export proteins (Montville and Winkowski 1997). Rather, they may be exported by a sec-independent pathway involving a transport protein encoded within the operon. Alternatively, the N-terminal extension may play a role in neutralizing bacteriocin activity within the cell to protect the producer strain. The structural genes of several LAB bacteriocins have been described and reviewed Jack *et al.* 1995, Klaenhammer 1993, Sahl *et al.* 1995, Montville and Winkowski 1997). These include the structural genes coding for nisin (*nisA*), pediocin PA-1 (*pedA*), lactococcin A and B (*lcnA*, *lcnB*), sakacin A (*sakA*), leucine A (*leuA*), lactacin F (*lafA*, *lafX*) and lactococcin M (*lcnM*, *lcnN*).

The immunity gene confers on Bac⁺ cells immunity to their own bacteriocin. Immunity is specific and seems to be coordinated with bacteriocin production. Several immunity genes have been sequenced, and their ability to confer immunity to the producer strains has been proven (Holo *et al.* 1991, Kuipers *et al.* 1993, Marug *et al.* 1992, van Belkum *et al.* 1991). In the case of nisin, the *nisI* gene product is predicted to be an extracellular lipoprotein which, by anchoring to the membrane through its lipid moiety, confers immunity to the producer cells. Additional genes (*nisE*, *nisF* and *nisG*) thought to be involved in immunity have been described in the nisin gene cluster (Siegers and Entian 1995).

The processing and export genes encode at least two proteins that insure that the mature bacteriocin is formed and exported from the cytoplasm. One of these

located in the membrane (R_1) senses an external signal and transduces it to the cell's interior by phosphorylating a second, cytoplasmic protein (R_2) referred to as a "response regulator". The phosphorylation of the response regulator activates the transcription of the bacteriocins' biosynthetic genes. Such regulatory genes occur in the nisin operon (*nisKR*) (Engelke *et al.* 1994, van der Meer 1993) and have been postulated for sakacin A operon (*sakKR*) (Axelson and Holck 1995), the carnobacteriocins Bm1 and B2 (Quadri *et al.* 1995), and the plantaricin operon (*plnBCD*) (Diep *et al.* 1994). The bacteriocin molecule itself may be the signal molecule, playing an autoinductive role in a cell-to-cell communication pathway (de Vos 1995).

N. Factors influencing bacteriocin production and activity

Production of bacteriocins is dependent on the composition and pH of the medium and on the phase of growth of the producer.

a) **Composition of medium:** Investigating 15 bacteriocin-producing *Lactococcus* strains, Geis *et al.* (1983) compared five different media for bacteriocin production and found many differences among these five media. Pucci *et al.* (1988) report that maximum production of pediocin PA-1 was achieved by addition of 2% yeast extract to MRS medium. Some components of the medium used seemed to protect bacteriocins. For instance, addition of M17 broth stabilized diplococcin (Terzaghi and Sandine 1975) that was extremely unstable in its purified form. For some

when pH rises from 4.6 to 7.0. Production of lactacin F (Muriana and Klaenhammer 1987) could be maximized in MRS broth cultures maintained at pH 7.0.

d) Incubation: Other factors affecting growth and production of bacteriocins are the length of incubation and incubation temperature. The optimum temperature for growth is normally the incubation temperature necessary for observation of the effects of bacteriocin activity. For lactic acid bacteria this is usually a temperature of 32°C. As noted by Tagg *et al.* (1976), bacteriocins may be cell surface components that are produced in excess under certain conditions and are released from cells with time. The maximal time in the growth cycle for production of bacteriocins is dependent on the type of bacteria and can occur from log phase through early stationary.

O. Application of bacteriocins in food system

There are several distinct applications for the use of bacteriocins in foods. Bacteriocins can be added directly to the food to inhibit spoilage or pathogenic organisms. Only nisin is commercially available for addition in pure form, but the efficacy of pediocin addition has also been demonstrated. Spoilage or pathogenic organisms can also be inhibited by bacteriocinogenic cultures added to the food. Bacteriocinogenic cultures can be added to non-fermented foods to improve safety and quality. Another application of bacteriocin is addition to the packaging materials (edible films). Bacteriocins and bacteriocin producing LAB can also be used to

al. 1986b).

While inactive against bacterial spores, pediocins inhibit *L. monocytogenes* vegetative cells. European patents cover the use of pediocin PA-1 in the form of a dried powder or culture liquid to extend the shelf life of salads and salad dressing (Gonzales 1988) and as an antilisterial agent in foods such as cream, cottage cheese, meats, and salads (Pucci *et al.* 1988, Vandenberg *et al.* 1989, Golf *et al.* 1997).

Pediocins are more effective than nisin when used in meat products and are even more effective in dairy products with lower pH values. Dipping meat in 5,000 AU of crude pediocin PA-1 per ml decreases the viability of attached *L. monocytogenes* 100- to 1000-fold. Pretreating meat with pediocin reduces subsequent *L. monocytogenes* attachment (Nielsen *et al.* 1990). Pediocin AcH at 1,350-2,400 AU/ml reduces listeria in ground beef, sausage, chicken and other products by between 1 and 7 log cycles (Degnan *et al.* 1992, Golf *et al.* 1996). Pediocin AcH is more effective at 4°C than 25°C against *L. monocytogenes* in hot dog exudate. Emulsifiers such as Tween 80, or the entrapment of the pediocin in multilamellar vesicles, increases pediocin effectiveness in fatty foods (Degnan *et al.* 1993, Degnan and Luchansky 1992, Degnan *et al.* 1992). In most cases, the bacteriocin rapidly reduces listeria viability and delays growth of the survivors.

P. Factors effecting the efficacy of bacteriocins in foods

The effective use of bacteriocins in foods requires consideration of many

decrease in nisin activity seen in red wines may be the result of nisin interactions with phenolic compounds that are absent in white wines.

Since bacteriocins are proteins they are susceptible to enzymatic degradation. This factor is important in food applications if the active enzymes are present either as the result of the presence of the proteolytic microorganisms or as native constituents of the food that have not been thermally processed.

Temperature is the major determinant of bacteriocin's inhibitory action. Nisin is much less effective at elevated temperatures than at refrigeration temperatures against *C. botulinum* 56A spores in a model food system (Rogers and Montville 1994). Eventually, growth in this system occurs when nisin activity falls below some threshold level. The threshold nisin levels are lower at decreasing temperatures. For example, botulinal growth occurred when residual nisin concentrations fell below 154 IU/ml at 35°C, but not until nisin levels were <12 IU/ml at 15°C. Nisin also inhibits listeria and staphylococcal vegetative cells better at refrigerated temperatures than at elevated temperatures (Chung *et al.* 1989).

The antimicrobial spectra of activities of bacteriocins from lactic acid bacteria are limited to gram-positive bacteria. This group includes many serious foodborne pathogens. For example, some *Clostridium*, *Staphylococcus* and *Listeria* species are sensitive to nisin (Harris *et al.* 1989), pediocin AcH (Bhunia *et al.* 1988), and pediocin A (Daeschel and Klaenhammer 1985, Harris *et al.* 1989). Since, bacteriocins such as helveticin J and lactocin B inhibit only closely related species

CHAPTER III
CHARACTERIZATION OF BIFIDOCIN B, A BACTERIOCIN
PRODUCED BY *Bifidobacterium bifidum* NCFB 1454

INTRODUCTION

Bacteriocins are bacteria-produced antimicrobial proteinaceous compounds that have general bactericidal activity, often towards bacteria that are closely related to the bacteriocin-producing species (Tagg *et al.* 1976). Also, some bacteriocins inhibit the growth of pathogens and spoilage organisms during food processing and food fermentation. Therefore, bacteriocins have found application as food biopreservatives. Bacteriocins produced by starter cultures are excellent candidates to improve the quality and safety of various fermented foods. In the USA, only nisin produced by *Lactococcus lactis* spp. *lactis* is permitted as a food preservative (FDA 1988).

Bifidobacteria are one of the predominant bacteria in the normal flora of the human intestinal tract. They constitute up to 25% of the total population in adults and 95% in newborns. Bifidobacteria are thought to exert a number of advantageous effects towards the health of the host in both the infant and adult gut. Claimed benefits are improvements in protein metabolism and lactose digestibility and suppression of putrefactive and pathogenic bacteria by the production of lactic and acetic acid, and bacteriocins (Anand *et al.* 1985, Kawase 1982, Rasic 1983).

MATERIALS AND METHODS

Bacterial cultures and media

Bifidobacterium bifidum ATCC 11863, ATCC 29521, NCFB 1453, 1454 and 1455 were tested. Only *B. bifidum* NCFB 1454 was shown to excrete a bacteriocin into the culture medium. The bacteriocin produced by *B. bifidum* NCFB 1454 was termed bifidocin B. *Lactobacillus plantarum* NCDO 955 was used as the indicator strain for antimicrobial assays. For production of antimicrobial agents from *B. bifidum* NCFB 1454, MRS broth with 0.05% cysteine (MRS-C) was used. *B. bifidum* NCFB 1454 was stored in MRS-C with 20% glycerol at -70°C freezer.

Preparation of crude bifidocin B

B. bifidum NCFB 1454 was inoculated into MRS-C broth at 1%. After 16 h incubation at 37°C, culture filtrate of *B. bifidum* was collected by centrifugation at 22,100 x g for 30 min. Filtrate was sterilized by filtration (0.45 and 0.22-μm pore size) and precipitated with 70% ammonium sulfate. Precipitate was dissolved in deionized water and exhaustively dialyzed through 1,000 molecular-weight-cutoff dialysis membrane against deionized water for 48 h. The resulting solution was designated crude bacteriocin.

Partial purification of bifidocin B by adsorption onto producer cells

B. bifidum NCFB 1454 was grown in MRS-C broth at 37°C for 16 h.

seeded with 30 ml of tempered MRS soft agar (MRS broth plus 0.8% agar) containing *L. plantarum* NCDO 955 and incubated at 30°C for 24 h.

Antimicrobial activity of bifidocin B

Antimicrobial activity assay of bacteriocin was performed by using the agar spot method (Fleming *et al.* 1975). Bacteriocin preparations were serially, two-fold diluted (1:2, 1:4, 1:8, etc.) and 10 µl from each dilution was spotted on MRS agar (MRS broth containing 1.5 % agar) pre-poured plates which were overlaid with about 10⁸ cell of *L. plantarum* NCDO 955 in 5 ml of soft MRS agar (MRS broth containing 0.8 % agar). These plates were incubated at 30°C for 24 h and examined for the presence of 2 mm or larger clear zones of inhibition. Arbitrary units per ml (AU/ml) were calculated as the reciprocal of the highest dilution, in a 10 µl amount, which gave a clear zone of growth inhibition on a lawn of sensitive cells (Bhunia *et al.* 1991, Pucci *et al.* 1988).

The agar spot method was used to determine the antimicrobial activity of crude bacteriocins against several food spoilage bacteria and foodborne pathogens as indicator strains (Fleming *et al.* 1975). Ten µl of the crude bacteriocin were spotted onto MRS agar (MRS broth plus 1.5% agar) and BHI agar (BHI plus 1.5% agar) pre-poured plates which were overlaid with indicator organisms in 5 ml of soft MRS or BHI agar containing 0.8% agar. MRS and BHI agar media were used for lactic acid bacteria or other bacteria, respectively.

at a concentration of 50 mg/ml. Samples from these preparations were adjusted with sterile 5 M NaOH or 5 M HCl to different pH levels between 2 and 12 and the final bacteriocin concentration was adjusted to yield 10 mg/ml. Samples were then held as follows : (i) 2 h at 25°C, (ii) 24 h at 25°C, and (iii) 20 min at 95°C. Each sample was adjusted to pH 7.0 with sterile 5 M phosphate buffer and assayed as above for activity using the appropriate controls (Bhunia *et al.* 1988).

Freeze-dried crude bacteriocins were dissolved in the organic solvents formaldehyde, chloroform, acetone, 2-propanol, ethyl alcohol, hexane, iso-butanol, and ethyl ether at a concentration of 10 mg/ml. The samples were incubated at 25°C for 1 h. Solvents were evaporated in a centrifugal concentrator. Dried samples were reconstituted with sterile deionized water to a concentration of 10 mg/ml and assayed for antimicrobial activity (Bhunia *et al.* 1988).

RESULTS AND DISCUSSION

Activities of crude bifidocin B in the cell-free culture supernatant fluid and after dialysis were 3,200 and 58,200 AU/ml, respectively. Antimicrobial activity increased 18-fold after dialysis. Bifidocin B formed an insoluble precipitate inside the dialysis bag.

Adsorption of bifidocin B onto heat-killed *B. bifidum* NCFB 1454 producer cells was strongly influenced by the pH of the suspending environment. Bifidocin B showed 100% adsorption to cells at pH 5.0 and 6.0 whereas at pH values below

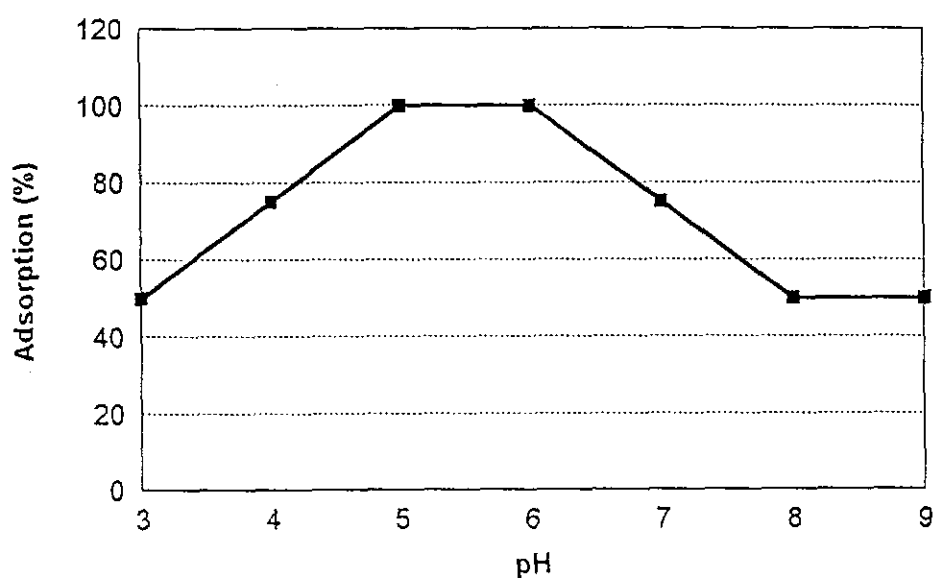


Fig. 3.1. Effect of pH on adsorption of bifidocin B to its producer cells.

