Silent Players of Health in a Broader Age-management Medicine Understanding: a Dissertation on Bacteriocin of Lactic Acid Bacteria.

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The preservation of foods in healthy and safe condition has long been used and still it remains an on-going challenge for food microbiologists. Drying, salting and fermentations were the traditional methods of preservation. Canning and freezing were relatively recent developments.

The role of fermented milk in human diet in well known since Vedic times but the scientific interest arose only after the publication of a book "*Prolongation of Life"* (Metchnikoff, 1908). In developed societies, food preservation is viewed as a 'convenience' of an efficient food system, and food preservation is the key to ensure the availability of food as vital benefit. Food fermentations, developed by default rather than by design. Lactic acid bacteria (LAB) play an important role in food fermentations, causing the characteristic flavor, changes and exercising a preservation effect on the fermented product (Caplice and Fitzgerald, 1999). It is estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Holzapfel *et al.,* 1995). The spice trade was the start of addition of the chemicals adjunct to foods. With the industrial revolution and subsequent development of food industries, food processing moved from kitchen or cottage industries to large-scale technological operations with increased need for food preservation. This stimulated the use of food additives, especially those that preserve the foods and enhance food quality. This has resulted in the emergence of a new generation of chill stored, minimally processed foods (de Souza *et al.*, 2005).

Hurst, (1973) reviewed the preservation of foods by the antagonistic growth of microorganisms. He showed the growth of lactic acid bacteria (LAB) in milk, saurkaut and vacuum packaged meats as examples of protective and antagonistic growth. In recent times this has been termed as 'biopreservation' to differentiate it from the chemical (artificial) preservation of foods.

Biopreservatives such as lactic acid bacteria (LAB) and their metabolites have been investigated by several authors (Buncic *et al.,* 1997; Pirttijarvi *et al.*, 2001; Sakhare and Narasimha Rao, 2003). Considerable research has been done on the ability of LAB to inhibit growth of pathogenic microorganisms (Winkowski *et al.,* 1993; Minor-Perez *et al.,* 2004). The capability of these bacteria to control growth of spoilage microorganisms has not been investigated to the same extent. To be successful in biopreservation, a bacteriocinogenic LAB culture must compete with the relatively high indigenous microbial load of raw meat, to actively inhibit pathogenic and spoilage bacteria (Sakhare and Narasimha Rao, 2003; Minor-Perez *et al.,* 2004).

Bacteria preserve foods as a result of competitive growth, products of their metabolism and bacteriocin production. Biopreservation refers to extended storage life and enhanced safety of foods using their natural or controlled microflora and (or) their antibacterial products. It may consist of (i) adding bacterial strains that grow rapidly and (or) produce their antibacterial products; (ii) adding purified antagonistic substance(s); (iii) adding the fermentation liquor or concentrate from an antagonist microorganism; or (iv) adding mesophilic LAB and other related bacteria as a

'fail-safe' protection against temperature abuse.

LAB and other related bacteria produce lactic acid or lactic and acetic acid, and they may produce other inhibitory substances such as diacetyl, hydrogen peroxide, reuterin (-hydroxypropionaldehyde) and bacteriocins (de Vuyst and Degeest, 1999).

Bacteriocins:-

Bacteriocins have been described as ribosomally synthesized extracellular macromolecular precursor polypeptides or proteins produced by one bacterium that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) and, as with host defence peptides (Jack *et al.,* 1995; Russell and Mantovani, 2002; Bowdish *et al.,* 2005). Bacteriocins have bacteriocidal activity (Tagg *et al.,* 1976) due to the combined action of the bacteriocin and the host autolysis (Martinez-Cuesta *et al.,* 2000), or bacteriostatic against other species, usually closely related to the producer strain (Russell and Mantovani, 2002). In some cases, they are also active against other species (Klaenhammer, 1993; Jack *et al.,* 1995).

Bacteriocins are heterogeneous group of bacterial antagonists that vary considerably in molecular weight, biochemical properties, range of sensitive hosts and mode of action. Klaenhammer (1988) defined them as, proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium. As peptides, bacteriocins are of low molecular weight, but larger than antibiotics. This makes them susceptible to biochemical reactions, which may limit their antimicrobial activity (Muriana, 1996). CAST (Council of Agricultural Science and Technology) (1998) reported that concentration, microorganisms, pH, temperature and interactions affect the activity of bacteriocins.

Bacteriocins are produced by both Gram-positive and Gram-negative bacteria including LAB, which are used in food fermentations (Klaenhammer, 1988; Daeschel, 1989; Ray and Daeschel, 1992; Nettles and Barefoot, 1993; Klaenhammer, 1993; Sahl *et al.,* 1995; Muriana, 1996) Two well-known representatives of bacteriocins produced by Gram-negative bacteria are colicins and microcins. The first desription of bacteriocin-mediated inhibition was reported 80 years ago, when antagonism between strains of *Escherichia coli* was first discovered (Gratia 1925), they were named as colicins (Fredericq, 1948), bacteriocins produced by *Escherichia coli* and usually showing activity against other strains of *E. coli* and very closely related members of the Enterobacteriaceae. Induction usually accurs under stressful conditions such as nutrients depletion or over crowding (Riley and Gordon 1999). Colicins have been studied for over six decades and are well characterized (Akutsu *et al.,* 1989). Colicins differ from bacteriocins that are preduced from Gram-positive bacteria in the sense that they have 3 general mechanisms of action: channel formation in the cytoplasmic membrane, (The common mechanism found with Gram-positive bacteriocins), degradation of cellular DNA, and inhibition of protein synthesis. It is estimated that about 30% of natural population of *E.coli* produce bacteriocins (Riley, 1998). Colicins are plasmid encoded bacteriocins and classified into groups on the basis of the receptor to which they bind. Over 25 colicin types have been identified (Pugsley, 1984). It is also estimated that about 65% of the cells in a population of *E. coli* are resistant to any one colicin, and 30% are resistant to all colicins produced in a population with the remaining cells colicin-sensitive (Smarda, 1992). The relative numbers of colicin-producing cells have been found dependent on the energy costs associated with colicin synthesis (Riley and Gordon, 1999).

The Gram-negative bacteriocins are colicin, which are produced by strain of E. coli (Braun et al., 1994; Riley and Gordon, 1999). These are large, complex proteins, that inhibit bacterial growth through the inhibition of cell synthesis, permeabilizing the cell membrane or inhibiting Rnase or Dnase activity (Cleveland et al., 2001) 20-90 Kda, with characteristic structural domains involved in cell attachment, translocation and bactericidal activity. They bind to specific receptors on the outer membrane of the target cell. The bacteriocins produced by Gram-positive bacteria are small peptides 3-6 Kda, in size (Nes et al., 1996), although there are exceptions (Joerger and Klaenhammer, 1990). They fall with in two broad classes, viz (namely) the lantibiotics (Jack et al.,

1995) and the non-lantibiotic bacteriocins (Nes et al., 1996). Most of the Gram-positive bacteriocins are membrane active compounds that increase the permeability of the cytoplasmic membrane (Jack et al., 1995). They often show a much broarder spectrum of bactericidal activity than the colicins. There is currently much interest in the application of bacteriocins in both food preservation and the inhibition of pathogenic bacteria (Liao et al., 1994; Delves-Broughton, 1996; Cleveland et al., 2001; de Souza et al., 2005). Most of the bacteriocins have been isolated from organisms involved in food fermentation. Bacteriocin production and resistance is considered as an important property in strains used as commercial inoculants to eliminate or reduce growth of undesirable or pathogenic organisms.

Microcins, produced by the Gram-negative bacteria of family Enterobacterioaceae, are post-translationally modified. They are active against other Gram-negative bacteria and act through inhibition of DNA replication or protein synthesis (Bacquero and Moreno, 1984).

Lactic acid bacteria (LAB):- Lactic acid bacteria (LAB) have played a long and important role in food technology. These microorganisms are industrially important and have been used as starter cultures in various foods–fermentation processes. Global production of cheese starter cultures, for example, already 1.5×10^6 tons per year (Fox, 2000). The LAB include a wide variety of cell types and physiological and biochemical characteristics (Yanagida *et al.,* 2005). Lactic acid bacteria (LAB) are a group of bacteria united by a constellation of morphological, metabolic and physiological characteristics. The currently recognized genera of LAB are *Aerococcus, Alloicoccus, Carnobacterium, Dolosigranulum, Enterococcus, Globicalella, Lactococcus, Lactosphaera, Leuconostoc, Melissioccus, Oenococcus, Pediococcus, Strepcoccus, Tetragenococcus, Vagococcus and Weissella* (Axelsson, 1998). This classification is largely based on phenotypic characteristics such as morphology, mode of glucose fermentation, growth at different temperature, configuration of lactic acid produced, ability to grow at high salt concentration, and acid or alkaline tolerance (Axelsson, 1998).

Generally, LAB are described as Gram-positive, non-motile, non-spore forming and microaerophilic rods (singly or in chains) or cocci (diplococci, tetracocci, streptococci). These bacteria usually belong to the family Lactobacteriacae and are characterized by the production of lactic acid as a major metabolic end product of carbohydrate fermentation (Axelsson, 1998)**,** hydrogen peroxide, diacetyl secondary reaction products and bacteriocins, which may be important for starter culture functions of the bacteria (Daeschel, 1989). The characteristics of LAB used as a starter culture are well documented (Tramer and fowler, 1964). Lactic acid bacteria (LAB) are low-GC-content, Gram-positive bacteria, which are found in nutrient-rich environments such as milk, meat, decomposing plant material and the mammalian gastrointestinal tract, (Carr *et al.,* 2002). Surface growth on most media is very poor. The nutritional requirement of this group is complex; they need amino acids and vitamins.

Lactic acid bacteria may be homofermentative or heterofermentative. Those bacteria which ferment only lactic acid from lactose are known as homofermentative and those that ferment other than lactic acid, *e.g.,* acetic acid alcohol and produce CO_2 are heterofermentative. They are catalase negative, acid tolerant, and lack cytochromes and porphyrins (Adams *et al.,* 1995).

Lactic acid bacteria (LAB) in the form of fermentative organisms are traditionally used to preserve food and feed. It is well known that many species of *Lactobacillus* and *Lactococcus* used in the manufacture of fermented dairy products inhibit the growth of other bacteria including intestinal pathogens like *Escherichia coli, Enterobacter faecalis, etc.* Bacteriocins produced by LAB are of great interest to the food industry because of their antagonistic effect against food borne pathogenic and spoilage microorganisms (Matchikoff, 1908; Ray *et al.*, 2001). The inhibitor part is a protein that could not be destroyed in milk even on heating at 100°C for 30 min and was inhibitory to several strains of *Streptococcus lactis*. Among the microorganisms inhibited by certain bacteriocins, numerous reports have included the fatal pathogen *Listeria monocytogenes* (Spelhang and Harlander, 1989; Muriana, 1996; Klaenhammer, 1993; Ennahar *et al.,* 2000; Hechard et al., 2002).

Over the years, several publications have reviewed colicins, bacteriocins, bacteriocins from LAB and applications of specific bacteriocins. Examples include, Reeves (1972), Franklin and snow (1975), Hardy (1975), Tagg *et al.,* (1976), Konisky (1982), Klaenhammer (1988, 1993), Jack *et al.,* (1995), de Vos *et al.* (1995b), Sahl *et al.* (1995), Venema *et al.* (1995), Abee *et al.* (1995), Nes *et al.* (1996), Cleveland *et al.* (2001).

Taxonomy of lactic acid bacteria:- The classification of LAB was initiated in 1919 by Orla Jensen and was until recently primarily based on morphological, metabolic and physiological criteria. Lactic acid bacteria comprise a diverse group of Gram positive, non-spore forming, non-motile rod and coccus shaped, catalase-lacking organisms. They are chemoorganotrophic and only grow in complex media. Fermentable carbohydrates and higher alcohols are used as the energy source to form chiefly lactic acid. LAB degrades hexoses to lactate (homofermentatives) or lactate and additional products such as acetate, ethanol, CO_2, formate or succinate (heterofermentatives). They are widely distributed in different ecosystems and are commonly found in foods (dairy products, fermented meats and vegetables, sourdough, silage, beverages), sewage, on plants but also in the genital, intestinal and respiratory tracts of man and animals whose they play important roles as symbionts.

Current methodolgies used for classification of LAB mainly rely on 16S ribosomal ribonucleic acid (rRNA) analysis and sequencing (Olsen *et al.* 1994). Based on these techniques, Gram-positive bacteria are divided into two groups depending on their G + C content. The Actinomycetes have a G + C content above 50 mol% and contain genera such as *Atopobium, Bifidobacterium, Corynobacterium* and *Propionibacterium.* In contrast, the *Clostridium* branch has a G + C content below 50 mol% and include the typical LAB genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus.*

Table 1. Orla-Jensen (1919) key to differentiation of the lactic acid bacteria and current taxonomic classification.

Genus[a]	Shape	Catalase	Nitrite reduction	Fermentation	Current genera
Betabacterium	Rod	-	-	Hetero	*Lactobacillus Weissella*
Thermobacterium	Rod	-	-	Homo	*Lactobacillus*
Streptobacterium	Rod	-	-	Homo	*Lactobacillus Carnobacterim*
Streptococcus	Coccus	-	-	Homo	*Streptococcus Enterococcus Lactococcus Vagococcus*
Betacoccus	Coccus	-	-	Heter	*Leuconostoc Oenococcus Weissella*
Microbacterium	Rod	+	+	Homo	*Brochothrix*
Tetracoccus	Coccus	+[b]	+	Homo	*Pediococcus Tetragenococcus*

[a]*According to Orla-Jensen (1919).*
[b]*In genera Pediococci are catalase negative but some strains produce a pseudocatalase that results in false positive reactions.*
+ =Positive result, - = Negative result

Bcteriocins from lactic acid bacteria:-

The bacteriocins from LAB are mostly small, heat-stable, hydrophobic and cationic peptides (Jack *et al.,* 1995). Several bacteriocins of LAB have been characterized biochemically and genetically and in a number of cases their mode of action has been studied (Hoover and steenson, 1993; Klaenhammer, 1993; Chen and Yanagida, 2006; Kabuki *et al.* 2007).

Ever since, the publication of the first review on the bacteriocins of Gram-positive bacteria by Tagg *et al.* (1976), there has been a renewed interest in the field of bacteiocins of Gram-positive bacteria. The researches on bacteriocins produced by a heterogeneous group of Gram positive bacteria comprising genera, *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Carnobacterium*, collectively known as lactic acid bacteria (LAB) has witnessed a tremendous growth in the past one and half decade as evident from the publications of several review articles (Klaenhammer, 1988; Piard and Desmazeaud, 1992; Klaenhammer, 1993; Nettles and Barefoot, 1993) and books (Ray and Daeschel, 1992; Hoover and Steenson, 1993) dealing with various aspects of bacteriocins produced by lactic acid bacteria. The interest in microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains (Leisner *et al.,* 1999).

In the dairy products, the species composition of lactic acid bacteria is more varying and inconsistent when compared with those of the trade products. In biotechnological aspects, the "wild" strains of the LABs are prospective bacteriocin producers (Niku-paavola *et al.,* 1999) and probiotics (Rinkinen *et al.*, 2003).