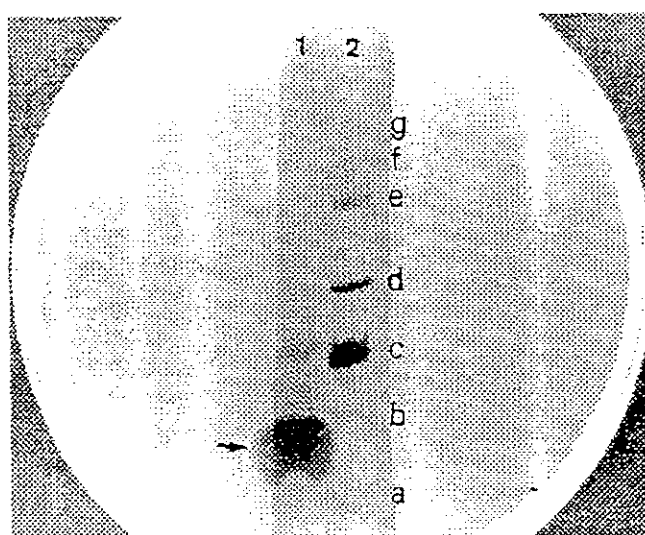


Fig. 3.2. The gel overlaid with *L. plantarum* to determine which band corresponds to the antimicrobial activity. Lane 1 shows the zone of growth inhibition (see arrow) corresponded with about 3.3 kDa; other bands did not show antimicrobial activity. Lane 2 contains low molecular weight standard (Bio-Rad): a, 1,423; b, 3496; c, 6,512; d, 14,437; e, 16,950; f, 26,625 Da.

the middle of the logarithmic (Fig. 3.3). This suggested that the production of bifidocin B was dependent on the cell number under these growth conditions. During extended incubation time at the stationary phase, activity decreased considerably. Bacteriocin titers at 18 or 72 h decreased by 50 and 75 %, respectively. These decreases may be due to the activity of endogenous extracellular proteases induced during this growth phase. The results suggest that bifidocin B is a primary metabolite which is produced at maximum levels at the mid-log phase. Similar profiles for production of several other bacteriocins produced by lactic acid bacteria have been reported previously (Atrih *et al.* 1993, Pucci *et al.* 1988).

Bifidocin B preparations initially at 51,200 AU/ml retained much of their activity after high temperature treatments. Activity of bifidocin B was undiminished after 15 min at 90°C; however, bacteriocin titers were decreased by about 25, 50 or 50%, respectively, when crude bifidocin B was heated at 90°C for 30 and 60 min, or autoclaved at 121°C for 15 min. Bifidocin B preparations at an initial activity of 51,200 AU/ml retained full activity when stored at -20 or -70°C for 1 or 3 months; samples stored at 4°C showed a decline from 51,200 to 30,000 AU/ml from 1 to 3 months (Fig. 3.4).

Bifidocin B lost its antimicrobial activity completely when treated with trypsin, α -chymotrypsin, papain, proteases, or pepsin (Table 3.2). Untreated samples, or samples containing heat-inactivated enzymes, showed no loss of activity. These results indicated that the inhibitory agent was a protein and therefore, a

bacteriocin. The inhibitory activity was not inactivated by lysozyme, ribonuclease A or glucose oxidase (Table 3.2). Also, treatment with lipase, amylase, dextranase, cellulase or organic solvents did not cause any apparent loss of bifidocin B activity, indicating that lipid and carbohydrate moieties were absent and not responsible for inhibitory action (Table 3.2 and 3.3). Antimicrobial activity of the preparation was not due to hydrogen peroxide or acidity since bifidocin B did not lose its activity after treatment with catalase or peroxidase or adjustment of pH to 7.0 with 5 M NaOH.

Bifidocin B retained biological activity after exposure to pH 2-12 (Fig. 3.5). When bifidocin B was kept at 25°C for 2 h, it was stable at pH levels between 2-12 (Fig. 3.5). However, its activity was lost at pH 10, 11 and 12 when samples were held at 25°C for 24 h or heated for 20 min at 90°C. Bifidocin B was relatively more stable at acidic than basic conditions. The activity of bifidocin B was decreased 50% at pH 3 or below and was decreased 50-100% at pH 8 or above.

The existence of a large number of bacteriocins is now well recognized (Anand *et al.* 1984, Bhunia *et al.* 1988, Brink *et al.* 1994, Fleming *et al.* 1975, Gonzalez *et al.* 1994, Holck *et al.* 1992, Itoh *et al.* 1995, Keppler *et al.* 1994). Bacteriocins generally inhibit only closely related bacteria whereas the bacteriocins nisin and pediocin inhibit strains of several different gram-positive genera. Bifidocin B has a wide antimicrobial spectrum, inhibiting the growth of more than three genera, namely *Listeria*, *Enterococcus*, *Bacillus*, *Lactobacillus*, *Leuconostoc*, and

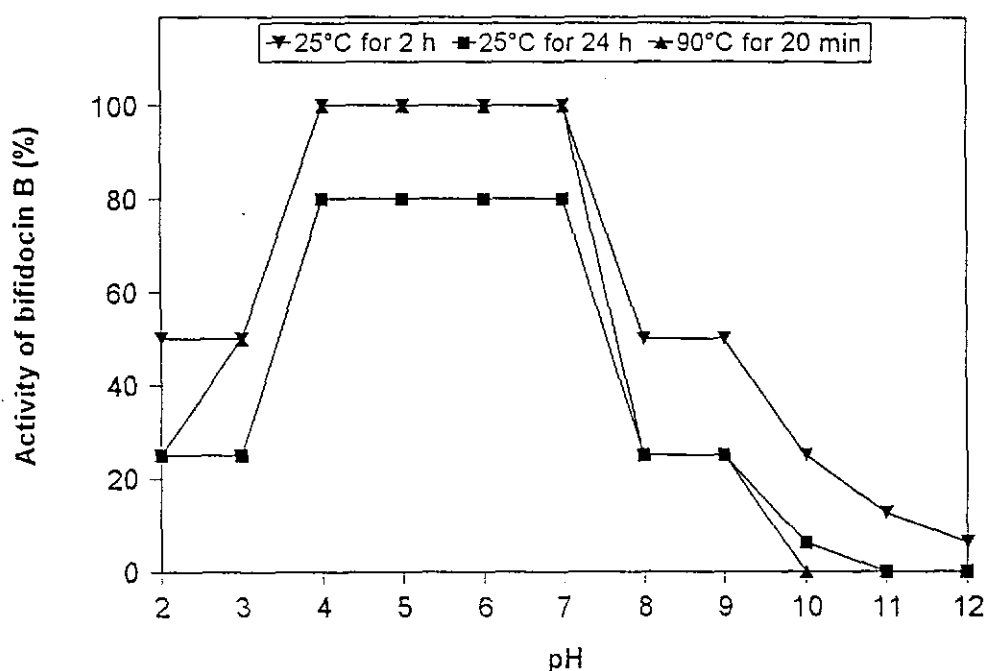


Fig. 3.5. Effect of pH on the activity of bifidocin B.

Pediococcus species. In contrast to previous reports about bacteriocins from *Bifidobacterium* strains, this is the first report a bifidocin B bacteriocin showing inhibition toward *Listeria* and *Enterococcus* species. Tests are underway to determine whether bifidocin B is useful as a biopreservative to prevent the growth of foodborne pathogens and food spoilage bacteria in certain food products.

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conserved N-terminal domain, -Tyr-Gly-Asn-Gly-Val-Xaa-Cys-, and a nonconserved hydrophobic C-terminal peptide of variable length. Another distinctive and important feature of these bacteriocins is their ability to kill listeria.

Yildirim and Johnson (1998a) have described a bacteriocin, termed bifidocin B, from *Bifidobacterium bifidum* NCFB 1454. Bifidocin B inhibits the growth of selected species of the genera *Listeria*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. It is sensitive to proteolytic enzymes but resistant to pH (2-10), heat and organic solvents. Bifidocin B is produced maximally during mid-log growth phase.

To use bacteriocins in the most effective ways, it is important to purify the active compounds and determine their mode of inhibitory action against food spoilage and food borne bacteria. The objectives of this study were to purify the protein, and determine its amino acid sequence and mode of action, and to determine whether the gene that encodes bifidocin B is located on a plasmid or within chromosomal DNA.

MATERIALS AND METHODS

Bacterial culture and media

For production of bifidocin B, *B. bifidum* NCFB 1454 was cultured in de Mann Rogosa Sharpe (MRS) broth with 0.05% cysteine (MRS-C). *B. bifidum* NCFB 1454 was stored in MRS-C with 20% glycerol at -70°C.

adsorption and desorption procedures.

Cation-exchange chromatography (CMC)

Crude bifidocin B prepared using Micro-Cel (15 ml) was applied to Whatman carboxymethyl cellulose column CM-52 equilibrated previously with sodium phosphate buffer (50 mM, pH 6.6). The column (30 cm x 2.5 cm) was then washed with the same buffer, followed by a 500 ml linear NaCl gradient (0-1 M) in sodium phosphate buffer. Fractions of 5 ml were collected at a flow rate of 1.5 ml/min, and monitored for absorption at 280 nm and for inhibitory activity against the indicator strain, *L. plantarum*. Fractions containing inhibitory activity were pooled, dialyzed against distilled water and freeze dried.

Purity of bifidocin B was analyzed by 16.5 % tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Bhunja and Johnson 1992, Schagger and von Jagow 1987).

Estimation of protein concentration

The protein content of the samples was determined by the method of Lowry *et al.* (1951) with BSA (bovine serum albumin) (Sigma) as standard.

Cell wall preparation

Cells of *L. plantarum* grown in 200 ml of MRS broth at 30°C for 18-20 h were

at 25°C for 30 min and centrifuged. The supernatant fluids were assayed for unbound bacteriocin activity.

For the effect of temperature, cell suspensions of *L. plantarum* in phosphate buffer (5 mM, pH 6.6) were held at different temperatures (0, 10, 25, 50 or 85°C). Each sample received bifidocin B at the level of 3,200 AU/ml and then the samples were incubated at each respective temperatures for 30 min. After centrifugation, the supernatant fluids were assayed for unbound bifidocin B.

To examine the effect of time on adsorption of bifidocin B, *L. plantarum* cells suspended in phosphate buffer were exposed to crude bacteriocins (5,120 AU) for 0.5, 5, 10, 20, 30 or 60 min. At each time interval, one portion of each sample was centrifuged and unbound bifidocin B activities were determined in the supernatants. The other portion of each sample was incubated at 37°C for 4 h and their absorbance values were periodically measured at 600 nm in a Beckman DU-50 spectrophotometer.

Effect of NaCl and sodium phosphate on adsorption of bifidocin B to *L. plantarum* NCDO 955

L. plantarum cells grown at 30°C for 18-20 h were harvested, washed and resuspended in sterile salt solutions (0-500 mM NaCl) or in phosphate buffer (0-500 mM sodium phosphate, pH 6.6). After addition of crude bifidocin B (1,280 AU/ml), the samples were incubated at 37°C for 1 h. A portion of each suspension was

Effect of detergents, solvents and enzymes on adsorption of bifidocin B to whole cells and cell walls of *L. plantarum* NCDO 955

The cells of *L. plantarum* were suspended in 1 % sodium dodecyl sulfate (SDS), 2 % Triton X-100 and 4 M guanidine-HCl and incubated at 25°C for 40 min. After the samples were centrifuged, the pellets were washed three times with phosphate buffer (5 mM, pH 5.5). They were resuspended to the original volume in the same buffer, mixed with bacteriocins and assayed for bacteriocin activity. Controls were untreated preparations.

Similarly, cells of *L. plantarum* were suspended in organic solvents such as acetone, methanol, hexane, butanol, ethanol or isopropanol (80% v/v) and incubated at 25°C for 40 min. After the solvents were removed by centrifugation and air drying, the dry pellets were resuspended in phosphate buffer (5 mM, pH 5.5), mixed with bacteriocins and assayed to determine the amount of bound bacteriocins. Controls were cells untreated with detergents and solvents.

Cell wall preparations were suspended in a mixture of methanol:chloroform (40:40, v/v) and incubated at 25°C for 40 min. The solvents were removed by air drying, and the pellets were resuspended in either a cold (4°C) or hot (93°C) 20% trichloroacetic acid solution for 15 min (Bhunja et al. 1991, Wicken and Know 1975). After centrifugation, the pellets were washed and resuspended in phosphate buffer (5 mM, pH 5.5). Crude bifidocin B was added into these preparations and they were incubated at 25°C for 30 min. After centrifugation, the bacteriocin

5 mM phosphate buffer (pH 6.6). Crude bifidocin B at different levels (0, 640, 1,280, 2,560 and 5,120 AU/ml) was added and the cells were incubated at 30°C. Samples (0.1 ml) were withdrawn at 0, 0.5, 1.0, 2.0 and 4.0 h, serially diluted, and plated on MRS agar. The plates were incubated at 30°C for 48 h and the colony forming units were counted.

Effect of bifidocin B on growing cells of *Listeria monocytogenes*

An actively growing *Listeria monocytogenes* ATCC 19113 was inoculated into tempered fresh BHI broth at 37°C until their absorbance reached about 0.25 at 600 nm. Crude bifidocin B (1,280 AU/ml) was added after 60 min. Samples were withdrawn every 30 min during the subsequent incubation period (4 h), cultures absorbances were read at 600 nm and cfu/ml were determined. The control was *L. monocytogenes* ATCC 19113 without bifidocin B added.

Effect of treatment of cells with bifidocin B on the release of cytoplasmic materials in the environment

L. plantarum cell suspensions in 5 ml of phosphate buffer (5 mM, pH 5.5) was mixed with pure bifidocin B (640 AU/ml). The controls were cells without bifidocin B and buffer with bifidocin B but no cells. After the samples were incubated at 37°C for 45 min, they were centrifuged and filtered through 0.22 µm pore size sterile membranes (Millipore). The UV- absorbances of the filtrates was

B band on membrane was excised and submitted for NH₂-terminal amino acid sequencing in a Applied Biosystem Model 473A Sequinator. Data management was achieved with Biosystems Model 610A Control Data Analysis Module (Applied Biosystems Inc., Culver City, CA, USA).

MIC estimation and plasmid curing

Minimal inhibitory concentration (MIC) value was determined by using different concentrations of acriflavine in MRS-C broth (0-40 µg/ml). Each MRS-C broth tube containing different levels of acriflavine received about 10⁶ cells of *B. bifidum* and was incubated at 37°C for 24 h. The MIC value was determined as the lowest concentration of acriflavine that prevented the appearance of turbidity (Schved *et al.* 1993).

To induce the curing of the plasmid, *B. bifidum* was grown on MRS-C broth containing 20 or 25 µg of acriflavine per ml. A sample from the culture was diluted and spread onto MRS-C agar containing 20 or 25 µg of acriflavine per ml and the plates were incubated at 37°C under anaerobic conditions by using gas pack. Five hundred colonies were picked from random stock cultures and then checked for plasmid content and bacteriocin production.

Plasmid DNA isolation

Plasmid DNA isolation was achieved using the method of Frere (Frere 1994)

Southern blot hybridization

The DNA was electrophoresed through a 0.8% agarose gel and transferred to a Nytran Plus membrane (Schleicher & Schuell). The probe, ZY-1, was synthesized to correspond to the first 8 amino acids of the bacteriocin, as determined by amino acid analysis and was as follows: 5' AAA TAC TAC GGT AAT GGG GTT ACT 3'. It was radioactively labeled at the 5' end using [γ - ^{32}P]-ATP and T_4 polynucleotide kinase. The membrane was prehybridized in 2 x SSC (saline-sodium citrate, 0.3 M sodium citrate, 3 M NaCl, pH 7.0), 5 x Denhardt's, 0.2% SDS and 50 μg Calf thymus DNA for 8 hours at 47°C. Hybridization was performed overnight, using the same solution with the addition of the radioactively labeled probe. After hybridization, the membrane was washed at 47°C for 15 min in 2 x SSC. This was followed by a second wash for 30 min in 2 x SSC. The membrane was then washed at room temperature for 30 minutes each in 2 x SSC with 0.1% SDS and in 0.2 x SSC with 0.1% SDS. The membrane was then exposed to X-ray film with an intensifying screen for 24 h at -70°C (Ausubel *et al.* 1987, Sambrook *et al.* 1989).

RESULTS

Purification of bifidocin B

The purification of bifidocin B was achieved by using Micro-Cel and cation exchange chromatography (CMC). Bifidocin B was adsorbed 100 % onto Micro-Cel

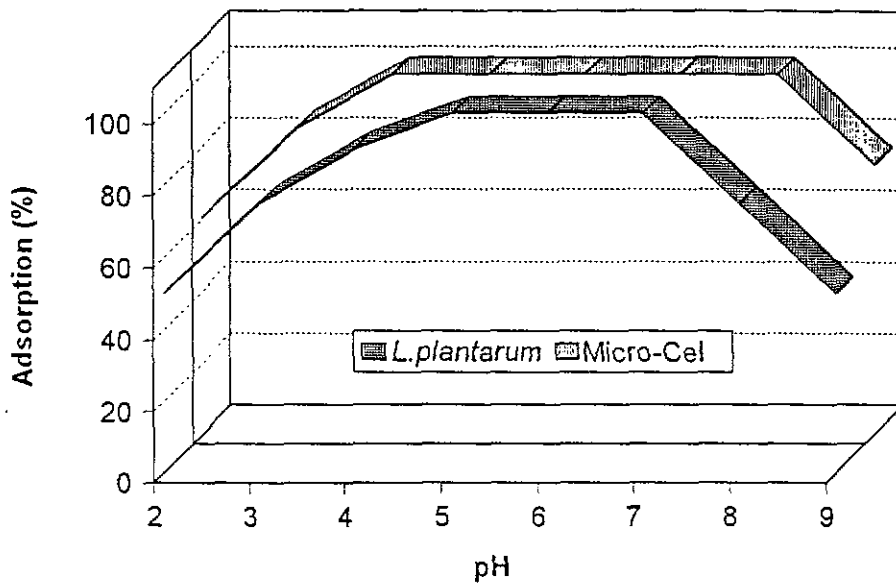


Fig. 4.1. Effect of pH on adsorption of bifidocin B to Micro-Cel or *L. plantarum*.

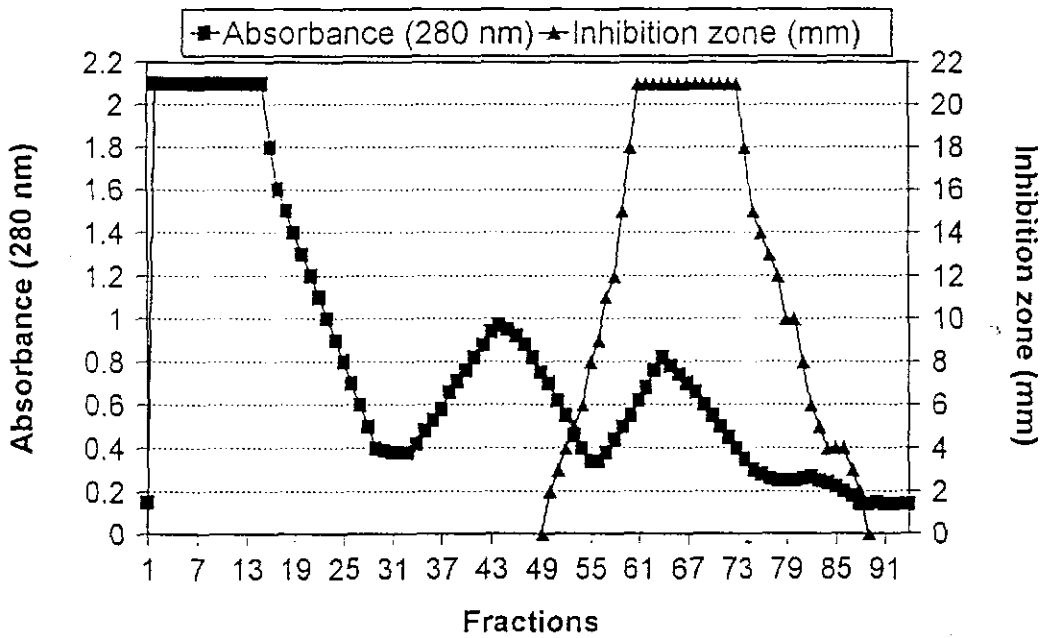


Fig. 4.2. Elution profile of bifidocin B at 0-1 M NaCl by cation exchange chromatography. The sample (15 ml) applied to the column was desorbed from Micro-Cel. The starting amount of culture was 1.5 L.

Table 4.2. Adsorption of bifidocin B to resistant and sensitive bacterial species.

Sensitive organisms	Adsorption (%)	Resistant organisms	Adsorption (%)
<i>Lactobacillus plantarum</i>	100	<i>Bifidobacterium bifidum</i>	95
<i>Pediococcus dextrinicus</i>	100	<i>Pediococcus pentosaceus</i>	100
<i>Leuconostoc oenos</i>	100	<i>Staphylococcus aureus</i>	90
<i>Enterococcus faecalis</i>	100	<i>Salmonella typhimurium</i>	0
<i>Listeria monocytogenes</i>	95	<i>Escherichia coli</i>	0
<i>Listeria ivanovii</i>	95	<i>Yersinia enterocolitica</i>	0
<i>Bacillus cereus</i>	95	<i>Pseudomonas fluorescens</i>	0

(100%) maintained at 25°C. High (85°C) and low temperature (0°C) treatments of cells did not reduce the adsorption of bifidocin B.

The binding of bifidocin B to *L. plantarum* was not time dependent. After *L. plantarum* was exposed to crude bifidocin B for 0.5, 5, 10, 20, 30 or 60 min, *L. plantarum* did not grow during the experiment time, 4 h. (Fig. 4.3). The controls under the same conditions grew very well and reached about 0.7 absorbance value. Also, 95% of bifidocin B was adsorbed within 0.5-10 min and adsorption was 100% complete after 20 min.

The adsorption of bifidocin B to *L. plantarum* cells was completely prevented by addition of purified lipoteichoic acid from *E. faecalis* (Table 4.3). Also, NH_4Cl , MgCl_2 , KCl , KI or Tris-HCl reduced bifidocin B adsorption. Conversely, NH_4 -citrate, Na-acetate, NaCO_3 , EDTA, bovine serum albumin or casein had no effect on adsorption (Table 4.3).

Effect of NaCl and sodium phosphate on adsorption of bifidocin B

The adsorption and lethal effect of bifidocin B was not affected in the presence of NaCl at 25 to 100 mM (Fig. 4.4A), but higher concentrations (above 100 mM) adversely affected the adsorption of bifidocin B and lead to higher viable cell counts. Also, phosphate concentration above 50 mM reduced the adsorption and lethal effect of bifidocin B (Fig. 4.4B). Bifidocin B at 1,280 AU/ml resulted in 99.5% death of control cells. The use of 500 mM NaCl or NaHPO₄ caused reduction in bifidocin B activity and lowered death rates to 36.9 or 20.6%, respectively. However, the protective effects of NaCl and phosphate could be eliminated by using a higher concentration of bifidocin B (5,120 AU/ml) (Table 4.4).

Effect of some detergents, solvents and enzymes on adsorption of bifidocin B

Whole cells and cell wall treated with guanidine-HCl (4M), SDS (1%), Triton X-100 (2%) and organic solvents (mercaptoethanol, ethanol, butanol, isopropanol, hexane, acetone and methanol) did not affect the adsorption of bifidocin B (Table 4.5). Treatment of cell walls with a mixture of methanol and chloroform followed by hot 20% TCA completely inhibited the adsorption of bifidocin B (Table 4.5) and, methanol:chloroform with heat and methanol:chloroform with cold TCA treatment decreased adsorption by 70%. However, these treatments on whole cells did not affect the binding of bifidocin B. Also, treatments of whole cells and cell walls with enzymes (protease, lipase, lysozyme) caused no reductions in adsorption (Table 4.5).

Table 4.4. Effect of bifidocin B concentration on the death of *L. plantarum* NCDO 955 at 37°C for 1 h in the presence of NaCl or NaH₂PO₄.

Bifidocin B (AU)	500 mM NaCl		500 mM NaH ₂ PO ₄	
	cfu/ml	cell death (%)	cfu/ml	cell death (%)
0	6.1x10 ⁸	0.00	6.1x10 ⁸	0.00
1280	3.9x10 ⁸	36.06	4.7x10 ⁸	22.95
2560	1.6x10 ⁸	73.77	2.1x10 ⁸	65.57
5120	5.9x10 ⁶	99.03	1.1x10 ⁷	98.20
10240	6.2x10 ⁵	99.90	6.4x10 ⁵	99.89

Table 4.5. Effects of some detergents, solvents, and enzymes on adsorption of bifidocin B to *L. plantarum*.

Treatments	Adsorption (%)	
	Whole cells	Cell walls
Control (untreated cells or cell walls plus bifidocin B)	100	100
Guanidine-HCl (4 M)	100	100
SDS (1 %), heat at 60°C for 15 min	100	100
Triton X-100 (2 %)	100	100
2-mercaptoethanol (0.5)	100	100
Ethanol (80 % v/v)	100	100
Methanol (80 % v/v)	100	100
Butanol (80 % v/v)	100	100
Isopropanol (80 % v/v)	100	100
Hexane (80 % v/v)	100	100
Acetone (80 % v/v)	100	100
Methanol:chloroform (40:40, v/v) and heat 93°C, 5 min	100	70
Methanol:chloroform (40:40, v/v) and TCA (20%) at 4°C, 2 h	100	70
Methanol:chloroform (40:40, v/v) and TCA (20%) at 93°C, 5 min	100	0
Trypsin	100	100
Protease	100	100
Lipase	100	100
Lysozyme	100	100

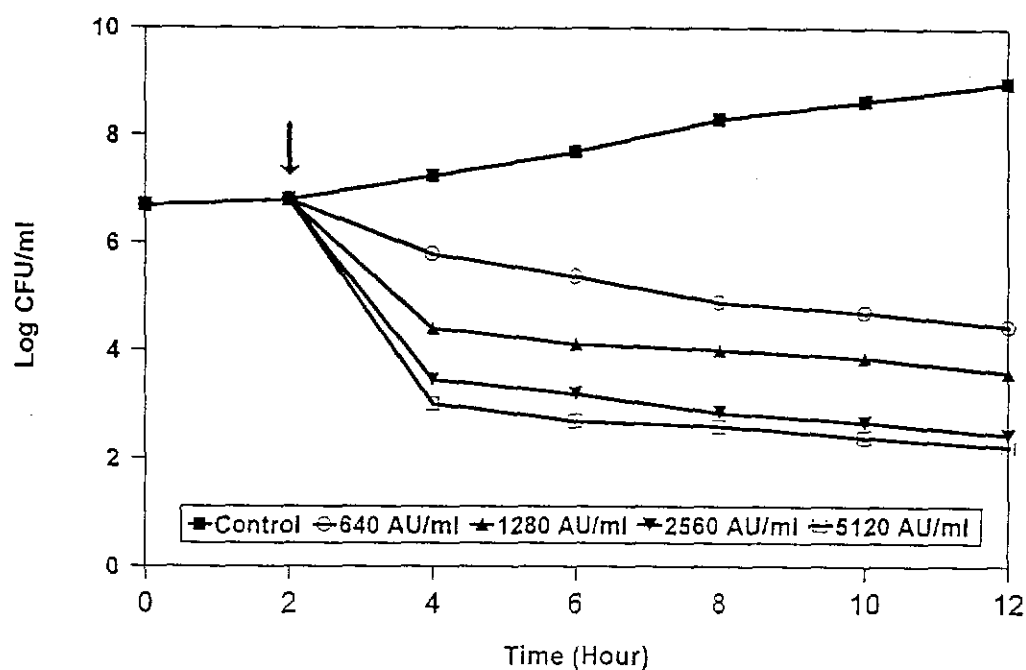


Fig. 4.5. Effect of bifidocin B on growing cells of *L. plantarum* NCDO 955. Bifidocin B was added at 2 h (arrow).