Bacteriocin producing LAB in food preservation

has led to the isolation and characterization of several bacteriocins. The bacteriocin producing LAB have been isolated from various sources such as vegetables, meat and meat products, milk and milk products *etc*. In some cases, an identical bacteriocin may be produced by different subspecies of the same species as observed for lactococcin A (Neve *et al.,* 1984; Holo *et al.,* 1991). There are also incidents where a single strain produces more than one bacteriocin as recorded for *L. lactis* subsp. *cremoris* 9B4 (van Belkum *et al.,* 1991a, 1992) and *Lactobacillus plantarum* LPC010 (Jimenez-Diaz *et al.,* 1993).

Numerous bacteriocins produced by species and strains of LAB were identified in 1980s and 1990s. These include lactocin and helveticin (*Lactobacillus helveticus*), lactocin B and F (*Lactobacillus acidophilus*), curvacins (*Lactobacillus curvatus*), propionicin (*Propinibacterium* spp.), plataricin A (*Lactobacillus plantarum*), Las 5 and diplococcin (*Streptococcus cremoris*), mesenterosins and leuconosins (*Leuconostoc* spp.) and pediocins (*Pediococcus acidilactici* and *Pediococcus pentasaceous*) (Klaenhammer, 1988; Daeschel, 1989; CAST, 1998).

Classification of bacteriocin from LAB:- During recent years, a large number of novel bacteriocins have been identified from several different LAB. Based on their amino acid sequences, stability to heat, size, mode of action, biological activities, secretion mechanism and the presence of modified amino acids, LAB bacteriocins have been classified into three classes of which the first two classes have further been subtyped (Klaenhammer, 1993; Nes *et al.,* 1996).

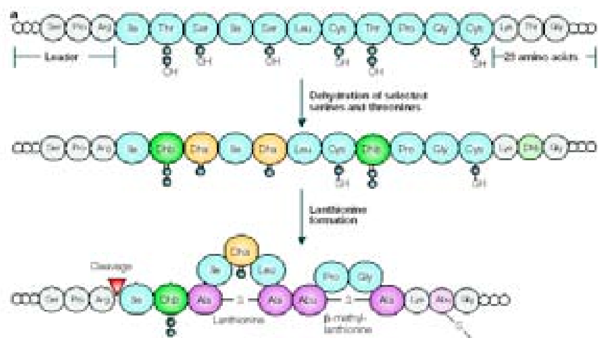

***Figure 1 Lanthionine synthesis. As shown in a, lanthionine residues are formed when an enzymatically dehydrated serine (dehydroalanine, Dha) condenses with the sulphydryl group of a neighbouring cysteine (Cys). This forms a bridge between the two residues, thereby creating a ring within the modified peptide or lantibiotic. When the partners are threonine (Thr) and cysteine, the novel residue is a β-methyllanthionine. The resulting lanthionine and β-methyllanthionine bridges are indicated in pink as Ala–S–Ala (alanine–S–alanine) and Abu–S–Ala (aminobutyrate–S–alanine), respectively. Many lantibiotics also contain dehydrated serines (Ser) and threonines (dehydrobutyrine, Dhb)*. *Source*:-** Cotter *et al.* (2005).

<u>Class I</u>– Lantibiotices (from lanthionine-containing antibiotic) are small (< 5kDa) peptides containing the unusual amino acids lanthionine (Lan), α-methyllanthionine (melan), dehydroalanine, and dehydrobutyrine. These bacteriocins are grouped in class I. Class I is further subdivided into type A and type B lantibiotics according to chemical structures and antimicrobial activities (Moll *et al.,* 1999). Type A lantibiotics are elongated peptides with a net positive charge that exert their activity through the formation of pores in bacterial membranes. Type B lantibiotics are smaller globular peptides and have a negative or no net charge; antimicrobial activity is related to the inhibition of specific enzymes. They are heat stable protein. *e.g.* nisin, lacticin 481, lactocin 5, Carnocin U 149 *etc.*

<u>Class II</u>– Small (< 10kDa), heat- stable, non-lanthionine containing peptides are contained in class II. The largest group of bacteriocins has been included in this classification system. These peptides are divided into 3 subgroups.

Class IIa includes pediocin-like peptides having N-terminal consensus sequence –Tyr-Gly-Asn-Gly-Val-Xaa-Cys. This subgroup has attracted much of the attention due to their anti-listeria activity (Ennahar *et al.,* 2000b).

Class IIb contains bacteriocins requiring two different peptides for activity, *e.g.* lactococcin G and lactocin F.

Class IIc contains the remaining peptides of this class, including sec-dependent secreted bacteriocins (Worobo *et.al.,* 1995). *e.g.* divergicin A.

<u>Class III</u>– These bacteriocins are not well characterized. This group contains large (> 30kDa) heat-labile proteins that are of lesser interest to food scientists. (Joerger and Klaenhammer, 1986;

Vaughan *et al.,* 1992) *e.g.,* helveticin I, caseicin 80, lacticins A and B.

A class IVth class consisting of complex bacteriocins that require carbohydrate or lipid moieties for activities has also been suggested by Klaenhammer (1993); however, bacteriocins in this class have not been characterized adequately at the biochemical level to the extent that the definition of this class requires additional descriptive information (Jimenez-Diaz *et al.,* 1995).

Cotter *et al.* (2006) have proposed a new scheme of classification for bacteriocins, which is reproduced below in Figure 2.

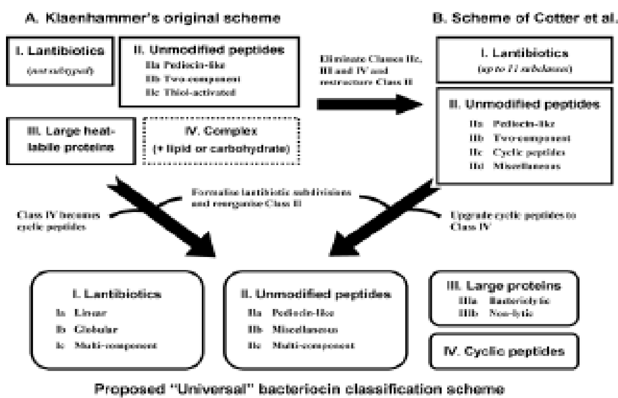

Figure-2: Proposed classification scheme for bacteriocins. ***Source*:-** Cotter *et al.* (2006) .

Chen and Hoover (2003) have summarized different classes of bacteriocins and their producer strain, which are reproduced below in Table 2.

Table 2:Examples of bacteriocin producing by lactic acid bacteria: -

BACTERIOCINS	PRODUCER	REFERENCES
CLASS I-typeA lantibiotics Nisin	*Lactococcus lactis*	Hurst 1981
lactocin S	*Lactobacillus sake*	Mortvedt *et al.*, 1991
Epidermin	*Staphylococcus epidermidis*	Allgaier *et al.*, 1986
Gallidermin	*Staphylococcus gallinarum*	Kellner *et al.*, 1988
lacticin 481	*Lactococcus lactis*	Piard *et al.*, 1992
CLASS I-typeB lantibiotics mersacidinj	*Bacillus subtilis*	Altena *et al.*, 2000
cinnamycin	*Streptomyces cinnamoneus*	Sahl and Bierbaum 1998
ancovenin	*Streptomyces* spp.	Sahl and Bierbaum 1998
duramycin	*Streptomyces cinnamoneus*	Sahl and Bierbaum 1998
actagardin	*Actinoplanes* spp.	Sahl and Bierbaum 1998
CLASS IIa pediocin PA-1/AcH	Pediococcus acidilactici	Henderson *et al.*, 1992
sakacin A	*Lactobacillus sake*	Holck *et al.*, 1992
sakacin P	*Lactobacillus sake*	Tichaczek *et al.*, 1992
leucocin A-UAL187	*Leuconostoc gelidum*	Hastings *et al.*, 1991
Mesentericin Y105	*Leuconostoc mesenteroides*	Hechard *et al.*, 1992
enterocin A	Enterococcus faecium	Aymerich *et al.*, 1996
divercin V41	Carnobacterium divergens	Metivier *et al.*, 1998
lactococcin MMFII	*Lactococcus lactis*	Ferchichi *et al.*, 2001
CLASS IIb lactococcin G	*Lactococcus lactis*	Nissen-Meyer *et al.*, 1992
lactococcin M	*Lactococcus lactis*	van Belkum *et al.*, 1991
lactacin F	*Lactobacillus johnsonii*	Allison *et al.*, 1994
plantaricin A	*Lactobacillus plantarum*	Nissen-Meyer *et al.*, 1993a
plantaricin S	*L. plantarum*	Jimenez-Diaz *et al.*, 1995
plantaricin EF	*L. plantarum*	Anderssen *et al.*, 1998
plantaricin JK	*L. plantarum*	Anderssen *et al.*, 1998
CLASS IIc acidocin B	*Lactobacillus acidophilus*	Leer *et al.*, 1995
carnobacteriocin A	*Carnobacterium piscicola*	Worobo *et al.*, 1994
divergicin A	*C. devergens*	Worobo *et al.*, 1995
enterocin P	*E. faecium*	Cintas *et al.*, 1997
enterocin B	*E. faecium*	Nes and Holo 2000
CLASS III helveticin J	*Lactobacillus heleveticus*	Joerger and Klaenhammer 1986
helveticin V-1829	*L. heleveticus*	Vaughan *et al.*, 1992

Bacteriocin produced by different group of lactic acid bacteria:-

1 *Lactococcus:* - *Lactococci* are coccibacteria, which form chains of variable length, They have a homo-fermentative metabolism and produce exclusively L (+) lactic acid (Roissart, 1994), although Akerberg *et al* (1998) reported that, D (−) lactic acid can also be produced specially at low pH values. Furthermore, *Lactococcus lactis* is sub-divided into other sub-species: *lactis, cremoris* and *diacetylactis*.(Schleifer and Kilpper-Balz, 1987; Kim *et al.,* 1999). Their most important habitat is untreated milk, fermented milk and cheeses. *Lactococcus lactis* subsp. *lactis*, either in pure form or associated with other microorganisms, is mesophilic strain most commonly used as a starter culture for lactic products, thus, they fulfill an irreplaceable role in ensuring the structure, taste, conservation and healthfulness of these products. (Jensen and Hammer, 1993; Salminen and Von wright, 1993; Roissart, 1994; Boonmee *et al.,* 2003; Ziadi *et al.,* 2005; Do-won *et al.,* 2006).

They also play an important role in aroma enhancement, the production of flavoured milk, and in milk and cheese flavouring, and recently a great deal of attention has been focused on their probiotic properties (Salminen and Von wright, 1993; Boonmee *et al.,* 2003). For these reason this microorganism, has great commercial potential, and that is why *Lactococcus*, and more especially *Lactococcus lactis*, isolated in the lactic industry, is still being studied exhaustively. The major product of fermentation is lactic acid, a compound with a high commercial value, with applications in the food, cosmetic, medical and pharmaceutical industries (Boonmee *et al.,* 2003.

Several scientists (Mattick and Hirsch, 1947; Neve *et al.,* 1984; Holo *et al.,* 1991 and Kojik *et al.,* 1991) have reported bacteriocinogenicity among the different strains of the three most economically important lactococcal species: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis biovar.diacetylactis.*

1.1*Lactococcus lactis* subsp. *lactis*-

Nisin: The most extensively characterized bacteriocin from lactic acid bacteria is produced by several strains of *Lactococcus lactis* subsp. *lactis.* Mattick and Hirsch coined the word 'nisin' to designate the group 'N' inhibitory substance in 1947. Nisin, is widely used bacteriocin, is normally ineffective against Gram negative bacteria, yeast and moulds, but effective against a wide range of Gram positive bacteria including other lactic acid bacteria, *Staphylococcus aureus* and *Listeria monocytogenes*. Gram-positive spore formers *i.e. Bacillus* spp. and *Clostridium* spp. are particularly sensitive to nisin with spores being more sensitive than vegetative cells (Ray, 1992). Hirsch *et al* (1951) first examined the potential of nisin as a food preservative. In 1957, nisin was reported to be commonly occurring in farmhouse cheese (Chevalier *et al.,* 1957). In that same year, Aplin and Barrett developed commercial preparations for use in foods (Delves-Broughton *et al.,* 1996). Nisin-like substances were found to be commonplace among cheese cultures (Hurst 1967), and now it is understood (that lactococci can produce other bacteriocins and inhibitory substances in addition to nisin.

First elucidated by Gross and Morell in 1971, nisin is a 34 amino, acid peptide. At least 6 different forms have been discovered and characterized (designated as A through E and Z), with nisin A, the most active type. Nisin Z is a natural variant of nisin differing from nisin A with substitution of a histidine residue for an aspartic acid. The most established commercially available form of nisin for use as, a food preservative is Nisaplin^{TM}, with the active ingredient 2.5% nisin A and the predominate ingredients NaCI (77.5%) and nonfat dry milk (12%, protein and 6% carbohydrate). Several companies market antimicrobial products containing nisin. It belongs to inhibitory substances called 'lantibiotics' a family of peptides containing unsaturated amino acids, dehydroalanine and dehydrobutyrine and thio-ether amino acids, lanthionine and -methyl lanthionine (Gross and Morell, 1967, 1971). It is a protein having 34 amino acids and molecular weight of 3.5 KDa (Klaenhammer, 1988). Nisin has five polypeptide variants, which are designated as A, B, C, D, and E. International acceptance of nisin was given in 1969 by the Joint Food and Agriculture Organization/World Heath Organizat*on* (FAD/ WHO) Expert Committee on Food Additives (WHO 1969). The only other antibiotic-like

compound with similar approval as a preservative is the surface-active antimycotic compound, pimaricin (Henning *et al.,* 1986). FAO/WHO Committee recommended a maximum daily intake of nisin for a 70-kg person to be 60 mg of pure nisin or 33000 Units (Hurst and Hoover 1993); however, nisin is permitted in processed cheeses in Australia, France, and Great Britain with no maximum limit. In the U.S., the maximum limit is 10000 IU/g; in Russia, the maximum limit is 8000 IU/g, while in Argentina, Italy, and Mexico, the limit is 500 IU/g for processed cheeses and other products (Chikinda and Montville 2002).

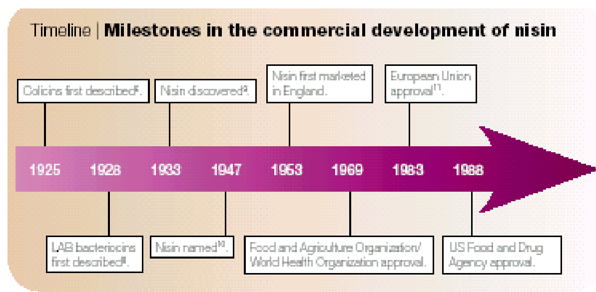

Figure 3: Milestones in the commercial delopment of nisin ***Source*:-** Cotter, *et al.* (2005).

In the USA, the FDA has affirmed a nisin preparation as a GRAS (Generally recognized as safe) substance for use in pasteurized cheese spreads for inhibition of outgrowth of *Clostridium botulinum*. Shtenberg and Ignatev already reported the toxicity of nisin in 1970. They suggested that the toxicity of nisin is low and it is not used in animal or human medicine.