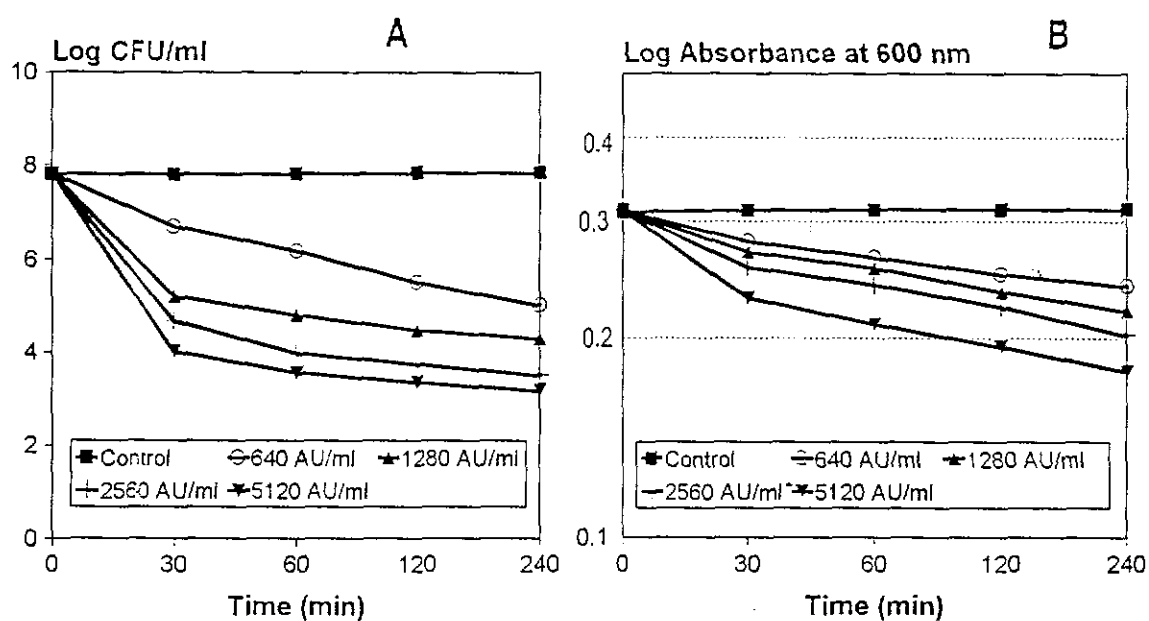


Fig. 4.6. Effects of bifidocin B on non-growing cells of *L. plantarum* NCDO 955. A, log CFU/ml; B, log absorbance at 600 nm.

grew very well.

Effect of bifidocin B on the release of cellular materials

L. plantarum treated with pure bifidocin B resulted in the leakage of UV-absorbance materials and potassium ions, and an increase in 260 nm absorbing materials compared with controls (Table 4.6). In bifidocin B treated cells, potassium ion efflux and UV-absorbing materials increased 9.8 and 12.4 times, respectively. Also, the treated cells became 6 times more permeable to ONPG and ONP than the untreated cells (Table 4.6).

Plasmid curing and southern hybridization

The MIC of acriflavine for *B. bifidum* was found to be 35 $\mu\text{g/ml}$. *B. bifidum* was screened for the occurrence of Bac^- derivatives after treatment with acriflavine. Acriflavine treatment at 20 or 25 $\mu\text{g/ml}$ gave 100 bacteriocin-non-producer isolates (Bac^-) from 500 colonies tested. These Bac^- variants did not differ from the parent strain in growth rate or carbohydrate fermentation patterns. They remained immune to bifidocin B. *B. bifidum* harbored two plasmids. The Bac^- derivatives did not have the 8 kb plasmid which was present in the parent strain (Fig. 4.8). Southern hybridization tests show that ZY-1 probe reacted with the 8.0 kb plasmid present in Bac^+ strain, but it did not yield any reactions with Bac^- variant (Fig. 4.8).

DISCUSSION

Bifidocin B was purified with a yield of 18% from 1,500 ml. It was strongly bound to cationic resin at pH 6.6. The behavior of this bacteriocin in the cation exchange chromatography and on Micro-Cel points out its cationic character.

The results for purification and characterization of bifidocin B indicate that this is a new member to be added to the group of class IIa bacteriocins from lactic acid bacteria (Klaenhammer 1993). An amino acid sequencing homology search revealed that bifidocin B shared significant homology with other class IIa bacteriocins (pediocin- like peptides). The N-terminal region of bifidocin B contained the Lys-Tyr-Tyr-Gly-Asn-Gly-Val consensus region common to several other class IIa bacteriocins, including bavaricin A (Larsen *et al.* 1993), curvacin A (Tichaczek *et al.* 1992), leucocin A (Hastings *et al.* 1991), pediocin PA-1 (Lozano *et al.* 1992), pediocin AcH (Motlagh *et al.* 1992), piscicocin VIa, piscicocin VIb (Bhugaloo-vial *et al.* 1996), sakacin A, sakacin P (Tichaczek *et al.* 1992), mesentericin Y105 (Hechard *et al.* 1992) and carnobacteriocin B2 (Quadri *et al.* 1994). The presence of this sequence in these bacteriocins produced by these different bacteria suggest that this consensus region is of fundamental importance for bacteriocin activity. The sequence may perhaps directly cause the bactericidal effect or it might be important in determining the bacteriocin's target-cell specificity. The conserved N-terminus motif is considered to be involved in the action against *Listeria* strains. However, many other bacteriocins which lack this structural homology with class IIa peptides

Adsorption of bifidocin B to the cell surface was decreased in the presence of NaCl and Na-phosphate, and the reduction was concentration dependent. These inhibition patterns show that the binding of bifidocin B with cell surface receptors may be mediated by ionic interactions. The protective effect of sodium cations or chloride and phosphate ions was overcome by increasing the concentration of bifidocin B. Cell or cell wall preparations from *L. plantarum* showed no loss in ability to adsorb bifidocin B after treatment with different enzymes (protease, trypsin, lipase, lysozyme), several organic solvents or detergents. These negative results suggest that the major binding sites or receptors for bifidocin B were probably not composed of proteins, carbohydrates or lipids. After treatment with methanol:chloroform and hot 20% TCA, cell wall preparations lost the ability to adsorb the bifidocin B. This suggested a possible involvement of cell wall lipoteichoic acid (LTA) in binding of bifidocin B (Bhunja *et al.* 1991, Wicken and Knox 1975). Also, the addition of purified LTA to cells completely blocked the adsorption of bifidocin B. Bifidocin B probably forms a complex with LTA and then becomes unavailable for adsorption to the cells (Bhunja *et al.* 1991). LTA is present in gram-positive bacteria but absent in gram-negative bacteria. This could also explain why bifidocin B was adsorbed to gram-positive bacteria and not to gram-negative bacteria. LTA molecules probably serve as the non-specific receptors and mediate binding of bifidocin B to both resistant and sensitive gram-positive bacteria. Secondly, in the sensitive gram positive cells, when these sites have been saturated,

a mutant which was unable to produce any inhibitory activity but which retained resistance to the parent bacteriocin. Concerning sugar fermentation patterns (API 20 and CHL 50), this Bac⁻ variant was not different from the Bac⁺ parent strain. Plasmid analysis of the Bac⁻ variant revealed a loss of a plasmid of about 8 kb. Therefore, this plasmid may encode bacteriocin production but does not seem to carry immunity nor any information related to utilization of the sugar tested. The locus of the gene coding for immunity trait in *B. bifidum* NCFB 1454 strain still remains unclear because the variant strain lacking the 8 kb plasmid remained immune to bifidocin B. Similar results were reported for other lactic acid bacteria (Schved *et al.* 1993). The presence of the gene encoding bifidocin B on the 8 kb plasmid was further confirmed by Southern blotting using an oligonucleotide synthesized based on the N-terminal amino acid sequence.

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cleavage site; active and stable in the acidic pH range; organization of the structural and regulatory genes in an operon; the existence of specific immunity peptides or proteins which protect the producer strains from the lethal action of their own bacteriocins; and the cytoplasmic membrane as the primary target of action (Vuyst *et al.* 1996).

Bacteriocins of *Lactococcus* subsp. *lactis*, *L. lactis* subsp. *diacetylactis* and *L. lactis* subsp. *cremoris* have been reported (Davey and Richardson 1981, Holo *et al.* 1991, Hurst 1981, Martinez *et al.* 1996, Mayer *et al.* 1992, Moll *et al.* 1996, Morgan *et al.* 1995, Parente *et al.* 1994, Piard *et al.* 1992). The best known is nisin, a 3.4 kDa polypeptide produced by *L. lactis* subsp. *lactis* that inhibits the growth of gram-positive bacteria, including *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Micrococcus*, *Staphylococcus*, *Clostridium*, and *Listeria* species. Diplococcin and lactococcin A, B, G and M from *L. lactis* subsp. *cremoris*, lactococcin 140 and 972, and lacticin 481 from *Lactococcus* subsp. *lactis* have been identified (Davey and Richardson 1981, Holo *et al.* 1991, Hurst 1981, Martinez *et al.* 1996, Mayer *et al.* 1992, Moll *et al.* 1996, Morgan *et al.* 1995, Parente *et al.* 1994, Piard *et al.* 1992). Diplococcin and lactococcin A, B, M, G, 972 and 140 have a narrow inhibition spectrum, inhibiting only sensitive lactococci. Also, the activity of lactococcin G and B depend on the complementary action of two peptides and a reduced state of the Cys-24 residue, respectively (Venema *et al.* 1993). Lacticin 481 had a bactericidal effect on some *Lactococcus*, *Lactobacillus*,

Preparation of crude lactococcin R

L. lactis subsp. *cremoris* R was inoculated into MRS broth (1 L) at the rate of 1 % (v/v) and incubated at 30°C. Preparation of crude lactococcin R from cell free-culture supernatant was performed as described in Chapter III (page 50).

Partial purification of lactococcin R by adsorption onto producer cells

Partial purification of lactococcin R from *L. lactis* subsp. *cremoris* R culture which was grown in MRS broth at 30°C for 22 h was given in Chapter III (pages 50-51).

Molecular weight of lactococcin R

Molecular weight of partially purified lactococcin R was carried out as described in Chapter III (pages 51-52).

Antimicrobial activity of lactococcin R

Antimicrobial activity assay of lactococcin R was performed by using the agar spot method (Fleming *et al.* 1975) which was mentioned in Chapter III (page 52).

Effect of growth conditions on production of lactococcin R

Culture samples were withdrawn every 2 h as *L. lactis* subsp. *cremoris* R

Mode of action of lactococcin R

An actively growing *Listeria monocytogenes* ATCC 19113 was inoculated into tempered fresh BHI broth and incubated at 37°C until absorbance at 600 nm reached about 0.25. Lactococcin R (1280 AU/ml) was added at 60 min. Samples were withdrawn every 30 min during the incubation period (4 h), their absorbances read at 600 nm and cfu/ml were determined. The control was *Listeria monocytogenes* ATCC 19113 without lactococcin R.

Statistical analysis

Data were analyzed using the general linear models procedure of SAS Institute, Inc. (1992) to determine differences between treatment means. Pairwise comparisons of all treatment means were performed using the Least Significant Difference (LSD) procedure at $P < 0.05$. Experiments were performed three times in a completely randomized design.

RESULTS

Strain identification

Colonies from the outer surface of radish causing inhibition were isolated. Cells of these strains were ovoid, present in pairs or short chains, catalase-negative, gram-positive, facultatively anaerobic cocci. They were able to grow between 10 and 40°C and pH 4.5-9.2, but not at 45°C or 50°C and at pH 4.2. They also grew in the

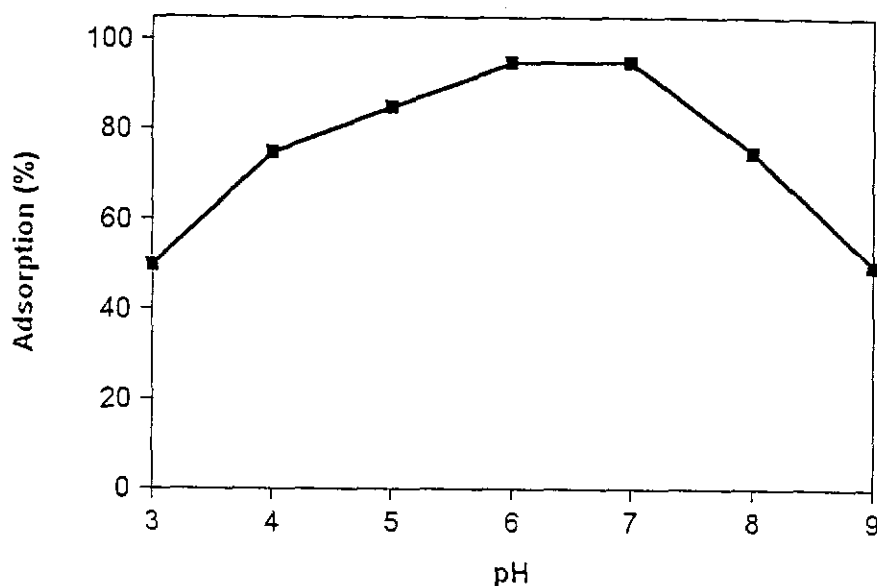


Fig. 5.1. Effect of pH on adsorption of lactococcin R to heat-killed *L. lactis* subsp. *cremoris* cells.



Fig. 5.2. Gel overlay with *L. plantarum* to determine which band(s) corresponds to the antimicrobial activity and molecular weight. Lane 1 contains low molecular weight standards (Bio-Rad): a, 1,423; b, 3,496; c, 6,512; d, 14,437; e, 16,950; f, 26,625 Da. The band in lane 2 which corresponds to the antimicrobial activity, the zone of growth inhibition (see arrow), had a mass of about 2.5 kDa. Other bands did not show antimicrobial activity.

Table 5.1. Antimicrobial spectrum of lactococcin R.

Bacteria & Source	Antimicrobial Activity ^a	Diameter of inhibition zone(mm)
<i>Listeria ivanovii</i> ATCC 19119	+	12±0.12
<i>Listeria murrayi</i> ATCC 33090	+	12±0.10
<i>Listeria innocua</i> ATCC 25401	+	14±0.05
<i>Listeria grayi</i> ATCC 19120	+	22±0.09
<i>Listeria seeligeri</i> ATCC 35967	+	13±0.05
<i>Listeria welshimeri</i> ATCC 35897	+	11±0.07
<i>Listeria monocytogenes</i> Scott A USDA	+	13±0.12
<i>Listeria monocytogenes</i> 587 CHRL	+	11±0.13
<i>Enterococcus faecalis</i> Dan Fung	+	13±0.15
<i>Enterococcus faecium</i> Dan Fung	+	12±0.11
<i>Bacillus cereus</i> ATCC 232	+	12±0.14
<i>Bacillus subtilis</i> Univ. Wisconsin	+	10±0.12
<i>Bacillus megaterium</i> Univ. Wisconsin	+	9±0.08
<i>Lactobacillus viridans</i> Univ. Wisconsin	+	15±0.15
<i>Lactobacillus acidophilus</i> NRRL B1910	+	17±0.10
<i>Lactobacillus plantarum</i> NCDO 955	+	19±0.20
<i>Lactobacillus casei</i> UMRL	+	18±0.07
<i>Leuconostoc oenos</i> UMRL	+	16±0.05
<i>Leuconostoc mesenteroides</i> Univ. Wisconsin	+	16±0.09
<i>Pediococcus acidolactici</i> UMRL	+	17±0.08
<i>Pediococcus dextrinicus</i> ATCC 19371	+	25±1.00
<i>Pediococcus parvulus</i> ATCC 19371	+	16±0.80
<i>Pediococcus urinaequi</i> ATCC 29723	+	17±0.25
<i>Streptococcus thermophilus</i> Univ. Wisconsin	+	25±1.10
<i>Streptococcus agalactiae</i> ATCC 13813	+	13±0.16
<i>Micrococcus roseus</i> Univ. Arkansas	+	11±0.09
<i>Micrococcus luteus</i> ATCC 4698	+	12±0.11
<i>Staphylococcus aureus</i> ATCC 25923	+	11±0.13
<i>Staphylococcus lentus</i> Univ. Arkansas	+	11±0.09
<i>Staphylococcus hyicus</i> Univ. Arkansas	+	11±0.11
<i>Clostridium perfringens</i> Univ. Wisconsin	+	11±0.15
<i>Clostridium sporogenes</i> Univ. Arkansas	+	12±0.17
<i>Lactococcus lactis</i> ATCC 11454	-	NA ^b
<i>Aerococcus viridans</i> Univ. Wisconsin	-	NA
<i>Pediococcus pentosaceus</i> ATCC 43200	-	NA
<i>Corynebacterium glutamicum</i> ATCC 31834	-	NA
<i>Klebsiella pneumoniae</i> Univ. Wisconsin	-	NA
<i>Shigella flexneri</i> ATCC 12022	-	NA
<i>E. coli</i> 0157:H7 ATCC 43895	-	NA
<i>Salmonella typhimurium</i> ATCC 14028	-	NA
<i>Enterobacter aerogenes</i> Univ. Arkansas	-	NA
<i>Pseudomonas aerogenes</i> ATCC 10145	-	NA
<i>Citrobacter freundii</i> ATCC 8090	-	NA

^a +, inhibition; -, not inhibition; ^bNA, not applicable

Table 5.4. Effects of different initial pH levels on final pH, maximal growth and lactococcin R production in MRS broth at 30°C for 22 h

Initial pH	Final pH	Absorbance (600nm)	AU/ml
4.50	4.43±0.01 ¹ b	0.07±0.01e	0±0d
5.00	4.45±0.03ab	0.47±0.01d	400±16.2c
5.50	4.44±0.03ab	0.94±0.06c	800±30.3b
6.00	4.46±0.01ab	1.25±0.03b	1600±30.3a
6.50	4.49±0.02a	1.36±0.04a	1600±26.3a
7.00	4.51±0.02a	1.40±0.01a	1600±28.3a
7.50	4.53±0.04a	1.32±0.01ab	800±16.2b

1-means of three replicates±standard deviation. Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$).

of *L. lactic* subsp. *cremoris* R cultivated in MRS broth at 30°C (Fig. 5.3). Its activity reached a maximum level (1,600 AU/ml) at 16-24 h of culture during late exponential and early stationary phases. After 30 h or 72 h of incubation, bacteriocin titers decreased by 50 or 88 %, respectively.

Stability of lactococcin R

Lactococcin R preparations retained much of their initial activity of 25,600 AU/ml after high temperature treatments. Activity of lactococcin R was only decreased by 25 % after 60 min at 90°C and 15 min at 121°C (Table 5.5). There was no decrease in initial activity of 25,600 AU/ml caused by storage at -20 or -70°C for 3 months, but activity at 4°C diminished by 25 and 75 % after 1 and 3 months (Fig. 5.4).

Table 5.5. Effects of heat treatments on the activity of lactococcin R

Heat Treatment	Activity of lactococcin R (AU/ml)
Control (unheated)	25,600 $\pm$ 22.22 ¹ a
90°C for 15 min	25,600 $\pm$ 36.37a
90°C for 30 min	25,600 $\pm$ 14.14a
90°C for 60 min	19,200 $\pm$ 28.28b
121°C for 15 min	19,200 $\pm$ 32.32b

1-means of three replicates $\pm$ standard deviation. Any two means in the same column followed by the same letter are not significantly different ($P > 0.05$).

The antimicrobial activity of lactococcin R was lost completely when treated with protease type IV, pronase E, proteinase K, pepsin and α -chymotrypsin. However, lactococcin R was resistant to papain and trypsin (Table 5.6). Untreated lactococcin R samples or samples containing heat-inactivated enzymes, showed no loss of activity. Lactococcin R was also not inactivated by treatments with lysozyme, ribonuclease A, catalase, peroxidase, amylase, dextranase, cellulase, lipase, glucose oxidase or organic solvents (Table 5.7).

Lactococcin R retained activity at pH levels between 2-9 when kept at 25°C for 2 h. However, after 2 h, activity was decreased 10,15 and 77 % at pH 8, 9 and 10, respectively ($P < 0.05$). After holding at 25°C for 24 h, or heating at 90°C for 20 min, lactococcin R lost 20-30, 20-35 and 100 % of its activity at pH's 8, 9 and 10, respectively ($P < 0.05$)-(Fig. 5.5).

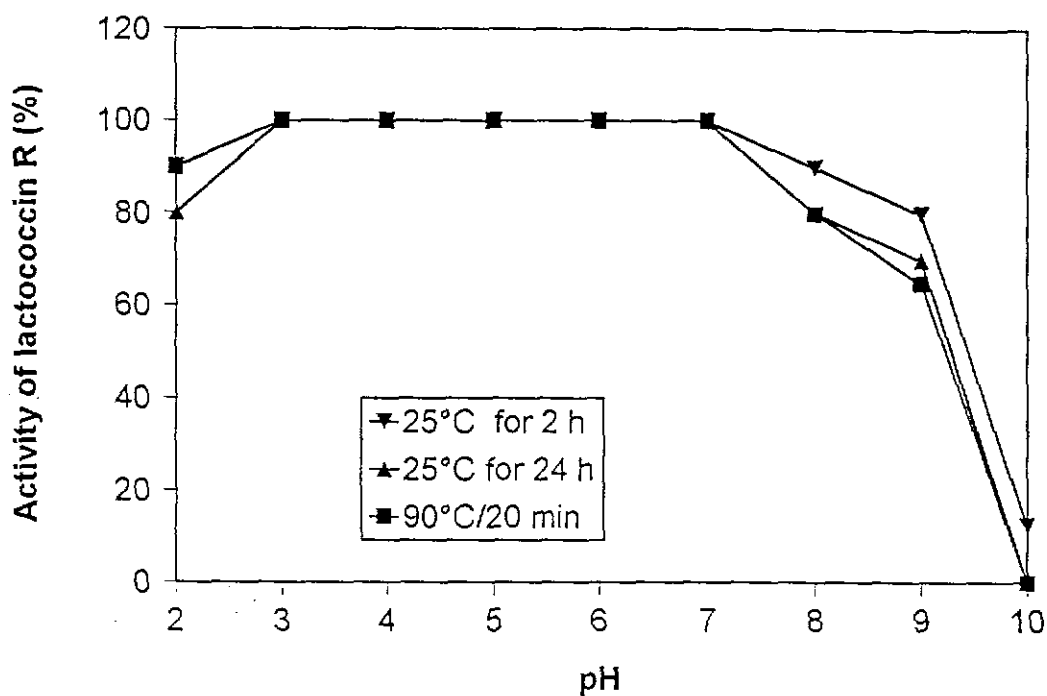


Fig. 5.5. Effect of pH on the activity of lactococcin R.

Mode of action of lactococcin R

Addition of lactococcin R at 1280 AU/ml to a growing culture of *Listeria monocytogenes* in BHI broth resulted in reductions of absorbance values and colony counts (Fig. 5.6). At 1 and 3 h, absorbance values at 600 nm decreased by 0.05 and 0.08 units (Fig.5.6A) while the colony counts decreased by 2.7 and 3.0 log cycles (Fig. 5.6B) for reductions of 99.79 and 99.98 %, respectively. Conversely, control cells without lactococcin R, under the same condition, showed increases of 0.07 or 0.40 absorbance units and of 44.78 or 87.24 % in colony counts.

Protease sensitivity is a key criterion for the characterization of an inhibitory substance such as a bacteriocin. Like other lactococcal bacteriocins, lactococcin R was not sensitive to all proteases (Holo *et al.* 1991, Hurst 1981). Lactococcin R is sensitive to protease, pronase E, proteinase K, pepsin and α -chymotrypsin. However, it was resistant to trypsin and papain. Treatment with lipase, amylase, dextranase, cellulase or organic solvents did not cause any apparent loss of lactococcin R activity. These results indicate that lipid and carbohydrate moieties were not responsible for its antimicrobial activity. Antimicrobial activity of the lactococcin R preparation was also not due to hydrogen peroxide or acidity since activity was not lost after treatment with catalase or peroxidase or adjustment of pH to 7.0.

Like other bacteriocins produced by lactic acid bacteria, crude lactococcin R seems to aggregate and precipitate with other proteins and to precipitate during dialysis against water to remove ammonium sulfate (Atrih *et al.* 1993, Bhunia *et al.* 1988, Gonzales *et al.* 1994). These aggregates had antimicrobial activity. Adsorption of lactococcin R onto heat killed *L. lactis* subsp. *cremoris* R cells was affected by the pH of the suspending environment with maximum adsorption occurring at pH 6.0-7.0.

Lactococcin R production displays secondary metabolite kinetics, i.e. the bacteriocin was produced during pre- and early exponential growth phases and reached a maximum level at early stationary phase. Extending stationary phase resulted in a decrease in bacteriocin activity. This decrease could be due to the

proteolytic enzymes, pepsin and pronase E, to which nisin is resistant.

A large number of bacteriocins have been reported (Bhunia *et al.* 1988, Davey and Richardson 1981, Fleming *et al.* 1985, Gonzales *et al.* 1994, Holo *et al.* 1991, Hurst 1981, Morgan *et al.* 1995, Piard *et al.* 1992, Vuyst *et al.* 1996). Bacteriocins usually inhibit only closely related bacteria, except for the bacteriocins nisin and pediocin, which inhibit strains of several different gram-positive genera. Similar to nisin and pediocin, lactococcin R has a wider antimicrobial spectrum since it inhibits the growth of at least ten genera; namely selected species of *Listeria*, *Clostridium*, *Staphylococcus*, *Micrococcus*, *Bacillus*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*. Collectively, the above data indicate lactococcin R may be useful as a biopreservative to prevent the growth of foodborne pathogens and food spoilage bacteria in certain food products.

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known lantibiotic which has been approved for use as a food preservative in cream cheese products (FDA 1988). Nisin is a 34-amino acid peptide containing lanthionine and β -methyllanthionine residues. Pronisin is synthesized ribosomally and undergoes posttranslational modification such that serine and threonine are dehydrated to dehydroamino forms, which then condense with cysteine to give lanthionine or β -methyllanthionine, respectively (Ingram 1970, Kaletta and Entian 1989). Other lactococcal strains produce bacteriocins with a more restricted spectra of inhibitory activity or different physical and chemical properties (Kozak et al. 1978, Davey and Richardson 1981, Geis et al. 1983, Piard et al. 1992).

Recently we reported that *L. lactis* subsp. *cremoris* R isolated from the outer surface of radish produced a bacteriocin, lactococcin R. Lactococcin R is sensitive to some proteolytic enzymes, resistant to heat, pH 2.0-9.0, storage temperatures (-20°C) and organic solvents (Yildirim and Johnson 1998b). Also, it is inhibitory to some species of *Listeria*, *Bacillus*, *Clostridium*, *Staphylococcus*, *Micrococcus*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. The objectives of this study were to purify lactococcin R, and determine its amino acid sequence, as well as factors affecting its mode of action and its adsorption to indicator organisms, and whether the gene(s) that encode lactococcin R are located on plasmid or chromosomal DNA.

described in Chapter IV (page 67).