A bacteriocin, lactacin 481, produced by *Lactococcus lactis* subsp. *lactis* CNRZ481 was found to be effective against *Lactococcus* spp., some *Lactobacillus* spp., *Leuconostoc* spp., and *Closrtridium* spp. reported by Piard *et al* (1990) The production of several lactococcins has been described in several other *Lactococcus lactis* subsp. *lactis* which include: Lactococcin by *Lactococcus lactis* subsp. *lactis* ADRI 85L030 (Dufour *et al.,* 1991) has been found to inhibit vegetative cells of *Clostridium tyrobutyricum*, strains of *Streptococcus thermophilus* and *Lactobacillus helveticus* but is rather inactive against other Gram positive and Gram negative genara (Thuault *et al.,* 1991); lactococcin G by *Lactococcus lactis* subsp. *lactis* LMG2081 (Nissen-Meyer *et al.,* 1992); lactococcin 972 by *Lactococcus lactis* subsp. *lactis* (Martinez *et al.,* 1996); lactococcin 484 by *Lactococcus lactis* subsp. *lactis* 484 has been reported to be effective against members of the *Lactococcus* group, *B. cereus*, *Staphylococcus aureus* and *Salmonella typhi* (Gupta and Batish, 1992).

Structure of bacteriocin: – Nisin is well known member of bacteriocin and is produced by strains of *Lactococcus lactis* subsp. *lactis*. Its structure is illustrated in Fig-4. (Kaletta and Entian, 1989). The name "nisin" for this bacteriocin is derived from the term "group N inhibitory substance" (where group N refers to sero group N of bacteria classified as member of the genus *Lactococcus*). Nisin consists of 34 amino acids, however, it is initially synthesized as prenisin, consisting of 23 amino acid leader peptide and the 34- amino acid pronisin peptide. (Nes, *et al.*1996).

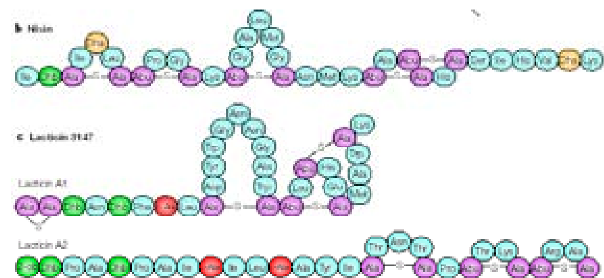

Figure 4 : Nisin structure. ***Source*:-** Cotter *et al.* (2005).

Variants of nisin differing in 1 amino acid are known. Certain serine and threonine residues in the pronisin are converted to dehydroalanine and dehydrobutyrine through dehydration. Thioether bonds are then formed by reaction with the sulfhydryl groups of cysteine residues in the pronisin (lanthionine, Ala-S-Ala; β-methyllanthionine; Ala-S –Aba; aminobutyric acid). Following these chemical modifications in the pronisin segment of prenisin, export and concomitant removal of the leader sequence yield active nisin.

Normally, two nisin molecules form a dimer. The early reports on the molecular weight of nisin as 7 kDa (approx) where, due to the formation of dimers rather than the approximately 3.5 kDa measured only (Joerger and Hoover, 2000), Sharma (2002) also reported 3.5 kDa molecular weight

of nisin isolated from *Lactococcus lactis* subsp. *lactis* CCSU1101.

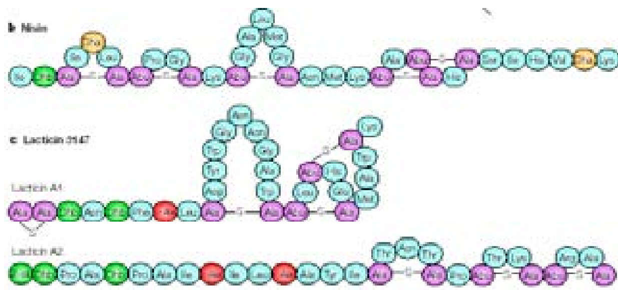

Figure 5: Lacticin A1 and A2 structure.

1.2 *Lactococcus lactis* subsp. *cremoris*- The first description of a proteinaceous inhibitor in lactococci was from *Lactococcus lactis* subsp. *cremoris*. The antimicrobial agent described by Whitehead in 1933 was later on partly purified and shown to be proteinaceous in nature. It was termed as 'Diplococcin' to signify the diplococcal arrangement of the producer cells. A number of lactococcins have been described for *Lactococcus lactis* subsp. *cremoris.* These include: lactococcin A from strain LMG2130 (Holo *et al.,* 1991) and strain 9B4 (Neve *et al.,* 1984). Later it was found that *Lactococcus lactis* subsp. *cremoris* strain 9B4 produced two more bacteriocins termed as lactococcin M (van Belkum *et al.* 1991) and lactococcin B (van Belkum *et al.* 1992). Huot *et al* (1996) described the production of a bacteriocin designated as Bacteriocin J46 by *Lactococcus lactis* subsp. *cremoris* J46.

Many substances have been reported from *lactococci, which* have been designated as lactococcin. These include lactococcin I, A, B and M, lactococcin I has been isolated from *Lactococcus lactis* subsp. *cremoris* strain A and C. Purified lactococcin has been reported to be heat stable (99°C, 33 min) peptide with a molecular weight of 6,000 Da. It is encoded by a 18.4 Kb fragment of DNA of a 60 Kb conjugative plasmid. It has been reported to inhibit other *Lactococci* and some *Clostridia*. (Geise *et al.* 1983).

Lactococcin A has been reported to be produced by three different *Lactococci*, which include 1.8 Kb region of plasmid p9 B4-6, from *Lactococcus lactis* subsp. *cremoris* 9B4, associated with production of another bacteriocin. It has also been cloned and analyzed (Van Belkum, *et al.,* 1991). Nucleotide sequence analysis of the resulting plasmid (pMB225) showed a ribosomal binding site followed by three ORFs designated ORFA-1 ORFA-2 and ORFA-3. The third ORF, ORFA-3 is suspected to encode an immunity protein for lactococcin M (Van Belkum *et al.* 1991).

Lactococcin B is a bacteriocin associated with a 1.2 Kb fragment present in plasmid p9B4--6 of *Lactococcus lactis* subsp. *cremoris* 9B4 (Van Belkum *et al.* 1991). The genes encoding this bacteriocin are present on the same fragment on which the genes for lactococcin M and lactococcin A are present. Active bacteriocin is a 5300 Da protein.

Diplococcin is one of the earliest bacteriocin isolated from LAB. It is protein with molecular weight of 5.3 kDa and produced by *Lactococcus lactis* subsp. *cremoris* in milk and M17 broth during early stationary phase. It was partially purified by Oxford (1944) and was found to be water-soluble and heat stable under acidic conditions. It differs from nisin in many of its characteristics. It does not conatain sulphur containing amino acids, lanthionine and -methyl lanthionine, which are the characteristics of lantibiotics (Davey and Richardson, 1981). The inhibitory spectrum of diplococcin from *Lactococcus lactis* subsp. *cremoris* was restricted to lactococci only (Davey and Pearce, 1980). *Lactococcus lactis* subsp. *cremoris* starin 9B4-secreting lactococcins A, B and M prevented the growth not only of other lactococci but also of some *Clostridia* (Geise *et al.* 1983). Holo *et al.* (1991) purified lactococcin A and found that it inhibited the growth of only lactococci. Out of over 120 strains of different *Lactococci* tested only one was insensitive to lactococcin A as was the case with all other Gram-positive bacteria. Bacteriocin J46 has a wide spectrum of antibacterial activity including anticlostridial activity (Gonzalaz *et al.* 1996).

Purified diplococcin is unstable at room temperature, rapidly inactivated by heat and degraded by proteolytic enzymes like chymotrypsin, trypsin and pronase (Klaenhammer, 1988). It has a narrow spectrum of activity against closely related *Lactococcus lactis* and other strains of *Lactococcus cremoris* (Davey, 1981). It is encoded by 54 Mda plasmid (Davey, 1984). In addition to nisin and diplococcin,

lactococci have been reported to produce some other bacteriocins. Hirsch and Grinsted (1951) have reported that streptococci produced a variety of bacteriocins but in comparision to nisin activity of other bacteriocins was much lesser.

1.3 *Lactococcus lactis* subsp. *lactis biovar. diacetylactis*-The bacteriocin described in *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* WM4 (Scherwitz *et al.,* 1983) has been found to be identical to the lactococcin A produced by *Lactococcus lactis* subsp. *cremoris* strains 9B4 and LMG2030 (Stoddard *et al.,* 1992). Kojic *et al* (1991) reported the production of bacteriocin S50 by *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* S50. A strain of *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* UL720 isolated from raw milk has been found to produce a bacteriocin termed as diacetin B (Ali *et al.,* 1995). Morgan *et al* (1995) isolated *Lactococcus lactis* subsp. *lactis biovar. diacetylactis* DPC398 from an Irish cheese factory and observed the effect of all the three lactococcins *viz.* A, B, and M in the strain DPC398.

Lactostrepsins are another group of inhibitory substances, which have been reported to be produced by non-nisin producing strains of *Lactococcus lactis* **subsp** *diacetylactis, Lactococcus lactis* **subsp.** *cremoris* **and** *Streptococcus lactis* **subsp.** *lactis* **(Kozak,** *et. al.,* **1978). These substances show maximum activity in the pH range of 4.6 to 5.0, whereas at pH 7.0 the activity is lost (Dobrzanski, 1982). Lactostrepsins are stable at 121°C for 10 minutes and produced in non-agitated broth cultures during early logarithmic phase. These are inactivated by proteolytic enzymes. Their molecular weight exceeds 10,000 Da. These show inhibitory action against other** *lactococci,* **group A, C and G** *streptococci*, *Bacillus cereus, Lactobacillus helveticus*, *Leuconostoc mesenteroides* **subsp.** *cremoris*, **and** *Leuconostoc paracitrovorum..* **Lactostrepsin 5 produced by** *Lactococcus lactis* **subsp.** *cremoris* **202 disrupts the cell membrane, interferes with uridine transport and inhibits DNA, RNA or protein synthesis (Dabrzanski, 1982). Information on genetic determinants responsible for production and immunity is inconclusive.**

2.2 Lactobacilli:- The bacteriocinogenicity has been described for several of the obligate homofermenters (*Lactobacillus acidophilus*, and *Lactobacillus helveticus*), facultative heterofermenters (*Lactobacillus plantarum* and *Lactobacillus sake*) and for the heterofermentative *Lactobacillus brevis*.

2.2.1 Dairy lactobacilli:-

2.1.1 *Lactobacillus helveticus*-The bacteriocins described from the species includes helveticin J by the strain *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1986). Helveticin J is sensitive to several proteolytic enzymes and heat and shows its action against limited related *Lactobacilli*. It is encoded by chromosomal determinants. Crude protein has molecular weight of 30,000 Da but purified protein has a molecular weight of 37,000 Da and helviticin V-1829 by the strain *Lactobacillus helveticus* 1829 (Vaughan *et al.,* 1992). This bacteriocin has been found to be heat labile (50°C for 30 min.), which is bactericidal against other *Lactobacilli*. The partially purified preparation is inactivated by proteinase K, trypsin, pronase, heat and pH above 7.0. It is chromosomally encoded and has no plasmids. (Vaughan, *et al.,* 1992). Thompson *et al.,* (1996) identified a bacteriocin in the culture supernatant of *Lactobacillus helveticus* CNRZ450.

2.1.2 *Lactobacillus acidophilus*:- Early investigations into the antimicrobial activities of *Lactobacillus acidophilus* suffered due to insufficient characterization of the antagonistic agents, to determine whether or not bacteriocins are responsible for the observed inhibition (Klaenhammer, 1988). Barefoot and Klaenhammer (1983) provided a more definitive characterization of bacteriocins from the species with the description of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus* N2. ten-Brink *et al.* (1994) reported the production of acidocin B, an atypical bacteriocin by *Lactobacillus acidophilus* strain M46 isolated from human dental plaque and *Lactobacillus acidophilus* TK9201 was found to produce a bacteriocin termed as acidocin A (Kanatani *et al.,* 1995).

Two bacteriocins, which have been named as lactacin F and B, are produced by *Lactobacillus acidophilus* 11088 and *Lactobacillus acidophilus* N_2 respectively (Muriana and Klaenhammer, 1987; Barefoot and Klaenhammer, 1983). HPLC purification of lactacin F preparations followed

by SDS-PAGE showed that activity is retained in a 2500 Da band, however, this finding does not correlate with amino acid composition analysis, which indicates that the protein possesses 56 amino acids. (Muriana and Klaenhammer, 1991). Lactacin F is sensitive to protinase K, trypsin, ficin and subtilisin and is heat stable at 121°C for 15 minutes. It is active against *Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus Lactobacillus acidophilus*, *Lactobacillus fermetum* and one strain of *Lactobacillus faecalis*. Genetic determinants for lactacin F production and immunity are present on an episome (Muriana and Klaenhammer, 1991).

Lactacin B is a 6,500 Da protein which is sensitive to proteinase K and heat stable at 100°C for 3 minutes at pH 5, (Barefoot and Klaenhammer, 1983). It is inhibitory to *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*. Lactacin B appears to be encoded by chromosomal genes, as the producing bacteria possess no plasmids. (Barefoot and Klaenhammer, 1983).

2.2.2. Non-dairy lactobacilli:-

2.2.1 *Lactobacillus plantarum:-* Daeschel *et al* (1990) reported the production of plantaricin A by *Lactobacillus plantarum* C-11 isolated from cucumber fermentations. Plantaricin A is produced by *Lactobacillus plantarum* C-11 and is inhibitory to other lactic acid bacteria. Its molecular weight has been estimated to be more than 6000 Da. It is heat stable at 100°C for 30 minutes and shows activity in the pH range of 4 to 6.5. Investigations indicate that it may not be encoded by plasmid. (Daeschel *et al.,* 1990). A protein with narrow spectrum of activity, called plantaricin B is produced by *Lactobacillus plantarum* NCDO 1193, which shows inhibitory action against other strains of *Lactobacillus plantarum*, *Lactobacillus mesenteroides* and *Pediococcus damnosus* (West and Waner, 1988). Plantaricin B is a protein complexed with carbohydrate and lipid moieties, as inhibitory activity is reduced by lipase and α amylases. It is interesting to note that *Lactobacillus plantarum* LPC010 isolated from a green olive fermentation elaborated into the growth medium by two bacteriocins designated as plantaricins S and T (Jimenez-Diaz *et al.,* 1993). A bacteriocin, plantaricin KW30, producing strain of *Lactobacillus plantarum* has recently been isolated from fermented maize (Kelly *et al.,* 1996).

Bacteriocinogenic *Lactobacillus plantarum* strains from dairy and meat products have also been reported. A *Lactobacillus plantarum* strain LTF154 isolated from a fermented sausage produced a bacteriocin designated as plantacin 154 (Kanatani and Oshimura, 1994). Rekhif *et al.* (1994) isolated a bacteriocin producing *Lactobacillus plantarum* strain LC74 from goat raw milk and named the bacteriocin as plantaricin LC74. Recently Ennahar *et al.* (1996) reported the production of a bacteriocin identical to pediocin AcH by a strain of *Lactobacillus plantarum* WHE92 isolated from a soft cheese.

2.2.2 *Lactobacillus curvatus:-* Curvacin A produced by *Lactobacillus curvatus* LTH 1174, an isolate from meat, produces this bacteriocin which shows inhibitory action against other *Lactobacilli, Leuconostoc*, *Cornybacteria, Listeria monocytogenes*, as well as a weak action against *Micrococci* and *Staphylococci*. (Tichaczek *et al.* 1992). Like sakacin P, curvacin A is destroyed by proteinase K and trypsin but stable when treated with pepsin or heat (100°C, 3 min). Its molecular weight has been estimated to be 3,000 to 5,000 Da.

2.2.3 *Lactobacillus sake*:– Bacteriocins produced by the strains of *Lactobacillus sake* isolated from meat and fermented sausages include: sakacin A by *Lactobacillus sake* 706 (Schillinger and Lucke, 1989), lactocin S by *Lactobacillus sake* L45 (Mortvedt and Nes, 1990, 1991; Skaugen *et al.,* 1994).

Sakacins are the bacteriocins produced by *Lactobacillus sake* and include sakacin, A, M, S and P. *Lactobacillus sake* is responsible for fermentation in sausages. Sakacin A and M inhibit other *Lactobacilli* as well as *Listeria monocytogenes* (Schillinger and Lucke, 1989; Schillinger, *et.al.,* 1991). Lactocin S inhibits strains of lactic acid bacteria (Mortvedt and Nes, 1990). Sakacin A is produced by *Lactobacillus sake* 706 and is heat stable (100°C for 20 min.) produced during the mid and late logarithmic growth phase in liquid medium and is associated with a 27.7 Kb plasmid. (Schillinger and Lucke, 1989).

Sakacin M was produced from *Lactobacillus sake* isolated from Spanish dry fermented sausages.

(Sobrino *et al.* 1991). It is produced maximally in a synthetic medium supplemented with 1.5% tryptone during growth at 32°C. Molecular weight of the bacteriocin has been estimated to be 4640 Da. Inhibitory activity of a partially purified compound is diminised by trypsin, pepsin, papain and protease XIV and II. Crude and partially purified compounds are heat stable at 80°C for 60 minutes and 150°C for 9 minutes. Bacteriocin shows inhibitory action against *Lactobacilli, Leuconostoc, Carbonobacteria, Listeria monocytogenes* and *Staphylococcus aureus* (Sobrino *et al.* 1991).

Another bacteriocin, sakacin P, was produced by *Lactobacillus sake* LTH 673, isolated from meat and it was found to inhibit *Lactobacilli* and spoilage organisms like *Leuconostoc, Cornybacteria, Enterococci, Brochothrix thermosphacta* and *Listeria* sp. (Tichaczek *et al.* 1992). Bacteriocin is sensitive to proteinase K and trypsin but insensitive to pepsin and heat at 100°C for 7 minutes. It is a protein of molecular weight 3,000 to 5,000 Da with 36 to 41 amino acid residues.

Lactocin S is a heat stable protein active against *Pediococcus, Leuconostoc*, and *Lactobacilli*. It has been isolated from *Lactobacillus sake* 245. (Mortvedt and Nes, 1990). Molecular weight of crude Lactocin S has been reported to be 30,000 Da, however, partially purified active proteins have molecular weight less than. 13,700 Da. Production is associated with an unstable 50 Kb plasmid. (Mortvedt *et al.,* 1991). Mode of action studies indicated that lactosin S acts bactericidally in a pH dependent fashion (Twomey *et al.,* 2002).

2.2.4 *Lactobacillus brevis:–* Production of brevicin 286 has been recently reported in *Lactobacillus brevis* VB286 that was originally isolated from vaccum packaged meat (Conventry *et al.,* 1996).

Lactobacillus brevis produced an antibacterial substance named brevicin 37, which was inhibitory to *Pediococcus* sp., *Leuconostoc* sp., *Lactobacillus* sp. and *Nocardia carolina*. The protein is stable at pH range of 1 to 11. It is also stable to heat at 121°C for 1 h and is retained on a 10,000 molecular weight cut off membrane. (Rammelsberg and Radler, 1990).

2.2.5 *Lactobacillus casei–* Rammelsberg and Radler (1990) isolated *Lactobacillus casei* B 80 from plants and fermenting materials. *Lactobacillus casei* B 80 produced a heat sensitive protein with a narrow spectrum of activity against other strains of *Lactobacillus casei*. (Rammelsberg *et al.,* 1990). Its molecular weight has been reported to be 40,000 to 42,000 Da.

Other lactobacilli:– Lactacin F producing *Lactobacillus acidophilus* 11088 (Muriana and Klaenhammer, 1987) has been renamed as *Lactobacillus johnsonii* as cited by Klaenhammer (1993).

2.3 *Leuconostoc*:– The first evidence for bacteriocin production in *Leuconostoc* spp. was provided by Harding and Shaw in 1990. They reported the production of a heat stable protein by a strain of *Leuconostoc gelidum* that was active against other lactic acid bacteria and three strains of *Listeria monocytogenes.* In recent years, a number of bacteriocin producing strains of *Leuconostoc* species have been isolated from various sources such as milk and meat products.

Hastings and Stiles (1991) reported the production of a bacteriocin-designated leucocin A-UAL187 by *Leuconostoc gelidum* UAL187 isolated from meat packed under elevated (30%) carbondioxide. *Leuconostoc paramesenteroides* OX isolated by Lewus *et al* (1991) from retail lamb was found to produce a bacteriocin named as leuconocin S (Lewus *et al.*, 1992).

Bacteriocins, carnosin 44A, carnocin LA54A and leucocin B-Talla, produced by *Leuconostoc carnosum* LA44A from vaccum packagedVienna-type-sausage (van Laack *et al.* 1992), *Leuconostoc carnosum* LA54A from meat (Keppler *et al.* 1994) and *Leuconostoc carnosum* Talla isolated from vacuum packaged processed meat (Felix *et al.* 1994), repectively, have been described in the strains of *Leuconostoc carnosum*. Yang and Ray (1994a) observed the predominance of *Leuconostoc carnosum* and *Leuconostoc mesenteroides* in the spoiled low heat processed vacuum packaged meat products. The notable feature of many of these *Leuconostoc* isolates is their ability to produce bacteriocins.

Bacteriocinogenic strains of *Leuconostoc* spp. have also been isolated from milk and milk products. Strains of *Leuconostoc mesenteroides* subsp. *mesenteroides*,

Y105 from goat milk and FR52 from raw milk were found to produce bacteriocins, mesentericin Y105 (Hechard *et al.,* 1992) and mesentericin 52 (Mathiew *et al.* 1993), respectively. Dextranicin J24 was a bacteriocin produced by an isolate of *Leuconostoc mesenteroides* subsp. *dextranicum* J24 from French soft cheese (Sudirman *et al.,* 1994). Malik *et al* (1994a) reported the detection and activity of a novel bacteriocin, leucocidin R1, produced by *Leuconostoc paramesenteroides* NM14 isolated from an aged cream sample.

2.4 Pediococci:-

2.4.1 *Pediococcus pentosaceus*:- The bacteriocin produced by *Pediococcus pentosaceus* FBB61 from cucumber fermentations was designated as pediocin A (Daeschel and Klaenhammer, 1985). Hoover *et al,* (1988) observed bacteriocinogenic activity in *Pediococcus pentosaceus* MC03 isolated from pepperoni, a fermented sausage. Bacteriocin production in Pediococcus *pentosaceus* strain N5p from wine has been reported and the bacteriocin was named as pediocin N5p (Strasser-de-Saad and Manca-de-Nadra, 1993).

2.4.2 *Pediococcus acidilactici*:-The most extensively characterized bacteriocins, pediocin AcH and pediocin PA-1, after nisin have been produced by strains of *Pediococcus acidilactici*. Gonzalez and Kunka, (1987) reported pediocin PA-1 production by *Pediococcus acidilactici* PAC1.0. Pediocin AcH producing *Pediococcus acidilactici* H was isolated by Bhunia *et al,* (1987) from fermented sausage. Hoover *et al,* (1988) observed the production of unnamed bacteriocins by *Pediococcus acidilactici* PO2 as pediocin PO2. Schved *et al* (1993) reported the isolation of *Pediococcus acidilactici* SJ1 from a naturally fermented meat product and designated its bacteriocin as pediocin SJ1 while pediocin L50 producing *Pediococcus acidilactici* L50 was obtained from Spanish dry fermented sausage (Cintas *et al.,* 1995).

2.4.3 Characteristics of bacteriocins:- Bacteriocins of LAB have been characterized with respect to their (i) sensitivity to various proteolytic and non-proteolytic enzymes (ii) stability to various heat treatments (iii) pH stability (iv) mode of action and (v) molecular weight etc. In most of these characterization studies, either crude or partially purified bacteriocin preparations have been used.

The fact that bacteriocins are proteins renders them sensitive to at least one of the proteolytic enzymes. Apart from protein moiety, some bacteriocins have been found to contain an active lipid or carbohydrate moiety which is also required for antibacterial activity as revealed by loss of bacteriocin activity upon treatment with lipases or amylases (Lewus *et al.,* 1992; van Laack *et al.,* 1992; Jimenez-Diaz *et al.,* 1993; Schved *et al.,* 1993; Keppler *et al.,* 1994)

The term bacteriocin has been restricted to those antibacterial proteins that exhibit a bactericidal mode of action (Tagg *et al.* 1976). Although, a vast majority of bacteriocins of LAB exert a bactericidal mode of action, but a few have been found to be bacteriostatic rather than bactericidal to the sensitive cells (Lewus *et al.,* 1992; Thompson *et al.,* 1996). Characteristic of bacteriocin and other conventional antibiotics shown In Table 3.

Table 3:Characteristic aspects of bacteriocins and other conventional antibiotics.

S.No.	Characteristics	Bacteriocins	Other antibiotics
		Foods	
1	Application	Ribosomally	Clinical
2	Synthesis	Limited spectrum	Secondary metabolism
3	Activity	Present	Wide spectrum
4	Presence of immune cells in the host	The most through the channel	Absent
5	Mode of action	formation in the cell	Specific target
6	Toxicity/other effects in eukaryotic cells	cytoplasmic membrane	Present
		Absent	

Most of the bacteriocins of LAB characterized to date are small (< 10kDa) heat stable peptides, however, the occurrence of large (> 30kDa) heat labile proteins has also been reported (Joerger and Klaenhammer, 1986; Vaughan *et al.,* 1992). Bacteriocins are extremely heat stable at low pH (Hurst, 1981; Hastings *et al.,* 1991; Felix *et al.,* 1994) becoming more sensitive to heat upon purification (Davey, 1981; Hastings *et al.,* 1991). Bacteriocins of LAB, in general, are active over a wide pH range with optimum being on acidic side.

Currently, over 20 bacteriocins of lactic acid bacteria have been sequenced. Most contain less than 60 amino acids, and all are devoid of lipid and carbohydrate moieties. The molecules are cationic,

hydrophobic and have isoelectric points ranging from 8.6 to 10.4. Their net positive charge varies with pH, and this is important for both bactericidal efficiency and purification (Ray *et al.,* 2001). They also reported that due to the hydrophobic nature of these molecules they have a tendency to aggregate, especially when stored at high concentration or for a long time. Antibacterial potency is greater at lower pH, destroyed at a pH above 10, relatively heat stable but partially destroyed by heating above 100°C, and not affected after treatment with many organic and inorganic chemicals. However, some anions interfere with activity in a concentration dependent manner, and many proteolytic enzymes can hydrolyze the molecules resulting in loss of antibacterial properties.

Bactericidal potency remains stable during storage in dried or liquid forms at frozen and refrigeration temperatures. However, it slowly decreases during storage at room temperature in the presence of air that can oxidize methionine residues to the inactive methionine sulfoxide form (Ray 1992; Klaenhammer 1993; Jack *et al.,* 1995; Yang *et al.,* 1992; Ennahar *et al.,* 2000b). Characteristics of some common bacteriocins produced from LAB are summarized in Table 4.

Table 4: Characteristics of some bacteriocins produced by LAB.

Bacteriocin	Produced by	Number of amino acids	Moecularlar Weight (kDa)	Isoelectric points	References
Nisin A	*Lactococcus lactis* subsp. *lactis*	34	3.5	10.1	Klaenhammer *et al.,* (1988)
Lactococcin A	*Lactococcus lactis* subsp. *cremoris*	55	5.8	8.6	Van Belkum *et al.,* (1991a)
Lactococcin B	*Lactococcus lactis* subsp. *cremoris*	47	5.3	9.1	Van Belkum *et al.,* (1991a)
Sakacin A	*Lactobacillus sake* 706	41	4.3	10.0	Schillinger and Luke (1989)
Sakacin P	*Lactobacillus sake* LTH 673	43	4.4	8.8	Tichaczek *et al.,* (1992)
Lactocin S	*Lactobacillus sake* 245	37	3.9	N.A	Mortvedt and Nes, (1990)
Pediocin ACH	*Pediococcus acidilacticin* H	44	4.6	9.6	Bhunia *et al.,* (1987)
Leucocin A	*Leuconostoc gelidium*	37	3.9	9.5	Ahn and Stiles, (1990)
Enterocin A	*Enterococcus faecium*	47	4.8	9.6	Dutta *et al.,* (1999)

The bacteriocins of lactic acid bacteria following translation usually undergo very little structural alteration, and thus, are regarded as ribosomally translated peptides. At the translation level, a molecule designated as prebacteriocin (such as prenisin and prepediocin) contains a leader peptide segment at the N-terminus and a propeptide segment at the C-terminus. The molecules are transported out of the cytoplasm through the membrane by specific ABC (ATP binding cassette) transporters with the expense of energy. (Figure 6).

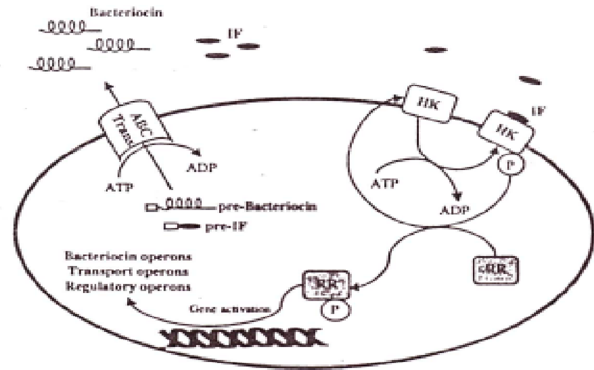

Figure 6: Schematic depiction of transport of bacteriocins and signaling pathway leading to bacteriocin expression. IF, induction factor; HK; histidine kinase;P, phosphate; RR, response regulator; ABC, Trans, ABC transporter system; (Nes *et al.* 1996).

During transport endopeptidase activity of the ABC transporter removes the leader peptide before the propeptide is released into the environment. (Jack *et al.,* 1995; Liu and Hansen, 1990; Ennahar *et al.,* 2000). Ennahar *et al.,* (2000) reported that the fate of excised leader peptide is not known but it has been speculated that they might act as signaling molecules for the production of bacteriocins. It was assumed earlier that leader peptides, besides helping prebacteriocin molecules to interact with ABC transporters, also inhibit activity of the attached probacteriocin. Recent studies have shown that this may be true for nisin, but is not the case for pediocin PA-1/AcH, as prepediocin as well as pediocin PA1/AcH with a fused protein at the N-terminus are biologically active. (De-Vos *et al.,* 1995). Following release of the matured bacteriocin, it either remains free or may bind via electrostatic attraction to the surface of the producer cells. At around pH 6, a large fraction of the molecules remain in the absorbed state while at pH 2 or below most are released into the environment (Yang *et al.,* 1992).