Effect of temperature, pH, time, sodium chloride and sodium phosphate on adsorption of lactococcin R to *L. plantarum*

The effects of temperature, pH, time, sodium chloride and sodium phosphate were analyzed using the methods given in Chapter IV (pages 67-69).

Effect of lipoteichoic acid, various chemicals, detergents, solvents and enzymes on adsorption of lactococcin R to whole cell or cell walls of *L. plantarum*

The effect of lipoteichoic acid and various chemicals on adsorption of lactococcin R was analyzed using the procedures given in Chapter IV (pages 69-71).

Effect of lactococcin R on growing and non-growing cells of *L. plantarum*

The effects of various levels of lactococcin R on growing and non-growing cells of *L. plantarum* was determined as described in Chapter IV (page 71-72).

Effect of lactococcin R treatment on the release of cytoplasmic materials in the environment

Analysis of the effect of pure lactococcin R treatment on the release of cytoplasmic materials from *L. plantarum* was given in Chapter IV (pages 72-73).

single peak of inhibitory activity. The peak of lactococcin R activity coincided with an absorption peak at 280 nm (Fig. 6.2). Fractions containing inhibitory activity were analyzed further by tricine SDS-PAGE and the purified lactococcin R showed a single band of protein which was indicative of the high purity (data not shown). The purification steps and recoveries of lactococcin R was given in Table 6.1.

Amino acid sequence analysis

Eleven residues, Ile-His-Gly-Ala-Leu-Met-Trp-Lys-Asn-Met-Lys, were sequenced from the N-terminus of lactococcin R. The remaining peptide sequence could not be further degraded by the Edman reaction. No sequence homologous to the above eleven amino acid residues in the lactococcin R N-terminal sequence was found in the literature.

Adsorption of lactococcin R to indicator organisms

Lactococcin R was adsorbed on cells of all gram-positive bacteria, both sensitive and resistant, and adsorption ranged from 75-100% (Table 6.2). However, lactococcin R did not adsorb to the gram-negative bacteria tested.

Effect of pH, temperature, time, lipoteichoic acid and various chemicals on adsorption of lactococcin R

The adsorption of lactococcin R to *L. plantarum* cells was pH dependent. It

Table 6.1. Purification steps of lactococcin R.

Sample	Volume (ml)	Total Protein (mg)	Total Activity (AU)	Specific Activity (AU/mg)	Activity Recovery (%)	Fold Purification
CFS ¹	1,500	16,860	2,400,000	142	100	1
FD ²	150	16,780	2,328,000	139	97	0.98
MC ³	15	124	1,653,000	13,900	69	98
CMC ⁴	1.5	1.7	345,000	203,300	14.4	1,432

¹ Cell free supernatant; ² Freeze dried; ³ Micro-Cel; ⁴ Cation exchange chromatography

Table 6.2. Adsorption of lactococcin R to resistant and sensitive bacterial species.

Sensitive organisms	Adsorption (%)	Resistant organisms	Adsorption (%)
<i>Lactobacillus plantarum</i>	100	<i>Lactococcus lactis</i>	94
<i>Pediococcus dextrinicus</i>	100	<i>Pediococcus pentosaceus</i>	100
<i>Leuconostoc oenos</i>	100	<i>Staphylococcus aureus</i>	90
<i>Enterococcus faecalis</i>	100	<i>Salmonella typhimurium</i>	0
<i>Listeria monocytogenes</i>	95	<i>Escherichia coli</i>	0
<i>Listeria ivanovii</i>	95	<i>Yersinia enterocolitica</i>	0
<i>Bacillus cereus</i>	100	<i>Pseudomonas fluorescens</i>	0

was found that the adsorption of lactococcin R was maximum between pH 5.0 and 7.0 (Fig.6.1). However, the adsorption was reduced to about 50 to 25 % below or above these pH values.

The amount of binding of lactococcin R to *L. plantarum* under different temperature conditions (0, 10, 50 and 85°C) were found the same as the control (100 %) maintained at 25°C. High (85°C) and low temperature (0°C) treatments of

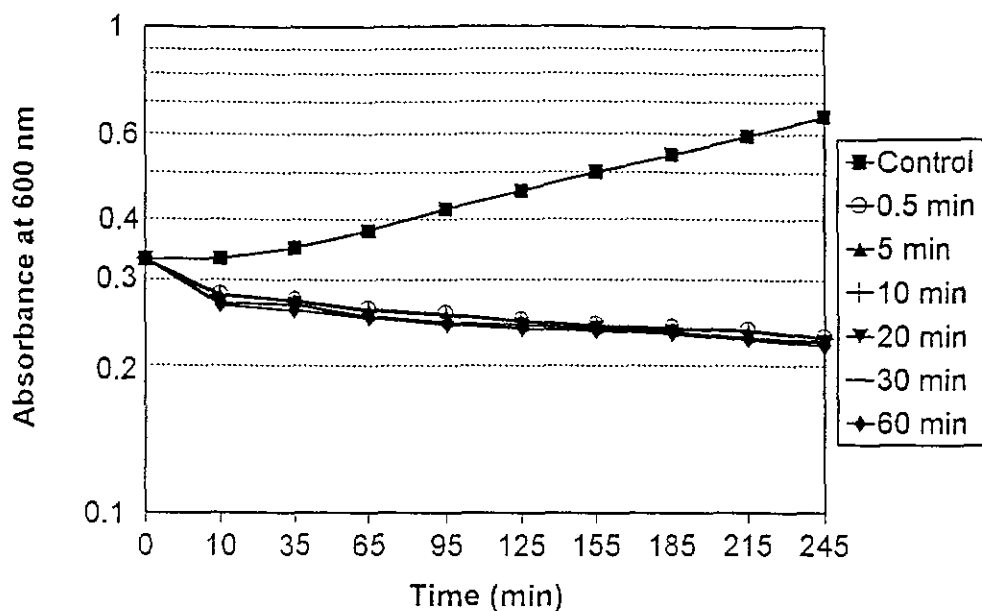


Fig. 6.3. Effect of lactococcin R (5,120 AU/ml) treatment for times varying from 0.5 to 60 min on the growth of *L. plantarum* NCDO 955.

Table 6.3. Effect of the presence of lipoteichoic acid and various chemicals on adsorption of lactococcin R to *L. plantarum*.

Treatments	Adsorption (%)
Control (cells+lactococcin R)	100
Lipoteichoic acid (1 mg/0.1 ml)	0
NH ₄ -citrate (0.1 %)	100
NH ₄ Cl (0.3 M, pH 5.5)	85
MgCl ₂ (0.3 M, pH 6.5)	75
KCl (0.3 M, pH 5.6)	85
KI (0.3 M, pH 6.6)	85
Na-acetate (0.5 %)	100
NaHCO ₃ (0.3 M, pH 7.6)	90
EDTA (0.01 M)	100
Tris-HCl (0.6 M, pH 6.8)	50
Tris-HCl (0.02 M, pH 6.8)	85
Bovine serum albumin (0.5 %)	100
Casein (2.0 %)	100

*At pH 7 and 8 adsorption of lactococcin R were about 100 and 75 % respectively.

protective effects of NaCl and phosphate could be overcome by increasing the concentration of lactococcin R to 5,120 AU/ml (Table 6.4).

Effect of some detergents, solvents and enzymes on adsorption of lactococcin R

Treatment of whole cells and cell walls with guanidine-HCl (4M), SDS (1%), Triton-X 100 (2%), organic solvents such as mercaptoethanol, ethanol, hexane, acetone and methanol, and enzymes (protease, trypsin, lipase, lysozyme) had no effect on adsorption of lactococcin R (Table 6.5). However, treatment of cell walls with a mixture of methanol and chloroform followed by hot 20% TCA prevented the adsorption of lactococcin R (Table 6.5). The later treatment of methanol:chloroform with heat or with cold 20% TCA treatment only reduced adsorption by 20%, whereas these treatments on whole cells had no effect on lactococcin R adsorption.

Effect of lactococcin R on growing and non-growing cells of *L. plantarum*

Addition of crude lactococcin R at various concentrations (0-5,120 AU/ml) to a growing culture of *L. plantarum* in MRS broth resulted in reductions of the colony counts in proportion to the amount of lactococcin R added (Fig. 6.5). After 2 h incubation period, the counts declined 98.1%, 99.98%, 99.99% and 99.99% at 640, 1,280, 2,560 and 5,120 AU/ml of lactococcin R, respectively. At the end of 12 h incubation, the colony counts decreased by 99.9-100% in all lactococcin R concentrations (Fig. 6.5).

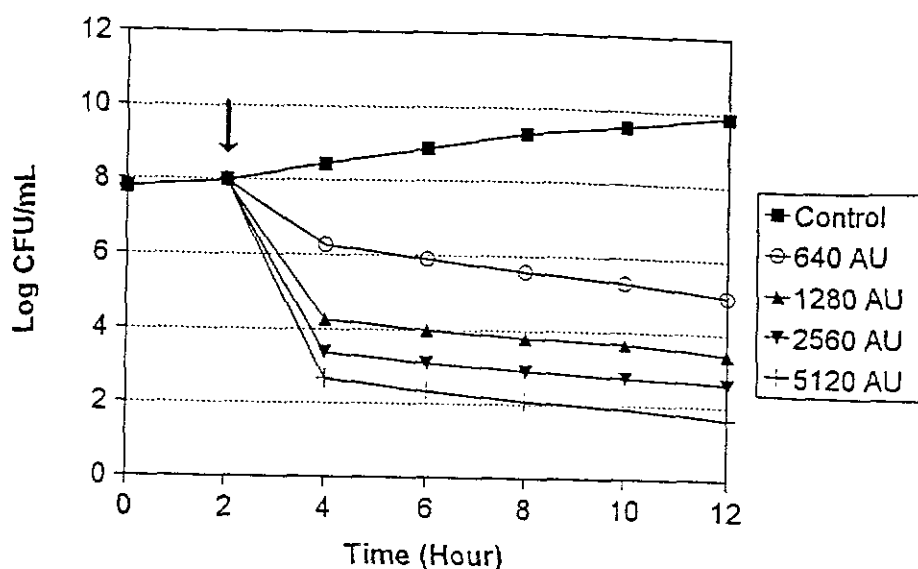


Fig. 6.5. Effect of lactococcin R on growing cells of *L. plantarum* NCDO 955. Lactococcin R was added at 2 h (arrow).

In studies on effects on non-growing cells, addition of lactococcin R to *L. plantarum* suspended in phosphate buffer at pH 5.5 decreased the colony forming units and absorbance values (600 nm) in proportion to the amount of lactococcin R added (Fig. 6.6). After 30 min incubation period, the counts had decreased approximately by 1.76, 3.78, 4.8 and 5.39 log in 640, 1,280, 2,560 and 5,120 AU/ml, respectively (Fig 6.6A). The colony forming units declined 99.9% in all lactococcin R concentrations. The respective absorbance values were decreased by 0.08, 0.10, 0.12, or 0.15 units (Fig 6.6B).

Effect of lactococcin R on the release of cellular materials

The treatment of *L. plantarum* with pure lactococcin R caused the leakage of

Table 6.6. Effect of lactococcin R on the release of cellular materials from *L. plantarum* NCDO 955 cells.

Parameters	Untreated cell supernatant	Lactococcin R solution	Treated cells supernatant
Absorbance (260 nm)	0.152	0.141	2.14
Potassium ions (mg/l)	36	2.84	468
ONP (mM)	113	0	872

growth rate in MRS broth at 30°C and they remained immune to lactococcin R. The parent strain, *L. lactis* subsp. *cremoris* R, and the Bac⁻ derivatives both harbor three plasmids with the same respective molecular masses (Fig. 6.8).

DISCUSSION

Purification of lactococcin R was achieved by using Micro-Cel adsorption/desorption and cation exchange chromatography. The final specific activity of pure lactococcin R was about 1,432-fold greater than in the crude culture supernatant, and the final recovery was 14%. The binding of lactococcin R to CMC at pH 6.6 and Micro-Cel is indicative of a cationic molecule. The lactococcin R bound Micro-Cel was desorbed 100% by using SDS (0.1%), whereas only 69% was desorbed by using a combination treatment of low pH (2.0), heat (80°C for 5 min) and NaCl (100 mM). These results strongly indicate that lactococcin R has quite hydrophobic regions.

portion of the lactococcin R. This result contrasts with most lantibiotics, i.e., nisin and subtilin (Sahl et al. 1995). The blockage of Edman degradation at 11-12 linkage residue in lactococcin R could be due to the presence of a dehydrated residue at twelve residue position. Similar blockages were reported for sequencing of lactacin 418 (Piard et al. 1992) and variacin (Pridmore et al. 1996) for which only the first seven and five residues respectively could be successfully sequenced. The N-terminal amino acid sequence of lactococcin R did not reveal homology with any known sequence for a lantibiotic bacteriocin, including nisin or lactacin 481, suggesting that lactococcin R is a new bacteriocin.

Lactococcin R was adsorbed to all sensitive or resistant gram-positive bacteria, but not to any of the gram-negative bacteria tested. Gram-negative bacteria probably do not have the necessary adsorption binding sites (receptors). Adsorption of lactococcin R caused cell death in sensitive gram-positive cells, but did not cause death when adsorbed to the gram-positive resistant cells (data not shown). The adsorption of lactococcin R to the gram-positive resistant cells could be due to the presence of non-specific (non lethal) receptors on such strains (Upreti and Hinsdill, 1975, Bhunia et al. 1991). The sensitive cells probably have both non-specific and specific (lethal) receptors. The adsorption and lethal action of lactococcin R on sensitive gram-positive strains was very rapid. In 0.5 min, lactococcin R was adsorbed to *L. plantarum* cells by 90% inhibited cell growth. In addition, adsorption of lactococcin R was dependent on pH rather than time. The highest adsorption

with binding of lactococcin R in both resistant and sensitive gram-positive bacteria. In the sensitive cells, when these sites have been saturated, lactococcin R binds to specific or lethal receptor(s) and caused cellular changes associated with cell death (Bhunia et al. 1991). The lethal action of lactococcin R on sensitive cells results in the loss of some cellular materials, UV-absorbing materials and potassium, associated with membrane damage. Pucci et al. (1988) and Bhunia et al. (1991) reported the similar results for action of pediocin on *L. monocytogenes* and *L. plantarum*.