The probacteriocins part of some bacteriocins may undergo nonenzymatic as well as enzymatic chemical modifications. Pre-nisin molecules, while

in the cytoplasm, undergo enzymatic dehydration of serine and threonine residues converting-

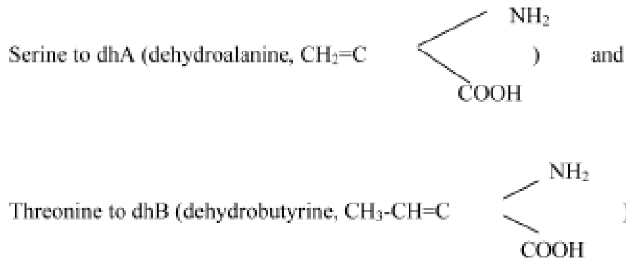

Figure 7: The conversion of serine residues to threonine residues in cytoplasm

They then form thioether linkages to cysteine creating lanthionine (Lan; -Ala-S Ala) or β-methyl lanthionine (MeLan; - Abu-S-Ala). Bacteriocins with thioether rings, *i.e.*, containing lanthionine and/or methyl lanthionine, are grouped as lantibiotics. In lantibiotics, these changes occur in the probacteriocin sequence while the prebacteriocin still resides in the cytoplasm. Lantibiotics can have different numbers of thioether rings *e.g.*, nisin has five rings while lacticin 481 has three rings. The thioether rings play a crucial role in the antibacterial properties of a lantibiotic; however, the presence of lanthionine does not necessarily make a bacteriocin more potent than bacteriocin that lack lanthionine. (Ray *et al.,* 2001).

2.4.4 Mode of action of bacteriocin: – Due to the great variety of their chemical structures, bacteriocins affect different essential functions of the living cell (transcription, translation, replication, and cell wall biosynthesis), but most of them act by forming membrane channels or pores that destroy the energy potential of sensitive cells. The different modes of action of various types of bacteriocin produced by gram-positive bacteria have been reviewed by several authors (Sahl and Brandis, 1982; Abee, 1995; Ennahar *et al.,* 2000).

"Nisin" (a compound belonging to group Ia, according to Klaenhammer's classification) is the bacteriocin whose mode of action has been studied the best. This cationic lantibiotic associates electrostatically with the negatively charged membrane phospholipids (Abee *et al.,* 1995; Driessen *et al.,* 1995), which favors subsequent interaction of bacteriocin's hydrophobic residues with the target cytoplasmic membrane. The interaction between the hydrophobic part of nisin and the bacterial target membrane generates un-specific ionic channels whose formation is aided by the presence of high transmembrane potentials, and by the presence of anionic and absence of cationic lipids (Hancock, 1997). Pore formation, on the other hand, decreases in the presence of divalent cations (Mg2+ or Ca2+) because they neutralize the negative charges of the phospholipids, reducing the fluidity of the membrane. Nisin generated membrane pores allow the passive efflux of ions (K + and Mg2+), amino acids (glutamic acid, lysin), and A TP, but not of larger cytoplasmic proteins, yielding membrane potential and proton-motiv-force dissipation and subsequent cell death (Boman *et al.,* 1994).

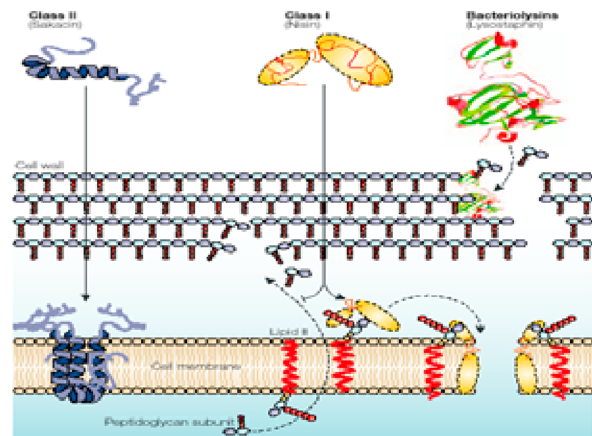

Figure 8: Mode of action of lactic acid bacteria bacteriocins. Lactic acid bacteria (LAB) bacteriocins can be grouped on the basis of structure, but also on the basis of mode of action. Some members of the class I (or lantibiotic) bacteriocins, such as nisin, have been shown to have a dual mode of action. They can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death. Furthermore, they can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. A two-peptide lantibiotic, such as lacticin 3147, can have these dual activities distributed across two peptides, whereas mersacidin has only the lipid-II-binding activity, but does not form pores. In general, the class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation and death. Large bacteriolytic proteins (here called bacteriolysins, formerly class III bacteriocins), such as lysostaphin, can functiondirectly on the cell wall of Gram-

positive targets, leading to death and lysis of the target cell.

***Source*: –** Cotter *et al.* (2005).

2.4.5 Antibacterial properties: – Many bacteriocin producing strains belonging to several genera and species of lactic acid bacteria have been isolated. Their bacteriocins are bactericidal to sensitive cells and death occurs very rapidly at a low concentration. Ray (1992) reported a range of Gram positive bacteria sensitive to a bacteriocin, while the producer strain is immune to its own bacteriocin and often is sensitive to other bacteriocins.

Most of the class I bacteriocins have a fairly broad inhibitory spectrum. They not only inhibit closely related bacteria, such as species from the genera *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus,* but also inhibit many less closely related Gram-positive bacteria, such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus,* and *Clostridium botulinurn*. Several bacteriocins in this class, such as nisin and thermophilin 13, prevent outgrowth of spores of *Bacillus cereus* and *Clostridium botulinum*. Interestingly, acidocin J1132 has a very narrow inhibitory spectrum and sensitive strains are limited to members of the genus *Lactobacillus* (Table 5), while at the other extreme, plantaricin LP84 (produced by *Lactobacillus plantarum* NCIM 2084) has demonstrated antagonism against *E. coli* (Suma *et al.,* 1998).

Compared to class I bacteriocins, most class IIa bacteriocins have comparatively narrow activity spectra and only inhibit closely related Gram-positive bacteria. In general, members *of* the genera *Enterococcus, Lactobacillus, Pediococcus* are sensitive to class lIa bacteriocins, and members of the genus *Lactococcus* are resistant (Table 5). For example, Eijsink and others (1998) found that pediocin PA-I was active against different species of *Enterococcus, Lactobacillus,* and *Pediococcus;* however, only 1 out of 11 *Lactococcus* strains tested (*Lactococcus lactis* LMG 2070) was sensitive to the bacteriocin. Some class lIa bacteriocins, such as pediocin PA-l, have fairly broad inhibitory spectra and can inhibit some less closely related Gram-positive bacteria, such as *Staphylococcus aureus* and vegetative cells of *Closlridium* spp. and *Bacillus* spp. Some class lIa bacteriocins, such as mundticin from *Enterococcus* mundtii, even prevent the outgrowth of spores of *Clostridium botulinum* (Table 5).

As evident in Table 5, class IIa bacteriocins are generally active against *Listeria*. Eijsink *et al* (1998) found that 9 strains of *Listeria* tested, including *Listeria monoeytogenes, Listeria innocua* and *Listeria ivanovii* were very sensitive to 4 class lIa bacteriocins (pediocin PA-1, enterocin *A,* sakacin P, and curvacin *A).* Moreover, the extent of sensitivity varied from strain to strain. The minimal inhibitory concentrations against *Listeria monocytogenes* for the above 4 bacteriocins varied from 0.1 to 8 mg/ml, however, some *Listeria* strains, such as *Listeria monocytogenes* V7 and *Listeria innocua* LB 1, have been found to be resistant to class lIa bacteriocins (enterocin A, mesentericin Y I05, divercin V41, and pediocin AcH) (Ennahar *et al.,* 2000).

It might seem that bacteriocins with broader activity spectra would always be preferable for use in food preservation, but under certain circumstances bacteriocins with narrower inhibitory spectra may prove more desirable. For example, sakacin P, which has limited activity against LAB but nearly as effective as pediocin PA-1 against *Listeria*, might find application in LAB fermentation products that are prone to contamination by *Listeria monocytogenes* (Eijsink *et al.*, 1998).

Table 5: Activity spectra of some Class I and Class IIa bacteriocins
(Chen, H. and Hoover, D.G. (2003).

Bacteriocins	Strain	Activity spectra	References
Class lIa	*Lactobacillus acidophilus* TK9201	Active against different species of *Enterococcus* (1/5), *Lactobacillus* (13/32), *Pediococcus* (2/7), *Streptococcus* (8/13), and *L. monocytogenes* (5/5). Not active against *Bacillus subtilis* (0/6) and *S. aureus* (0/2).	Kanatani *et al* 1995
Acidocin A	*Lactobacillus* sake MI401	Active against different species of *Enterococcus* (2/2), *Lactobacillus* (11/25), *Lactococcus* (5/15), *Leuconostoc* (4n), *Pediococcus* (215), and *L. monocytogenes* (9/10).	Nissen-Meyer *et al* 1993
Bavaricin A	*Lactobacillus* curvatus LTH1174	Not active against *Carnobacterium* (0/1), *Streptococcus* (0/2), *Brochothrix thermosphacta* (0/1), *Bacillus* spp. (On), and *Staphylococcus* spp. (0/5).	Eijsink *et al* 1998
Curvacin A	Carnobacterium divergens V41	Active against different species of *Carnobacterium* (3/3), *Enterococcus* (1/2), *Lactobacillus* (10/23), *Lactococcus* (1/12), *Pediococcus* (5/8), *L. monocytogenes* (7n), *L. innocua* (1/1), and *L. ivanovii (*1/1). Not active against *Leuconostoc* (0/3) and *Clostridium* spp. (0/12).	Guyonnet *et al* 2000
Divercin V41	Enterococcus faecium CTC492	Active against different species of *Enterococcus* (4/4), *Lactobacillus* (2/5), Pediococcus (2/2), L.monocytogenes (1/1). *L. innocua* (1/1), and *L. ivanovi* (1/1). Not active against *Lactococcus* (0/1) and *Leuconostoc* (0/3).	Aymerich *et al* 1996
Enterocin A	Lactococcus *lactis* MMFII	Active against different species of *Enterococcus* (4/4), *Lactobacillus* (2/2), *Pediococcus* (2/2), *L.monocytogenes* (4/4), and *L. innocua* (2/2).	Ferchichi *et al* 2001
Lactococcin MMFII	*Leuconostoc* mesenteroides Y105	Active against different species of *Enterococcus* (3/3), *Lactobacillus* (2/2) , *Lactococcus* (2/6), and *L. lanovi* (1/1),	Guyonnet *et al* 2000
Mesentericin Y105	Enterococcus mundtii ATO6	Active against different species of *Enterococcus* (3/4), *L.actobacillus* (1/5),_*Leuconostoc* 2/3, *ediococcus* (2/2).*L.* monocytogenes (1),*L. innocua* (1/1), and *L. ivanovi*(1/1). Not active against *Lactococcus* (0/1)	Bennik *et al* 1998
Mundticin	Pediococcus acidilactici PAC 1.0	Active against different species of *Carnobacterium* (1/1), Enterococcus (2/2), *Lactobacillus* (2/2), *Leuconostoc* (2/2), *Pediococcus* (2/2), *L. monocytogenes* (1/1), and *L. innocua* (1/1). Prevents the outgrowth of spores and vegetative cells of C. botulinum.	Eijsink *et al* 1998
Pediocin PA-1	Carnobacterium piscicolaV1	Active against different species of *Carnobacterium* (3/3), *Enterococcus* (2/3), *Lactobacillus* (23/31), *Lactococcus* (1/14), *Leuconostoc* (3/4), *Pediococcus* (8/11), L. *monocytogenes* (12/12), *L. innocua* (2/2). *L. ivanovii* (1/1), *Staphylococcus* spp. (2/6), *B. cereus* (1/1), and *Clostridium* spp. (4/17).	Bhugaloo-Vial *et al* 1996
Piscicocin V1a	Carnobacterium piscicola V1	Active against different species of *Carnobacterium* (2/2), *Enterococcus* (1/1), *Lactobacillus* (3/3), *Leuconostoc* (1/1), *Pediococcus* (1/1), *L. monocytogenes* (1/1), and L. innocua (1/1). Not active against *Lactococcus* (0/1), B. cereus (0/1), *Clostridium* spp. (0/3), and *S. aureus* (0/1).	Bhugaloo-Vial *et al* 1996
Piscicocin V1 b	Carnobacterium piscicola JG126	Active against different species of *Carnobacterium* (2/2), *Enterococcus* (1/1), *Lactobacillus* (3/3), *Leuconostoc* (1/1), *Pediococcus* (1/1), *L. monocytogenes* (1/1), and *L. innocua* (1/1). Not active against *Lactococcus* (0/1), *B. cereus* (0/1), *Clostridium* spp. (0/3), and *S. aureus* (0/1).	Jack *et al* 1995
Piscicolin 126	*Lactobacillus* sake LB706	Active against different species of Carnobacterium (1/1), Enterococcus (2/2), *Lactobacillus* (2/3), *Leuconostoc* (2/3), Pediococcus (1/2), Streptococcus (2/2), L. monocytogenes (2/2), L. grayi (1/1), L. ivanovii (1/1), L. seeligeri (1/1), and B. thermosphacta (1/1). Not active against Bacillus spp. (0/5), Clostridium spp. (0/2), Lactococcus (0/3), Listeria denitrificans (0/1), and Staphylococcus spp. (0/3).	Aymerich *et al* 1996; Guyonnet *et al* 2000
Sakacin A	*Lactobacillus* sake LB674	Active against different species of *Enterococcus* (7/8), *Lactobacillus* (317), *Pediococcus* (1/4), *L. monocytogenes* (5/5), *L. innocua* (3/3), and *L. ivanovi* (1/1). Not active against *Lactococcus* (0/1) and *Leuconostoc* (0/3).	Aymerich *et al* 1996;
Sakacin P		Active against different species of *Enterococcus* (7/8), *Lactobacillus* (317), *Pediococcus* (2/4), *L. monocytogenes* (5/5), *L innocua* (3/3), and *L. ivanovi* (1/1). Not active against *Lactococcus* (0/1) and *Leuconostoc* (0/3).	Guyonnet *et al* 2000

Several other characteristics of bacteriocins and bacteriocin producing lactic acid bacteria have been noted (Ray 1992; Ennahar 2000; Ray and Miller, 2001). A strain can sometimes produce more than one type of bacteriocin (*e.g.* lactoccin A, B and M by *Lactococcus lactis* subsp. *cremoris*). Strains of the same species generally produce the same bacteriocin (*e.g.* pediocin PA-1/AcH by different *Pediococcus acidilactici* strains), however, strains of the same species can also produce different bacteriocins (*e.g.* Sakacin A and Sakacin P produced by two strains of *Lactobacillus sake*), and the strains from different species and different genera can produce the same bacteriocin (*e.g.*, pediocin PA-1/AcH produced by *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Pediococcus parvulus,* and *Lactobacillus plantarum* strains). Strains from different subspecies of the same species can produce different bacteriocins (*e.g.* nisin A and lactocin 481 produced by different strains of *Lactococcus lactis* subsp *lactis)* different species in a genus can produce different bacteriocins (*e.g.* Enterococcin EFS2 and enterocin 900 produced by strains of *Enterococcus faecalis* and *Enterococcus faecium*, respectively). Natural variants of the same bacteriocin can be produced by different strains of the same species (*e.g.* nisin A and nisin Z by *Lactococcus lactis* subsp *lactis,* strains ATCC 11454 and ATCC 7962, respectively) and also by different species (*e.g.* leucocin A and mesenterocin by *Leuconostoc gelidum* and *Leuconostoc mesenteraides*, respectively). These generalizations are drawn based on analysis of the amino acid sequences of numerous bacteriocins.

2.4.6 Production of bacteriocins:- One of the most important steps in the study of bacteriocins is their production. The composition of culture medium and cultural conditions such as temperature, pH and time of incubation have profound effect on the production of bacteriocins. In general, conditions that provide high cell density favour high bacteriocin concentration.

The culture media generally employed for the growth of lactic acid bacteria such as MRS, APT, TGE, M17G, ELB *etc.* have also been found to support good bacteriocin production. Although, bacteriocin production occurs over a wide temperature range, it is greater at the optimum temperature for the growth of the producer.

The production of bacteriocins by lactic acid bacteria is strongly influenced by the pH of the culture medium. The regulation of pH at a certain value during the course of fermentation has been found to have favourable (Hurst, 1981; Piard *et al.,* 1990) and detrimental (Biswas *et al.,* 1991; Coventry *et al.,* 1996) effects on the final yield of bacteriocins of lactic acid bacteria.

The maximum production of bacteriocins occurs at different phases in the cell growth cycle. Most of the bacteriocins of lactic acid bacteria are secreted during the logarithmic growth phase with a slight decline in the activity of some of them during the stationary phase of the producer culture. However, some bacteriocins for *e.g.* nisin (Hurst, 1981), pediocin SJ-1 (Schved *et al.,* 1993) are secreted as secondary metabolites. The termination of the incubation at appropriate time is essential to prevent the loss of bacteriocin activity.

2.4.6.1 Growth medium: - Commonly used media for the production of bacteriocins by lactic acid bacteria include MRS (Ten Brink *et al.,* 1994; Coventry *et al.,* 1996; Holo *et al.,* 2001;Vaughan *et al.,* 2001;Yanagida *et al.,* 2005),TGE (Biswas *et al.,* 1991;Yang and Ray, 1994),APT (Lewus *et al.,* 1992), GM17 (Parente et al. 1997), M17(Achemchem *et al.,* 2005), ELB (Geis *et al.,* 1983; Piard *et al.,* 1990), BHI (Achemchem *et al.,* 2005) *etc.* with or without modifications. Although, a large number of bacteriocins have been found to be identified and several media have been used for the production of bacteriocins, very few studies are available on the comparision of bacteriocin production in different media.

Geis *et al.* (1983) compared various media including ELB, GM17, BHI, a synthetic medium and milk for their ability to support bacteriocin production by various lactococcal strains. All the strains produced antibiotic activities in milk. Highest bacteriocin activities were found in unbuffered ELB followed by BHI, buffered M17 and synthetic medium (Geis *et al.,* 1983).

Lactococcus lactis subsp. *lactis* CNRZ481 produced maximum bacteriocin (12800 AU/ml) in ELB buffered with sodium β–glycerophosphate. The

observed titre was double than the value recorded when the culture was grown in M17 or unbuffered ELB (Piard *et al.,* 1990).

Parente et al. (1997) formulated three media (Tryptone-Yeast Extract-Tween) TYT10, TYT11 and TYT30 and compared with seven different media [ELB, M17, M17 dialysate, Tryptose phosphate (TP), tryptone yeast extract broth (TYB), yeast glucose lemco (YGL) broth and MRS] for the growth of and bacteriocin production by *Lactococcus lactis* subsp. *lactis* DPC3286 and *Lactococcus lactis* subsp. *cremoris* LMG2130. Good growth and bacteriocin production were obtained for both in the TYT, M17 and MRS media. Bacteriocin production was very poor in YGL. It was also observed that *Lactococcus lactis* subsp. cremotis LMG2130 could not grow or produce bacteriocins in M17 dialysate and TP media (Parente et al, 1997). Although the cell mass was greater in MRS broth, 15% less pediocin AcH production by P. acidilactici LB42-923 produced higher pediocin AcH titres in TGE broth than in buffered TGE broth (Yang and Ray, 1994).

In contrast to pediocin AcH, higher levels of nisin, sakacin A and leuconocin Lm1 were observed in TGE buffer broth than in TGE (Yang and Ray, 1994). Earlier Hechard *et al* (1992) observed consistently higher levels of (x16) mesentericin Y105 in MRS broth than in a semi-defined medium.

2.4.6.2 Effect of pH on bacteriocins production: -

2.4.6.2.1 Lactobacilli bacteriocins:- Barefoot and Klaenhammer (1984) reported maximum lacticin B production when *Lactobacillus acidophilus* N2 was grown in MRS broth regulated at pH 6.0. In contrast, lacticin F was produced maximally in MRS broth held at a constant pH of 7.0 rather than 7.5, contrast, lacticin F was produced maximally in MRS broth held at a constant pH of 7.0 rather than 7.5, 6.0 or 5.0 (Muriana and Klaenhammer, 1987). Production of heveticin J and helviticin V-1829 was observed to be greatest in anaerobic MRS cultures maintained at a pH 5.5 than at other pH values tested in the range of 5.0 to 7.0 (Joerger and Klaenhammer, 1986;Vaughan *et al.,* 1992).Vaughan *et al.* (1992) also reported a two-fold increase in helveticin V-1829 when MRS broth was held at a pH 5.0 than in pH-unregulated cultures.

Ten-Brink *et al.* (1994) observed that growth of *Lactobacillus acidophilus* M46 in five fold concentrated MRS broth held at a constant pH 5.5 resulted in eight fold increase in acidocin B activity than that obtained after growth in normal MRS broth without pH control. Regulation of MRS broth at pH 5.0 resulted in maximum yield of acidocin A produced by *Lactobacillus acidophilus* TK9201 (Kanatani *et al.,* 1995).

Maximum production of plantaricin S was obtained in a fermenter system in unregulated pH in MRS broth containing 4% NaCl. It was also reported that regulation of pH at 4.0-7.0 during fermentation had a detrimental effect on the production of plantaricin S by *Lactobacillus plantarum* LPC010 (Jimenez-Diaz *et al.,* 1993).

Coventry *et al.* (1996) studied the effect of pH on the production of brevicin 286 by *Lactobacillus* brevis VB286. No substantial cell growth or brevicin 286 activity was detected in MRS broth with an initial pH 4.5. In spite of substantial cell growth, brevicin 286 production was minimal at pH 5.0. Optimum production of brevicin 286 was observed in MRS broth at an initial pH of 6.0-6.5. It was also observed that regulation of pH at either 6.0 or 6.5 had no advantage over stirred culture without pH control with respect to brevicin 286 (Coventry *et al.,* 1996).

2.4.6.2.2 Lactococcal bacteriocins:- Nisin production was maximum when medium was maintained at pH 6.0 alongwith a large cell mass (Hurst, 1981). Piard *et al* (1990) observed maximum lacticin 481 production when the producer strain *Lactococcus lactis* subsp. *lactis* CNRZ481 was grown in buffered ELB 6.5 or growing the producer in pH non-regulated medium resulted in decreased bacteriocin yields (Piard *et al.,* 1990). Bacteriocin production by *Lactococcus lactis* subsp. *lactis* ADRI 85L030 was reported to be independent of the initial pH of the medium in the range 5.0 to 7.0 (Thuault *et al.,* 1991). Cock and Stouvenel in 2006 reported MRS liquid culture medium, after 48 h at 36C and 45C in anaerobic condition for lactic acid production by a strain of *Lactocoocus lactis* subsp. *lactis* isolated from sugarcane plants.

2.4.6.2.3 *Leuconostocs* bacteriocins:- *Leuconostocs* gelidum UAL187 produced leucocin A-UAL187

maximally in APT broth at pH 6.0 and 6.5. At a lower initial pH, growth of the producer organism was slower and a concentration maximum was lower (Hastings and Stiles, 1991). Lewus *et al.* (1992) studied the effect of initial pH of APT broth on the growth of and bacteriocin production by *Leuconostoc paramesenteroides* OX. Leuconocin Swas produced in detectable amounts at pH 6.0 and appeared to be optimal (400 AU/ml) at pH 6.5 and 7.0. They observed slight depression in growth and leuconocin S production at pH 7.5. Recently Baker *et al* (1996) reported that the production of leuconocin S was maximum (2000 AU/ml) in fermenters maintained at pH 7.0 than at 6.0, 6.5 and 7.5. Van Laack *et al.* (1992) observed a 50% decrease in the production of carnosin 44A when the initial pH of MRS broth was lowered from 6.0 to 5.1.

Although, *Leuconostoc carnosum* Talla produced leucicin B-Talla in MRS broth with an initial pH in the range 4.5 to 7.5, the bacteriocin concentration was found to be optimal at pH 6.0 to 6.5 (Felix *et al.,* 1994).

2.4.6.2.4 Pediococcal bacteriocins:- *Pediococcus acidilactici* H produced maximum pediocin AcH when grown in TGE broth with an initial pH of 6.5. Pediocin AcH was produced in negligible amounts when the pH of TGE broth was maintained at pH 5.0 or above. It was concluded that a terminal pH below 4.0 alongwith a large cell mass was essential for the production of pediocin AcH (Biswas *et al.,* 1991). High titres of pediocin N5P were observed when *P. pentosaceus* N5P was grown in TGE broth with an initial pH of 6.5 (Strasser-de-saad and Manca-de-Nadra, 1993). It was also reported that pediocin N5P could not be detected in TGE broth at an initial pH below 5.0. Liao *et al* (1993) reported optimum production of pediocin PO2 in whey permeate medium with an initial pH of 6.5 without pH regulation during incubation.

2.4.6.3 Effect of temperature on bateriocins production:-

2.4.6.3.1 Lactococcal bacteriocins:- Nisin production was maximum when the culture was incubated between 25 and 30°C as opposed to 37°C. Incubation of nisin producer at 37°C resulted in 386 AU/ml of nisin as compared to 542 AU/ml at 26°C (Hurst, 1981). Thuault *et al.* (1991) reported that the bacteriocin production by *Lactococcus lactis* subsp. *lactis* ADRI 85L030 was not significantly dependent on the incubation temperature in the range of 30 to 42°C.

2.4.6.3.2 *Leuconostoc*s bacteriocins:- *Leuconostoc carnosum* Talla produced bacteriocin, leucocin B-Talla over a wide range of temperature *i.e.* 0°C to 30°C, but the optimal production was observed at 25°C (Felix *et al.,* 1994). Van Laack *et al.* (1992) reported that *Leuconostoc carnosum* LA44A could grow and produce bacteriocins in the temperature range of 4-10°C. Although bacteriocins by various *Leuconostoc* spp. was observed both at 4°C and 25°C, the bacteriocin titres, in general, were 2-3 times higher at 25°C than at 4°C (Yang and Ray, 1994a). Leucocin A-UAL187 production by *Leuconostoc gelidum* UAL187 was observed over a wide range of incubation temperatures (1-25°C) with more time taken at low temperatures (Hastings and Stiles, 1991).

2.4.6.3.3 Pediococcal bacteriocins:- *Pediococcus* acidilactici H produced same amounts of pediocin AcH after 16 h of growth in TGE broth both at 30°C and 37°C. The cell mass and bacteriocin production were slightly reduced at 40°C (Biswas *et al.,* 1991). Schved *et al.* (1993) observed the production of pediocin SJ-1 at 20°C, 30°C, 40°C and 45°C with optimal production in the range of 35 to 40°C. It was reported that the maunt of pediocin L50 fromed at 16°C was comparable to that formed at 32°C, while considerably less amount was produced at 8°C. The organism failed to produce detectable amounts of bacteriocin at 45°C (Cintas *et al.,* 1995).

2.4.6.4 Growth phase:-

2.4.6.4.1 Lactobacilli bacteriocins:- Joerger and Klaenhammer (1986) observed accumulation of helveticin J between late log phase and stationary phase of growth of *Lactobacillus helveticus* 481. Helveticin V-1829 was produced from the middle log phase into the stationary phase of growth of *Lactobacillus helveticus* V-1829 (Vaughan *et al.,* 1992).

Barefoot and Klaenhammer (1984) observed the production of lacticin B during the logarithmic

phase of growth of *Lactobacillus acidophilus* N2. *Lactobacillus acidophilus* M46 produced acidocin B continuously during the logarithmic growth phase. The level of inhibition reached maximum at the beginning of the stationary phase and maintained constant for at least 24 h (Ten Brink *et al.,* 1994).

Lactobacillus plantarum C-11 was found to accumulate maximum amount of plantaricin A during the mid log phase of growth with a decrease in activity thereafter (Daeschel *et al.,* 1990). Maximum production of plantaricin S was obtained in log phase cultures of *Lactobacillus plantarum* LPC010. It was also observed that *Lactobacillus plantarum* PLC010 secreted another bacteriocin-designated plantaricin T in the late-stationary phase (Jimenez-Diaz *et al.,* 1993). Rekhif *et al.* (1994) reported plantaricin LC74 production in exponential phase of growth of *Lactobacillus plantarum*, however, the bacteriocin, plantaricin KW30, was maximally produced at the beginning of stationary phase culture of *Lactobacillus plantarum* KW30 (Kelly *et al.,* 1996).

It was reported that the concentration of brevicin 286 was highest at the late exponential growth phase (Coventry *et al.,* 1996).

2.4.6.4.2 Lactococcal bacteriocins:- Davey and Pearce (1980) observed diplococcin production by *Lactococcus lactis* subsp. *cremoris* 346 throughout the exponential growth phase. Nisin is synthesized as a secondary metabolite at a high rate when the cells have reached mid-exponential phase, and continues to be synthesized during a greater part of the stationary phase when the cells are grown at a constant pH of 6.8 at 30°C for 20 to 24 h (Ray, 1992a).

Bacteriocin S50 by *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* S50 was produced continuously during the growth, but the highest production was observed after 8 h of incubation (Kojic *et al.,* 1991). Lacticin 481 production occurred in late-log phase of growth of *Lactococcus lactis* subsp. *lactis* 481 (Piard *et al.,* 1990).

2.4.6.4.3 *Leuconostocs* bacteriocins:- Mathieu *et al* (1993) reported that the biosynthesis of mesenterocin 52 and its secretion into the medium started early in the growth phase, continued over the whole of that phase before reaching a maximum at the end. A decrease upto one to two orders of magnitude in the activity of carnocin LA54A was recorded during the stationary phase (Keppler *et al.,* 1994). Yang and Ray (1994a) observed the termination of bacteriocins production by various *Leuconostoc* spp. in the stationary phase of their growth. Leucocin B-Talla production occurred during the exponential phase of growth of the producer *Leuconostoc carnosum* Talla (Felix *et al.,* 1994). Production of leucocin A-UAL187 occurred early in the growth cycle of the producer organism, rather than as secondary metabolites of growth (Hastings and Stiles, 1991).