Lactococcin R is bactericidal to sensitive bacterial cells and this effect is produced within a few minutes. Cell death appears to be associated with lysis of the cell membrane or subsequent cytosol leakage. The mode of action of lactococcin R, similar to that of pediocin AcH (Bhunia et al. 1991), first involves the binding of lactococcin R to some non-specific receptors like lipoteichoic acid and then, followed by binding to specific receptors and forms pores which can cause changes in the membrane integrity. As a result, the cells lose potassium ions, UV-absorbing materials and other small molecules. The cell membrane also loses structural integrity and lyses. In the resistant gram-positive bacteria, the specific receptors leading to disruption of cell membrane are either absent or not available for binding, while in the gram-negative bacteria both specific and non-specific receptors for lactococcin R are absent.

In lactococci, the genetic determinant for bacteriocin production can be either plasmid or chromosomally encoded as reported for bacteriocin genes in *L. lactis*

CHAPTER VII

EFFECTS OF BIFIDOCIN B AND LACTOCOCCIN R ON GROWTH OF
***Listeria monocytogenes* AND *Bacillus cereus* ON CHICKEN BREAST**

INTRODUCTION

Listeria monocytogenes is a gram-positive, non-spore forming, and psychrotrophic bacterium. It grows at temperatures between 1 and 45°C, with optimal growth occurring between 30 and 37°C. *L. monocytogenes* is widely distributed in the environment and has been isolated from a variety of sources including soil, vegetation, fecal, sewage, and water. Therefore, humans can come in contact with this pathogen. *L. monocytogenes* also can survive longer under adverse environmental conditions than most other non-spore forming bacteria of importance in foodborne disease, which makes this organism a particular threat to the food industry (Ryser and Marth 1991). *L. monocytogenes* is present in a wide variety of foods, both raw and processed such as milk and milk products, beef, pork, poultry, smoked and fermented sausage, salami, fresh vegetables, and seafoods (Lowett *et al.* 1987, Bailey *et al.* 1989, Beuchat and Brackett 1990 and 1991, Johnson *et al.* 1990, Farber 1991). It is able to grow in many foodstuffs during storage. Listeriosis outbreaks have been linked to the consumption of processed and ready-to-eat contaminated foods (Rocourt and Cossart 1997).

Bacillus cereus is a gram-positive, spore forming, aerobic rod, but it grows well anaerobically. *B. cereus* is a common soil saprophyte and easily spread to many types of

foods. Its presence on or in raw and processed grain, vegetable, meat, poultry, egg, and dairy products has been reported by several researchers (Johnson 1984, Sooltan *et al.* 1987, Konuma *et al.* 1988, Kramer and Gilbert 1989, Dufrenne *et al.* 1994, Sutherland and Murdoch 1994, Hassan and Nabbut 1996, Granum 1997). Certain strains of *B. cereus* are capable of producing a heat-labile diarrheal enterotoxin and a heat-stable emetic enterotoxin, as well as other toxins. Desserts, meat dishes, and dairy products are the foods most frequently associated with diarrheal illness, whereas rice is the most common vehicle of emetic illness (Kramer and Gilbert 1989). Some *B. cereus* strains are psychrotrophic and capable of growing and producing enterotoxins at refrigeration temperature. These variants raise concern about the safety of cooked, refrigerated foods with extended shelf life.

Bacteriocins are antimicrobial proteinaceous compounds produced by bacteria. Within the past several years there has been an increased interest in the development of bacteriocins as food preservatives. The authorization by the U.S. Food and Drug Administration (FDA) in 1988 for use of nisin as a food preservative in pasteurized processed cheese spreads opened the door for the potential application of bacteriocins in U.S. foods. A contributory factor for the increased interest in bacteriocins is the great concern that has grown in the food industry regarding *L. monocytogenes* (Hoover 1993).

Bifidocin B produced by *B. bifidum* is inhibitory to some strains of *Listeria*, *Bacillus*, *Micrococcus*, *Enterococcus*, *Lactobacillus*, and *Leuconostoc* (Yildirim and

Johnson 1998a). Lactococcin R produced by *L. lactis* subsp. *cremoris* R is active against *Listeria*, *Bacillus*, *Staphylococcus*, *Clostridium*, *Micrococcus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* (Yildirim and Johnson 1998b). The mode of action of both bacteriocins against *L. monocytogenes* in a model broth system is bactericidal. The objectives of this study were to determine the effects of bifidocin B or lactococcin R against growth of *L. monocytogenes* or *B. cereus* cells on irradiated-sterilized chicken breast under refrigeration and room temperature conditions.

MATERIALS AND METHODS

Cultures

Pure cultures of *L. monocytogenes* Scott A or *B. cereus* ATCC 232 were grown on BHI broth (Difco) at 37°C for 18 h, and *B. bifidum* and *L. lactis* subsp. *cremoris* R were grown on lactobacilli MRS plus cystein (0.05%) (MRS-C) at 37°C for 18 h and MRS broth at 30°C for 24 h, respectively. The indicator organism *L. plantarum* was grown on lactobacilli MRS. Working cultures were inoculated from frozen stocks as needed and incubated at their optimum growth temperatures.

Preparation of irradiated chicken breast

Fresh, whole, boneless chicken breast was cut in 5 g pieces and pieces were packed flat in 0.47-liter re-sealable polyethylene plastic bags (Glad-Lock, First Brand Corp.) and frozen overnight at -70°C. Frozen packages were packed in dry ice, shipped by overnight express, and irradiated (30 kGy) in a linear accelerator unit at Iowa State

University, Ames, IA. The sterile chicken pieces were confirmed to be free of vegetative bacterial cells by plating samples on BHI agar and incubating at 37°C for 24-48 h. Sterile chicken meat was stored at -70°C.

Bacteriocin preparation

For the production of bifidocin B or lactococcin R, *B. bifidum* NCFB 1454 or *L. lactis* subsp. *cremoris* R (1% v/v) were grown in MRS-C broth (2 L) at 37°C for 12 h or MRS broth (2 L) at 30°C for 22 h, respectively. After culture supernatants (or cell free extract) were collected by centrifugation at 1,000 x g for 20 min, they were filter sterilized (0.45 µm pore size) and freeze dried. The Freeze dried supernatants were reconstituted with 150 ml of distilled water, and their pH values were adjusted to 6.0 with 5 M phosphoric acid or 5 M NaOH. Micro-Cel (food grade diatomite calcium silicate) was added to yield 2 % (wt/v) (Coventry *et al.* 1996). The samples were stirred overnight at 4°C for bacteriocin adsorption onto Micro-Cel and then centrifuged at 1,000 g for 20 min. Bacteriocin adsorbed Micro-Cels were washed with sterile distilled water and resuspended to the original volume of 50 ml with 100 mM NaCl. For desorption of the bound bacteriocin, the pHs of the Micro-Cel samples were adjusted to 2.0 with 5 M phosphoric acid. The samples were stirred for 2 h at 4°C and heated at 80°C for 5 min. After centrifugation at bacteriocins 1,000 g for 20 min, the pHs of the supernatants were adjusted to 4.0 with 5 M NaOH, freeze dried and storage at -20°C until used.

Analysis of the effect of bifidocin B or lactococcin R on viability of *L. monocytogenes* or *B. cereus*

Five g irradiated chicken was used for each sample. Treatments were as follows: control-1 (only irradiated chicken, CC), control-2 (10^6 CFU of *L. monocytogenes* per g of chicken, CLm), control-3 (10^6 CFU of *B. cereus* per g of chicken, CBc), control-4 (3,200 AU of bifidocin B per g of chicken, CB), control-5 (3,200 AU of lactococcin R per g of chicken, CR), other samples received 10^6 CFU of *L. monocytogenes* or *B. cereus* per g of chicken plus 800, 1,600, or 3,200 AU of bifidocin B or lactococcin R (B8Lm, B16Lm, B32Lm, B8Bc, B16Bc, B32Bc, R8Lm, R16Lm, R32Lm, R8Bc, R16Bc, R32Bc). Sodium phosphate buffer (5 mM PBS) was added to all samples to make volume 5 ml. Samples were stored at refrigeration temperature (5-8°C) for 0, 7, 14, 21, or 28 days or at room temperature (22-25°C) for 0, 6, 12, or 24 h. Samples were randomly withdrawn during experiment time and stomached with a Stomacher Lab Blender 400 (Tekmar) for 30 sec. The filtered liquid was assayed for *L. monocytogenes* or *B. cereus* counts on spread plates containing BHI agar (1.5% agar) and plates were incubated at 37°C for 48 h.

Statistical analysis

Data were analyzed using the general linear model procedure of SAS Institute, Inc. (1992) to determine differences between treatment means. Pairwise comparisons of all treatment means were performed using the Least Significant Difference (LSD) procedure at $P < 0.05$. Experiments were repeated three times in a completely randomized design.

RESULTS

Effect of bifidocin B or lactococcin R on growth of *L. monocytogenes* or *Bacillus cereus* at 5-8°C

The inhibitory effects of bifidocin B or lactococcin R on the growth of *L. monocytogenes* or *B. cereus* at refrigeration temperature (5-8°C) were given in Fig. 1. Chicken samples inoculated with *L. monocytogenes* or *B. cereus* without bifidocin B or lactococcin R showed increases of 2.03 or 2.13 log units over 28 days in colony counts, respectively ($P < 0.05$). Negative controls consisting of chicken samples containing only PBS, bifidocin or lactococcin R were bacteria free over 28 days at 5-8°C or 24 h at 22-25°C. All three bifidocin B treatment levels produced significant decreases in *L. monocytogenes* counts at day 0 which ranged from 1.05 to 2.67 log units/g of chicken ($P < 0.05$) (Fig. 1A). Bifidocin B at 800 AU/g continued to cause inhibition from day 7 to day 14, causing a further 0.85 log unit decrease in *L. monocytogenes* counts by day 14. However, this treatment level ceased to inhibit growth after day 14 and allowed a 2.24 log unit increase by day 28. Conversely, bifidocin B concentrations of 1,600 or 3,200 AU/g produced inhibitory effects over 28 days, reducing counts by 3.65 or 5.13 log units, respectively (Fig. 1A).

The lactococcin R at level of 800 AU/g showed inhibitory effect from day 0 to day 21, causing 1.62 and 3.62 log unit decreases at these respective days ($P < 0.05$), while the lactococcin 1,600 AU/g level produced inhibitory effects at all experiment times, from 0 to 28 days, reducing colony counts by 2.44 log units at 0 day and 5.39 log units at day 28 ($P < 0.05$) (Fig. 1A). The 3,200 AU/g level of lactococcin R caused even more significant reductions in the colony forming units of *L. monocytogenes* from 6.43

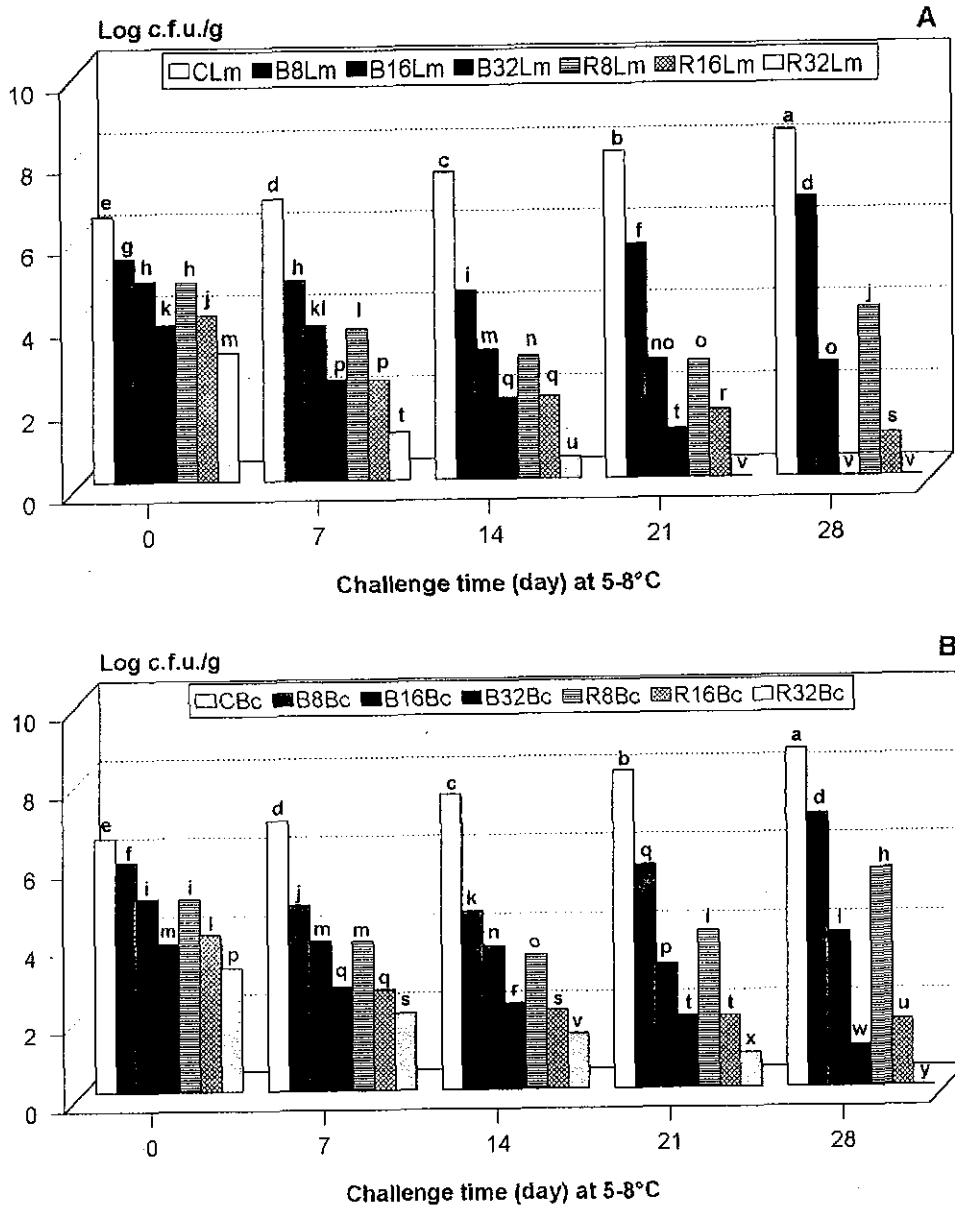


Fig. 7.1. Effect of bifidocin B or lactococcin R on growth of *L. monocytogenes* Scott A (Lm) (A) or *B. cereus* (Bc) (B) at 5-8°C. C with Lm or Bc was control chicken containing only Lm or Bc; B8, B16, and B32 with Lm or Bc were Lm or Bc plus 800, 1,600, or 3,200 AU of bifidocin B (B) per g of chicken, respectively; R8, R16 and R32 were Lm or Bc plus 800, 1,600 or 3,200 AU of lactococcin R (R) per g of chicken, respectively. Any two means followed by the same letter are not significantly different ($P > 0.05$).

log units to non-detectable levels after 21 and 28 days storage at 5-8°C ($P<0.05$).

It was found that both bifidocin B and lactococcin R had inhibition action on *B. cereus* on chicken breast at refrigeration temperature (Fig. 1B). Bifidocin B or lactococcin R at the level of 800 AU/g of chicken caused respective decreases of 0.62 and 1.56 log CFU/g by day 0, and 1.93 and 3.05 log CFU/g by day 14 ($P<0.05$). However, both bacteriocins at this low treatment level ceased to cause inhibition after day 14 and allowed both challenge pathogens to increase. At 1,600 AU/g of chicken, bifidocin B showed inhibition from day 0 to day 21 (reductions of 1.55 and 3.32 log units) ($P<0.05$), whereas lactococcin R at 1,600 AU/g caused decreases in colony counts of *B. cereus* from day 0 to day 28 (reductions of 2.48 and 4.8 log units) ($P<0.05$). At 3,200 AU/g of chicken, both bifidocin B and lactococcin R produced inhibitory effects over the entire 28 days ($P<0.05$) (Fig. 2), resulting in reductions of 2.7 or 3.4 log units by day 0, and 5.44 or 6.49 log units by day 28, respectively.

Effect of bifidocin B or lactococcin R on growth of *L. monocytogenes* or *Bacillus cereus* at 22-25°C

Both bacteriocins were less effective against *L. monocytogenes* or *B. cereus* at 22-28°C than at 5-8°C. Control irradiated chicken breast samples inoculated *L. monocytogenes* without bacteriocin showed an increase in counts from 6.08 to 8.12 over 24 hours at 22-25°C ($P<0.05$) (Fig. 2A). Conversely, control chicken containing *B. cereus* showed an increase in the colony forming units from 6.11 to 8.39 log CFU/g over 24 hours ($P<0.05$) (Fig. 2B). A 800 AU/g level of bifidocin B or lactococcin R reduced the colony counts of *L. monocytogenes* by 1.02 or 1.28 log units at 0 hour and 1.51 or 2.53 log units at 6 hours, respectively ($P<0.05$); however, both bacteriocins at this low

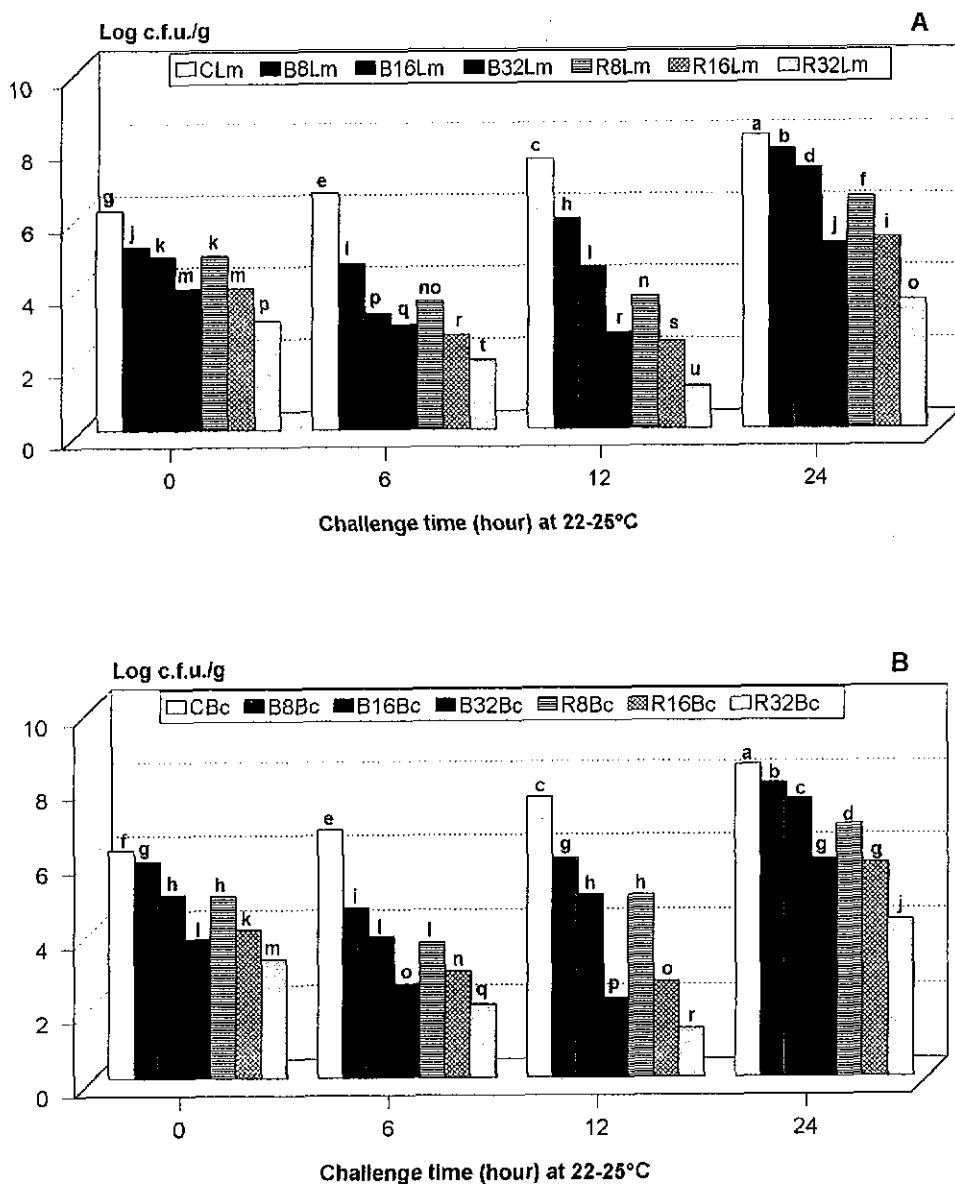


Fig. 7.2. Effect of bifidocin B or lactococcin R on growth of *L. monocytogenes* Scott A (Lm) (A) or *B. cereus* (Bc) (B) at 22-25°C. C with Lm or Bc was control chicken containing only Lm or Bc; B8, B16, and B32 with Lm or Bc were Lm or Bc plus 800, 1,600, or 3,200 AU of bifidocin B (B) per g of chicken, respectively; R8, R16 and R32 were Lm or Bc plus 800, 1,600 or 3,200 AU of lactococcin R (R) per g of chicken, respectively. Any two means followed by the same letter are not significantly different ($P > 0.05$).

level had no inhibitory effect after 6 hours (Fig. 2A). At 1,600 AU/g of chicken, bifidocin B showed inhibition against *L.monocytogenes* over 6 hours, reducing counts by 2.98 log units ($P<0.05$), but allowed significant increases from 6 to 24 h (Fig. 2A). Conversely, lactococcin R at the same level caused in reductions in colony counts from 0 to 12 h of 1.3 to 3.61 log units ($P<0.05$) before allowing a significant increase at 24 h. At a concentration of 3,200 AU/g of chicken, bifidocin B or lactococcin R each had inhibitory effects on *L. monocytogenes* from 0 to 12 hours, causing counts decrease of 2.17 to 3.44 or 3.07 to 4.91 log units respectively ($P<0.05$) (Fig. 2A).

Challenge tests with *B. cereus* gave results similar to these for *L. monocytogenes*. Bifidocin B or lactococcin R concentrations of 800 AU/g of chicken produced an inhibitory effects on *B. cereus* from 0 hour to 6 hours at 22-25°C ($P<0.05$), reducing counts by 0.3 to 1.57 or 1.24 to 2.50 log unit, respectively (Fig. 2B). At 1,600 AU/g, bifidocin B reduced the colony units by 1.19 log CFU/g by 0 hour and 2.32 log CFU/g by 6 h ($P<0.05$), while lactococcin R at the same concentration caused inhibition at 0, 6 or 12 h ($P<0.05$), reducing the counts by 2.14, 3.26 and 3.55 log CFU/g, respectively (Fig. 2B). A 3,200 AU/g treatment of bifidocin B or lactococcin R produced reductions in colony counts at 0, 6, or 12 h at 22-25°C ($P<0.05$) (Fig. 2B). At this highest level, bifidocin B at 0, 6, or 12 h decreased the count by 2.37, 3.6, or 4.02 log units, while lactococcin R caused 2.92, 4.13 or 4.80 log unit decreases, respectively. However, both bacteriocins failed to inhibit the growth of *B. cereus* from 12 to 24 h at this abuse temperature.

DISCUSSION

In this study, the effects of bifidocin B and lactococcin R on the growth and

survival of *L. monocytogenes* or *B. cereus* were examined. Growth characteristics of *L. monocytogenes* and *B. cereus* in chicken breast were affected by storage temperature and by treatment levels of the bacteriocin bifidocin B or lactococcin R tested.

It was found that the inhibitory effect of bifidocin B or lactococcin R was more pronounced at 5-8°C than 22-25°C. A concentration of 3,200 AU/g of bifidocin B or lactococcin R prevented growth of *L. monocytogenes* or *B. cereus* for 28 days at 5-8°C, whereas at the same concentration these bacteriocins remained inhibitory for 12 h at 22-25°C. Other researchers have reported that nisin was more inhibitory at 8°C than at 15°C against *B. cereus* and at 4°C than at 20°C against *L. monocytogenes* (Fang and Lin 1994, Beuchat *et al.* 1997). The magnitudes of the reductions in the numbers of cells were increased as the concentrations of bifidocin B or lactococcin R were increased from 800 to 3,200 AU/g. Golf *et al.* (1996) reported the same trends for pediocin treatments used against *L. monocytogenes* on irradiated chicken meat at 5-8°C.

The reinitiation of growth by *L. monocytogenes* or *B. cereus* on chicken breast pieces containing bifidocin B or lactococcin R after an initial inhibition period may be due to losses in bacteriocin activity which are known to occur as temperature and pH are increased (Delves-Broughton and Gasson 1994). Also, it is possible that *L. monocytogenes* and *B. cereus* produced some proteolytic enzymes which could have inactivated bifidocin B or lactococcin R. Studies have shown that retention of nisin activity is dependent upon the storage temperature, time, and pH (Delves-Broughton *et al.* 1996).

It has been estimated that the minimal infective doses for *L. monocytogenes* or *B. cereus* are 10^5 to 10^6 CFU/g (Kramer and Gilbert 1989, Ryser and Marth 1991). Using these estimates, our results indicate that treating chicken breast with 1,600 to 3,200 AU/g of bifidocin B or lactococcin R provide a good margin of safety by preventing growth of these two pathogens for 3-4 weeks at 5-8°C or 6-12°C at 22-25°C.

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ISOLATION, PURIFICATION, CHARACTERIZATION, MODE
OF ACTION, AND AMINO ACID SEQUENCE OF
BIFIDOCIN B AND LACTOCOCCIN R

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of the requirements for the degree of
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ABSTRACT

Ten dairy products and ten fresh vegetables products were researched for bacteria that produce bacteriocins. Also, *Bifidobacteria* species from ATCC (11863, 29521), and NCFB (1453, 1454, 1455) were examined for possible production of bacteriocins. Only *Bifidobacterium bifidum* NCFB 1454 and a radish isolate, *Lactococcus lactis* subsp. *cremoris* R, produced bacteriocins designated bifidocin B (BB) and lactococcin R (LR), respectively. BB and LR were sensitive to some proteolytic enzymes tested. Both bacteriocins were resistant to organic solvents, pH, or heating (90-121°C) and were active against *Listeria*, *Enterococcus*, *Bacillus*, *Micrococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* species tested. Also, LR was inhibitory to *Clostridium*, *Staphylococcus* and *Streptococcus* species. Their activity (1,280 AU/ml) on *L. monocytogenes* was bactericidal, reducing colony counts by 99.9% in 3-h.

BB and LR were purified to homogeneity by a simple purification procedure including freeze-drying, Micro-Cel adsorption/desorption and cation-exchange chromatography. These bacteriocins caused sensitive cells to lose intracellular K^+ ions, UV-absorbing materials and become more permeable to ONPG. Bacteriocins adsorbed to the gram-positive bacteria but not gram-negative bacteria tested. Several salts inhibited their binding. Also, addition of purified lipoteichoic acid to sensitive cells and methanol:chloroform plus hot 20% TCA treatment on cell wall preparations blocked bacteriocin adsorption. The N-terminal amino acid sequence of BB yielded