2.4.6.4.4 Pedicoccal bacteriocins:- Biswas *et al.* (1991) repoted that about 60% of the pediocin AcH was produced by 8 h and the rest 40% was produced during the next 8 h (stationary phase). The authors have suggested that pediocin AcH appeared to be a secondary metabolites. Later studies have shown that post translational processing of prepediocin to active pediocin AcH occurred efficiently at a pH below 5.0 (Johnson *et al.,* 1992). After 24 h of growth, the pediocin AcH was slightly reduced at all the temperatures studied (Biswas *et al.,* 1991). Production of pediocin during the logarithmic and early stationary phases of growth suggested that pediocin SJ-1 was a secondry metabolite and after reaching maximum levels, in contrast to many bacteriocins, the antibacterial activity of pediocin SJ-1 remained stable in broth cultures over a period of upto 48 h (Schved *et al.,* 1993). *Pediococcus acidilactici* L50 produced highest bacteriocin from the onset of stationary phase and it remained stable at 8°C and 16°C while at 32°C, a decrease in antibacterial activity was seen throughout the stationary phase (Cintas *et al.,* 1995). Daba *et al.* (1991) observed the secretion of pediocin 5 from *P. acidilactici* UL5 during the late exponential phase of growth and the activity dropped sharply (>90% in 24 h) during the early stationary phase, however, experiments with pH controlled at 5.0 did not show this large decrease in activity during the stationary phase.

2.4.7 Purification of bacteriocins: - An extensive characterization with respect to physical and chemical properties of bacteriocins is necessary

before considering them for application in foods. The availability of bacteriocins in a pure form is essential for characterization studies.

Purification of bacteriocins is a difficult task for several reasons. Firstly, protein concentration in the supernatant is very high while bacteriocin concentration is low, meaning a very low specific activity. Secondly, bacteriocins form a heterogeneous group of substances, and the specific purification protocol has to be developed by trial and error for each bacteriocin. An additional problem encountered with the purification of bacteriocins of lactic acid bacteria is the use of media containing tween 80, a surfactant that has been shown to interfere with the precipitation procedures (Muriana and Klaenhammer, 1991a; van Laack *et al.,* 1992). Vaughan *et al.* (2001) purified the bacteriocin to homogeneity by ammonium sulphate precipitation, cation exchange, hydrophobic interaction and reverse-phase liquid chromatography.

During the recent years, the above mentioned problems have been overcome and several bacteriocins of lactic acid bacteria have been purified to homogenecity by growing the producers in semi-defiend media by minimizing the level of contaminating proteins and peptides (Joerger and Klaenhammer, 1986; Hastings *et al.,* 1991; Hechard *et al.,* 1992). Also MRS broth has been generally modified by omission of tween 80 (van Laack *et al.,* 1992; Mortvedt *et al.,* 1991).

2.4.7.1 Lactobacilli bacteriocins:- Barefoot and Klaenhammer, (1984) purified lacticin B by ion-exchange chromatography, ultrafiltration and gel filtration chromatography. Later, as mentioned by Nettles and Barefoot, (1993) a simpler purification protocol was devised for lacticin B. The protocol involved lyophilisation of culture supernatants followed by ultrafiltration and preparative electrofocussing. Muriana and Klaenhammer, (1991a) achieved a 474-fold increase in specific activity of lactacin F by ammonium sulfate precipitation, gel filtration and HPLC.

Lactocin S produced by *Lactobacillus* sake L45 was purified to a 4000-fold increase in specific activity with a recovery of just 3.0% by ammonium sulfate precipitation, and sequential anion and cation exchange, hydrophobic interaction, gel filtration, phenyl superose and reverse-phase chromatographies (Mortvedt *et al.,* 1991). Holck *et al.* (1992) purified sakacin A to a 9000-fold increase in specific activity and a very good recovery of about 80% was achieved by ammonium sulfate precipitation, ion exchange, hydrophobic interaction and reverse-phase chromatography.

Plantaricin S from *plantarum* LPC010 was purified to homogeneity by ammonium sulfate precipitation, binding to SP-sepharose fast flow, phenyl sepharose CL-4B and C2/C-18 reverse-phase chromatographies. The purification protocol resulted in a final yield of 91.6% and 352, 617-fold increase in specific activity (Jimenez-Diaz *et al.,* 1995).

A purification protocol comprising ammonium sulfate precipitation and sequential cation exchange and reverse-phase chromatographies has been used for the purification of acidocin A with a recovery of about 10% (Kanatani *et al.,* 1995). The protocol resulted in a more than 3000-fold increase in the specific activity of acidocin A.

2.4.7.2 Lactococcal bacteriocins:- Diplococcin was purified from the supernatant of *Lactococcus lactis* subsp. *cremoris* 346. The procedure employed included ammonium sulfate precipitation (60% saturation) and cation exchange chromatography on carboxy methyl cellulose (CMC) resulting approximately 1000-fold purification (Davey and Richardson, 1981). Dufour *et al.* (1991) purified lactococcin from culture supernatant of *Lactococcus lactis* subsp. *lactis* as a single band by dialysis, cation exchange and gel filtration chromatographies. The procedure employed resulted in a 14.5-fold purification with about 3000-fold increase in specific activity.

Ammonium sulfate precipitation of culture supernatant obtained from *Lactococcus lactis* subsp. *lactis* CNRZ481 resulted in a 455-fold increase in the total lacticin 481 activity. Subsequent purification by gel filtration chromatography and C18 reverse-phase high performance liquid chromatography (HPLC) lead to a 107, 506-fold increase in the specific activity of lacticin 481 (Piard *et al.,* 1992). Holo *et al.* (1991) purified lactococcin A with about 2300-fold purification and yield of 16% by a sequential protocol including ammonium sulfate

precipitation, cation exchange chromatography and reverse-phase HPLC. Lactococcin G was similarly purified to homogeneity by a four step protocol which included ammonium sulfate precipitation, binding to a cation exchanger and octyl-sepharose CL-4B and reversed-phase chromatography leading to a recovery of about 20% of the original activity and a 7000-fold increase in specific activity (Nissen-Meyer *et al.,* 1992). The bacteriocin diacetin B produced by *Lactococcus lactis* subsp. *lactis* biovar.diacety*lactis* UL720 was purified by a pH dependent adsorption-desorption procedure followed by a reverse-phase HPLC with a yield of just 1.25% of the original activity (Ali *et al.,* 1995).

2.4.7.3 *Leuconostocs* bacteriocins- Leucocin A-UAL187 from *Leuconostoc gelidium* UAL-187 was purified by ammonium sulfate precipitation followed by a sequential hydrophobic interaction, gel filtration and reverse-phase HPLC with a yield of 58% of the original activity and a purification fold of 4500 (Hastings *et al.,* 1991). Hechard *et al.* (1992) employed a three-step protocol for the purification of mesentericin Y105. The protocol included affinity chromatography on a blue agarose column, untrafiltration through a 5-kDa cut off membrane and finally reverse-phase HPLC on a C4 column. The purification procedure resulted in a very low yield of 0.7% with a purification fold of about 420. The purification procedure consisting of ammonium sulfate precipitation, and a sequential gel filtration, cation exchange and hydrophobic interaction chromatography resulted in a satisfactory increase of specific activity (1,135-fold) but a very low recovery of 8% of mesenterocin 52 produced by *Leuconostoc* mesenteroides FR52. Keppler *et al.* (1994) reported the purification to homogeneity of carnocin LA54A by single step hydrophobic interaction chromatography using amberlite XAD-2. Revol-Juneless and Lefebvre, (1996) reported the purification of dextrancin J24 to homogeneity by desorbing the bacteriocin from the producer cells at pH 2.0 followed by a reverse-phase HPLC.

2.4.7.4 Pediococcal bacteriocins: - Pediocin AcH from the culture supernatant of *P. acidilactici* H was purified by ammonium sulfate precipitation (70% saturation), fast protein liquid chromatography (FPLC), gel filtration and anion exchange chromatography leading to a 98.8-fold purification with a single band on SDS-PAGE gel (Bhunia *et al.,* 1988). Yang *et al.* (1992) reported the purification of pediocin AcH to homogeneity as revealed by a single sharp band on SDS-PAGE gel by a pH dependent adsorption/desorption procedure. Pediocin AcH was adsorbed to the producer cells at a pH of 6.0- 6.5, centrifuged; the bacteriocin adsorbed onto the cells was extracted at a low pH of 1.5-2.0. The purification protocol resulted in the recovery of almost all the bacteriocin produced. Henderson *et al.* (1992) reported a 470-fold purification of pediocin PA-1 by gel filtration, ion-exchange chromatography, dialysis and HPLC, whereas Lozano *et al.* (1992) achieved a 80,000-fold increase in specific activity of pediocin PA-1 by employing ammonium sulfate precipitation, chromatography with a cation exchanger and octyl sepharose and reverse-phase HPLC.

Daba *et al.* (1994) employed the pH dependent adsorption/desorption procedure developed by Yang *et al.* (1992) for the recovery of pediocin 5 produced by P. acidilactici UL5. The procedure resulted in a partial recovery of the cell associated bacteriocin fraction and even longer desorption times exceeding 24 h could not result in the recovery of more than 10% of the original activity. Further purification to homogeneity was, however, achieved by reverse-phase HPLC (Daba *et al.,* 1994).

Schved *et al.* (1993) reported a 262-fold purification with a recovery of 50% of pediocin SJ-1 by the direct application of cell free supernatant containing crude bacteriocin to a cation exchange chromatography column. The homogeneity of pediocin SJ-1 thus purified was confirmed by SDS-PAGE. Cintas *et al.* (1995) purified pediocin L50 to homogeneity by ammonium sulfate precipitation, and sequential cation exchange, hydrophobic interaction and reverse-phase chromatographies resulting in the recovery of more than 80% of the starting material with a 114, 112-fold increase in specific activity.

2.4.8 Genetic determinants of bacteriocin production and immunity:- An understanding of the genetic control for bacteriocin production

and host immunity might be beneficial for their effective use. This is also necessary for cloning and sequencing of genes involved, and application of genetic methods for the construction and improvement of bacteriocin producing strains of LAB.

The original criteria laid down for bacteriocins specify the plasmid borne genetic determinants of bacteriocin production and host cell immunity (Tagg *et al.,* 1976). Although, most of the bacteriocins of lactic acid bacteria analysed to date adhere to this criterion, a very few, especially those produced by lactobacilli, have been found to have chromosomal borne genetic determinants (Barefoot and Klaenhammer, 1983; Joerger and Klaeanhammer, 1986). The bacteriocin immunity genes are generally borne on the plasmids that encode bacteriocin production, however, bacteriocin plasmids that do not carry immunity genes have also been found in lactic acid bacteria (Gonzalez and Kunka, 1987; Schved *et al.,* 1993; Kanatani and Oshimura, 1994).

2.4.9 Chemical nature of bacteriocins: – All bacteriocins, which have been studied in sufficient detail, are found to be macromolecular particulate in nature and include, if not consist of polypetides or protein, Currently, over 20 bacteriocins of lactic acid bacteria have been sequenced. Most contain less than 60 amino acids and all are devoid of lipid and carbohydrate materials. The molecules are cationic and hydrophobic. Due to the hydrophobic nature of these molecules they have a tendency to aggregate, especially when stored at high concentration or for a long time (Ray and Miller, 2001).

Their high isoelectric point (Jack *et al.,* 1995) allows thcm to interact at physiological pH values with the anionic surface of bacterial membranes. This interaction can suffice, in the case of broad-spectrum bacteriocins, or facilitate, in the case of receptor-requiring compounds, insertion of the hydrophobic moiety into the bacterial membrane. Later, the cooperation between a number of bacteriocin molecules will build up the transmembrane pore responsible for gradient dissipation and cellular death, These features have favored the development of general purification protocols for bacteriocins that include hydrophobic interaction, cationic exchange and reverse-phase chromatographic steps. The complex pattern of monosulfide and disulfide intramolecular bonds helps in the stabilization of secondary structures by reducing the number of possible unfolded structures (entropic effect). From a structural point of view, the effect of the intramolecular bonds is additive, and the higher their number, the higher the global stability of the peptide. These facts and the sensitivity of nisin to digestive enzymes discouraged the clinical application of this compound but made it a product of choice as food preservative (Barnby-smith, 1992).

The studies on the kinetics of killing by bacteriocins show that killing begins as soon as the bacteriocin is added to a culture and suggest that all bacteriocins are bacteriocidal, as opposed to bacteriostatic (Reeves, 1965). Bacteriocidal effect remains stable during storage in dried or liquid forms at frozen and refrigeration temperatures. It slowly decreases during storage at room temperature in the presence of air that can oxide methionine residues to the inactive methionine sulfoxide form Molecules with disulphide bond can undergo bond exchange in the presence limited amounts of reducing agent and form dimer and trimers that retain bactericidal activity. (Klaenhammer, 1993). The first stage is specific irreversible adsorption of the bacteriocion and that in some instances at least; only one molecule may be required to kill a sensitive cellBactericidal nature of bacteriocins has also been observed by Toora *et al.* (1994), as inhibition zone remained clear of indicator colonies for two weeks in case of *Yersinia enterocolitica.*

2.4.10 Bacteriocin biosynthesis: – Bacteriocins are synthesized as pre-propeptide which are processed and externalised by dedicated transport machinery (Nes *et aI.,*1996). Bacteriocin production in LAB is growth associated: it usually occurs throughout the growth phase and ceases at the end of the exponential phase or sometimes before the end of growth (Parente *et aI.,* 1997). Bacteriocin production is affected by type and level of the carbon, nitrogen and phosphate sources, cations surfactants and inhibitors. Bacteriocins can be produced from media containing different carbohydrate sources. Nisin Z can be produced

from glucose, sucrose and xylose by *Lactococcus lactis* IO-1 (Matsuaki *et aI.*, 1996) but better results were obtained with glucose compared to xylose. Glucose followed by sucrose, xylose and galactose were the best carbon sources for the production of Pediocin AcH in an unbuffered medium Biswas *et aI.*, 1991).

All bacteriocins are synthesized with an N terminal leader sequence and until recently only the double glycine type of leader was found in class II bacteriocins (Holo *et at.*, 1991; Muriana and Klaenhammer, 1991; Klaenhammer, 1993). However, it has now been disclosed that some small, heat stable and non modified bacteriocins are translated with sec dependent leaders (Leer *et al.,* 1995). The structural bacteriocin gene encodes a preform of the bacteriocin containing an N-terminal leader sequence (termed double glycine leader) whose function seems to prevent the bacteriocin from being biologicalhy active while still inside the producer and provide the recognition signal for the transporter system.

A number of genes, often found in close proximity to each other are required for production of lantibiotics. These genes include:

(a) The structural gene, Ian A,

(b) Immunity genes (Lan I and in some cases Lan E, Lan F and Lan G) encoding proteins that protect the producer from the producer lantibiotic,

(c) A gene Lan T encoding what appears to be a membrane associated ABC transporter that transfers, the lantibiotic across the membrane,

(d) A gene, Ian P, encoding a serine proteinase, which removes the leader sequence of the lantibiotic prepeptide,

(e) Two genes, Ian B and Lan C (or in some cases only one gene, Lan M), with no sequence similarity to other known gens thought to encode enzymes involved in the formation of lanthionine and methyllanthionine, and

(f) Two genes Ian k and Ian R encoding two component regulatory proteins that transmit an extracellular signal and therby inducing lantibiotic production.

Each gene cluster appears to contain all the genes necessary for translation and post- translational modifications, when necessary, of a prebacteriocin, secretion and removal of the leader peptide, and self-immunity. (Ray *et al.,* 2001).

The promoter upstream of the nis A gene is activated by the nis R and nis K proteins which induce transcription of the gene cluster. (de vos *et al.,* 1995). Following translation of prenisin, the leader peptide directs the precursor to the membrane located nis B protein that dehydrates serine to dhA and threonine to dhB. Nis C, which forms a complex with nis B, then forms thioether linkages between dehydration residues and cysteines (Ray *et al.,* 2001).

The modifying enzymes, nis B and nis C are encoded directly downstream of nis A. These enzymes act on the prepeptide, modifying only the mature protein, which is then transported (Allison and Klaenhammer, 1999). Nis T encodes a protein that shares significant homology with ATP-dependent translator proteins, and is involved in the translocation of fully modified precursor nisin across the cytoplasmic membrane (Qiao and Saris, 1996). Once outside the membrane, the leader peptide is removed from the biologically active precursor by the nis P which is an extracellular serine protease. With the removal of leader peptide, the matured pronisin (or nisin) molecule becomes biologically active and is released into the environment. Nis I encode a lipoprotein that is involved in immunity. Proteins F, E, and G also provide cells with additional protection against nisin. (Bukhtiyarova and Yand, 1994).

2.4.11 Storage studies:- Gandhi and Nambudripad (1981) reported that crude and partially purified antibiotic from *Lactobacillus acidophilus* was stored at –25°C for 6 months without any loss of activity. While lactacin B was stable during storage at room temperature or at -20°C for several months (Barefoot and Klaenhammer, 1984) without any loss of activity. ten-Brink *et al.* (1994) found that filter sterilized culture supernatant fluids containing acidocin B could stored at –20°C or 4°C for at least 90 days without loss of acidocin B activity while during storage at 37°C some inactivation occurred, possibly caused by the action of proteolytic enzymes present in culture supernatant. Dave and Shah (1997) repoted that acidophilicin LA-1 was

stable for >15 days at 37°C, >3 months at 4°C and >8 months at -18°C.

2.4.12 Applications: – The single most important reason behind the recent interest in isolating bacteriocin producing lactic acid bacteria and in studying bactericidal effectiveness of bacteriocin is their potential applications as food biopreservations (Ray *et al.,* 2001).

2.4.12.1 Food applications: – Food processors face a major challenge in an environment in which consumers demand safe foods with a long shelf life, but also express a preference for minimally processed products that do not contain chemical preservatives. Bacteriocins are an attractive option that could provide at least part of the solution. They are produced by food-grade organisms, they are usually heat stable and they can inhibit many of the primary pathogenic and spoilage organisms that cause problems in minimally processed foodstuffs, however, at present, only nisin and pediocin PAl/AcH have found widespread use in food. The form of nisin used most widely in food is Nisaplin (Danis co), which is a preparation that contains 2.5% nisin with NaCl (77.5%) and non-fat dried milk (12% protein and 6% carbohydrate). The use of pediocin PAl for food biopreservation has also been commercially exploited in the form of ALTA 2431 (Quest), which is based on LAB fermentates generated from a pediocin PAl-producing strain of *Pediococcus acidilactici* (Rodriguez *et al.,* 2002). Its use is covered by several US and European patents (Ennahar *et al.,* 2000; Rodriguez *et al.,* 2002) when screening for a bacteriocin With a food application in mind, there are several important criteria: first, the producing strain should preferably have 'generally recognized as safe' (GRAS) status; and second, the bacteriocin should have a broad spectrum of inhibition that includes pathogens, or have activity against a particular pathogen. Third, the bacteriocin should be heat stable; fourth, have no associated health risks; fifth, its inclusion in products should lead to beneficial effects such as improved safety, quality and flavour; and sixth, it should have high specific activity (Holzapfel *et al.,* 1995). Bacteriocins have been shown to have potential in the biopreservation of meat, dairy products, canned food, fish, alcoholic beverages, salads, egg products, high-moisture bakery products, and fermented vegetables, either alone, in combination with other methods of preservation, or through their incorporation into packaging film/food surfaces (Chen and Hoover, 2003).

Although, bacteriocins with a wide spectrum of activity are usually the most sought after, other factors including pH optima, solubility and stability are as important and are major considerations in choosing a particular inhibitor for a particular food or target bacterium. Furthermore, the antimicrobial spectra of a variety of LAB bacteriocins can be extended to encompass Gram-negative bacteria through their use in combination with measures that affect the integrity of the outer membrane, such as temperature shock, high pressure, chelators and eukaryotic antimicrobial peptides (Stevens *et al.,* 1991; Delves-Broughton *et al.,* 1996; *Suma et al.,* 1998). There are also rare natural and bioengineered bacteriocins (Yuan *et al.* 2004) that possess inherent activity against Gram-negative microorganisms.

Bacteriocins can also be used to promote quality, rather than simply to prevent spoilage or safety problems. For example, bacteriocins can be used to control adventitious non-starter flora such as non-starter lactic acid bacteria (NSLAB) in cheese and wine. The uncontrolled growth of NSLAB can cause major economic losses owing to calcium-D-lactate formation and slit defects in cheeses, and the production of detrimental compounds in wine. Bacteriocins producing starters and adjuncts (one- or two-strain strategies) have been found to significantly reduce these problems (Daeschel *et al.,* 1991; Ryan *et al.,* 1996). However, as some NSLAB such, as lactobacilli and other starter adjuncts in cheese, and *Leuconostoc oenos* and *Pediococcus damnosus* in some red wines can improve flavour, the complete elimination of NSLAB is not always desirable. This problem has been overcome through the use of a three-strain system in which an adjunct strain with reduced bacteriocin sensitivity (obtained on repeated exposure to increasing concentrations of the bacteriocin) is used with a bacteriocin-producing starter (Ryan *et al.,* 2001) Figure 9.

Bacteriocins can also be applied in other ways to enhance food fermentation. This has been shown during semi-hard and hard cheese manufacture

in which bacteriocin production brings about the controlled lysis of starter LAB, which results in the release of intracellular enzymes and ultimately accelerated ripening and even improved flavour (Martinez-Cuesta *et al.,* 2000).

Although traditionally, the use of bacteriocins is associated with the preservation of food, in the near future food might merely act as a vehicle for the delivery of bacteriocin-producing probiotic bacteria. The production of antimicrobials by a probiotic culture is a desirable trait as they are thought to contribute to the inhibition of pathogenic bacteria in the gut (Dunne *et al.*, 1999), whereas bacteriocins in food are degraded by the proteolytic enzymes of the stomach, probiotic bacteria might be ingested in a form that facilitates gastric transit, allowing the *in vivo* production of the bacteriocin in the small or large intestine. It has also been speculated that recombinant probiotic strains that can be induced to produce bacteriolysin could be developed to facilitate the *in vivo* delivery of bioactive compounds that are produced intracellularly (Hickey *et al.*, 2004).

Three approaches are commonly used in the application of bacteriocins for biopreservation of foods (Schillinger *et al.,* 1989):

1) Inoculation of food with LAB that produces bacteriocin in the products. The ability of the LAB to grow and produce bacteriocin in the products is crucial for its successful use.

2) Addition of purified or semi-purified bacteriocins as food preservatives.

3) Use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing.

Unlike most other preservation methods, such as heat or low pH, which are essentially indiscriminate in their antimicrobial effect, it is this ability to precisely influence the developing flora in an otherwise perishable food that led us to describe the use of bacteriocins as a form of 'innate immunity' for food. As already described, the inclusion of *Listeria*-active class IIa bacteriocins can specifically prevent the growth of this pathogen, without affecting harmless LAB, or bacteriocin-tolerant strains can be introduced into an otherwise hostile food environment. It is unlikely that the use of bacteriocins in food will negatively impact on the natural flora of either the human (or animal) host, or on the environment. The low level of bacteriocins required eliminating or reducing small numbers of pathogenic or spoilage organisms in food are unlikely to have an impact on more microorganism-rich environments. In any event, bacteriocins are unlikely to survive gastric transit, as they are sensitive to proteolytic degradation.

2.4.12.2 Clinical applications:- In particular, the elucidation of the precise mechanism of action of some lantibiotics and their activity against multidrug resistant pathogens by a novel mechanism makes them an attractive option as possible therapeutic agents.

The broad-spectrum lantibiotics could theoretically be of use against any clinical Gram-positive human or animal pathogen. For example, the two-peptide lantibiotic lacticin 3147 has *in vitro* activity against *Staphylococcus aureus* [including methicillin-resistant S. *aureus* (MRSA)], enterococci (including VRE), streptococci *(S. pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus mutans), Clostridium botulinum,* and *Propionibacterium acnes* (Galvin *et al.*, 1999). Initial *in vivo* trials with animal models have demonstrated the success of lantibiotics in treating infections caused by S. *pneumoniae* (Goldstein *et al.*, 1998), and MRSA (Niu and Neu, 1991; Kruzewska *et al.*, 2004), and in preventing tooth decay and gingivitislss (Howell *et al* 1993; Blackburn and Goldstein, 1995, Ryan *et al.*, 1999).

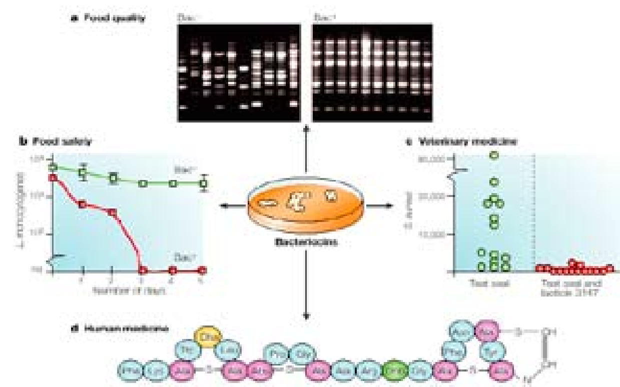

Figure 9. Selected applications of bacteriocins. a | Food quality. A cheese made with a commercial starter culture (Bac

–) will develop an undefined flora called non-starter lactic acid bacteria (typified by different fingerprints generated by random amplified polymorphic DNA patterns). However, a cheese inoculated with the same commercial strain that can produce a bacteriocin (Bac +) (lacticin 3147, in this example) and a resistant adjunct strain of *Lactobacillus*, chosen for a flavour attribute, will develop a single defined culture once the starter culture has died off, offering the cheese manufacturer control over previously adventitious flora development. **b** | Food safety. A simple example of the role of bacteriocins in food safety is the production of cottage cheese with a starter culture that produces a bacteriocin with activity against *Listeria monocytogenes*, which results in a cheese that is inherently anti-*Listeria*. **c** | Veterinary medicine. A teat seal is a physical barrier against infection. Here, a bacteriocin was incorporated into the teat seal and the teat was challenged with *Staphylococcus aureus.* The number of staphylococci recovered from 14 teats with or without bacteriocin is shown. **d** | Human medicine. A *Streptococcus mutans* strain that cannot produce acid, but that produces the lantibiotic mutacin (shown), can competitively exclude acidogenic *S. mutans*, thereby offering protection against tooth decay (Hilman, 2002). ***Source*:-** Cotter *et al.* (2005).

The use of nisin for human clinical applications has been licensed to Biosynexus Incorporated by Nutrition 21 and Immu Cell Corporation has licensed the use of the anti-mastitic nisin-containing product Mast Out to Pfizer Animal Health. *Bovine mastitis* is defined as an inflammation of the udder and is the most persistent disease in dairy cows. Nisin is also used as an active agent in Wipe-Out (a teat wipe), and lacticin-3147-containing Teat Seals (Cross Vetpharm Group Ltd) have been shown to prevent deliberate infection by mastitic staphylococci and streptococci in animal challenge trials (Ryan *et al.*, 1999) Figure 8. A strain that produces the lantibiotic mutacin 1140 is entering Phase I clinical trials in the US with a view to replacement therapy, and the dietary supplement BUS K12 throat guard, which contains a *Streptococcus salivarius* that produces two lantibiotics salivaricin A2 and B, is sold in New Zealand as an inhibitor of the bacteria responsible for bad breath (Tagg, 2004). From a nonantimicrobial medical perspective, the cinnamycin-like lantibiotics have attracted interest owing to their novel activities against the functions of medically important specific human enzymes, such as phospholipase A2 and angiotensin-converting enzyme, and nisin has also been found to have contraceptive efficacy (Aranha *et al.*, 2004;). The effectiveness of probiotics as agents in the treatment of various gastrointestinal disorders has also been shown in several recent studies (Fedorak and Madsen, 2004).

For commercial and industrial reasons, the selected probiotics and starter cultures must be produced under the most stringent fermentation and manufacturing conditions. In general, the selection criteria for starter cultures are at their acidification rate and flavour-producing characteristics. For probiotics, selection is based on a detectable health effect on the host. In addition, the use of low-cost industrial growth substrates and microbial strains with low phage sensitivity are regularly considered as important objectives for both strain and process improvement. Most of these strain and process-improvement strategies are based on screening and trial-and-error approaches.

Genome-sequencing and functional-genomics studies that focus on LAB which are used as part of industrial starter cultures (Kleerebezem *et al.,* 2003) or exploited for their probiotic properties are rapidly revealing the molecular basis of relevant traits, including stress-response-adaptation mechanisms and amino-acid and vitamin auxotrophies (Siezen *et al.,* 2004).

Some LAB secretes vitamins, including riboflavin (vitamin B_2), folate (vitamin B_{11}) and cyanocobalamine (vitamin B_{I2}). This unique characteristic offers the food industry the possibility to fortify raw food materials such as soy, milk, meat and vegetables with B vitamins without adding food supplements (Kleerebezem et al., 2003). Currently, in the fermentation industry, starter cultures are selected on the basis that they can produce and secrete high levels of vitamins B. In addition to natural strain selection, overproduction of vitamin

B_2 and vitamin B_{11} has been achieved by genetic engineering of the corresponding biosynthesis pathways of *L. lactis* (Sybesma *et al.,* 2003). However, metabolic engineering of such complex biosynthesis pathways can often lead to unexpected phenotypes because the products, being co-factors in various biochemical reactions, impact directly on many other pathways. LAB are also good candidates for the production and delivery of heterologous proteins and peptides that have potential therapeutic activity (Miyoshi *et al.,* 2002). Many LABs are acid and bile resistant, and are therefore, well adapted to function as vehicles for the oral delivery of vaccine antigens (Mercenier *et al.,* 2000). The best-studied LAB used, as vaccine vectors are *Lactococcus lactis* and *Lactobacillus plantarum*. Robinson *et al,* (1997) showed that intragastric or intranasal administration of recombinant lactococci expressing tetanus toxin fragment C resulted in the induction of systemic antibody responses in mice at levels sufficient to be protective against a lethal challenge with tetanus toxin. Lactococcal immunization was also found to induce mixed immunoglobulin-G and T-helper responses, allowing the induction of protection against various infectious agents and potentially at several mucosal surfaces (Robinson *et al.,* 2004),The impact of overproduction of heterologous proteins on the lactococcal host cells can be significant, as evident from the general stress response usually associated with protein overproduction (Schweder *et al.,* 2002). In addition, the drain on aminoacids used in heterologous protein biosynthesis can cause global effects on the metabolism of the host cell.

Thus, keeping in view the wide application of bacteriocins in food industry and clinical microbiology, it was felt desirable to undertake the present studies with an aim to isolate and characterize high bacteriocin producing strains of LAB from milk and milk products. It was also aimed to standerize various parameters for cheaper and optimum production of bacteriocin from the selected strain. The effects have also been made to purify and identify the bacteriocin during the present investigation.

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