CHAPTER 2

LITERATURE REVIEW

2.1 Lactic Acid Bacteria

2.1.1 Classification of lactic acid bacteria

Early definitions of LAB as a group, based on the ability to ferment and coagulate milk, included the coliform bacteria with the lactics. According to Orla-Jensen (1919) the 'true lactic acid bacteria' from a natural group of gram-positive, nonmotile, non-sporeforming, rod and coccus-shaped organisms that ferment carbohydrates and higher alcohols to form chiefly lactic acid. He propose the seven genara namely *Betabacterium, Thermobacterium, Streptobacterium, Streptococcus, Betacoccus, Microbacterium* and *Tetracoccus* (Table 2.1). Orla-Jensen (1919) monograph on LAB emphasised the importance of the streptococci in milk and dairy products. The first systematic classification of the streptococci was proposed by Sherman (1937). Strictly anaerobes and the pueumococci were excluded from the classification. The remaining facultatively anaerobic streptococci were dividing into the four groups as shown in Table 2.2

Today we have the means to examine, in detail, macromolecules of the cell, belived to be more accurate in defining relationships and phylogenetic position. These are, of course, the nucleic acid. Fortunately, nature has provided us with different kinds of nucleic acid for different type of taxonomic studies (Johnson, 1984). For determining phylogenic positions of species and genera, ribosomal RNA (rRNA) is more suitable because the sequence contains both well-conserved and less conserved regions (Lane *et al*, 1985). Thus, application of molacular genetic

technigue to determine the relatedness of food-associated LAB has resulted in significant changes in their taxonomic classification (Stile and Holzapfel, 1997).

Table 2.1 The 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-	*Carnobacterium*
Streptococcus	Coccus	-	-	Homo-	*Streptococcus* *Enterococcus* *Lactococcus* *Vagococcus*
Betacoccus	Coccus	-	-	Hetero-	*Leuconostoc* *Oenococcus* *Weissella*
Microbacterium	Rod	+	+	Homo-	*Brochothrix*
Tetracoccus	Coccus	+[b]	+	Homo-	*Pediococcus* *Tetragenococcus*

[a] According to Orla-Jensen (1919)

[b] In general pediococci are catalase negative but some strains produce a pseudocatalase that results in false positive reactions.

Source: Orla-Jensen (1919)

The LAB associated with food now comprises species of the genera following.

2.1.1.1

Lactobacillus

The genus *Lactobacillus* is by far the largest of the genera included in LAB. It is also very heterogenous, encompassing species with a large variety of phenotypic,biochemical and physiological properties. The heterogeneity is reflected by the range of mol % G+C of the DNA of species included in the genus. This range is 32-53%,

Table 2.2 Classification system for the streptococci

	Sherman group			
	Pyogenic	Viridans	Lactic	*Enterococcus*
Lancefield's group	A, B, C, G	-[a]	N	D(Q)
Haemolysis	β	α	-	-
Growth				
10°C	-	-	+	+
45°C	(+)	+	-	+
pH 9.6	-	-	-	+
Survive 60°C for 30 min	-	+	Variable	+
Methylene blue (0.1%)	-	-	+	+(-)

[a] Various group or negative.

Source: Sherman (1937)

which is twice the span usually accepted for a single genus (Schleifer and Stackebrandt, 1983). The classical division of the lactobacilli was based on their fermentative characteristic, obligately homofermentative, facultatively heterofermentative and obligately heterofermentative shown in Table 2.3. This division suited the interests of food microbiologists. Several lactobacilli of group 1, 2 and some of the heterofermentative group 3 lactobacilli are either used in fermented foods, but group 3 are also commonly associated with food spoilage (Sneath *et al*, 1986).

Group 1 includes the obligately homofermentative lactobacilli that ferment pentose or gluconate.

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

Table 2.3 Arrangement of the Genus *Lactobacillus*

Character	Group I obligately homofermentative	Group II facultatively heterofermentative	Group III obligately heterofermentative
Pentose fermentation	-	+	+
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+[a]	+[a]
FDP aldolase present	+	+	-
Phosphoketolase present	-	+[b]	+
	Lb. acidophilus	*Lb. casei*	*Lb. brevis*
	Lb. delbrückii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantalum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sake*	*Lb. reutrri*

[a] When fermentd.

[b] Inducible by pentoses.

Adapted from Sharpe (1981) and Kandler and Weiss (1986).

Group 3 includes the obligately heterofermentative lactobacilli that ferment hexoses to lactic acid, acetic acid and/or ethanal and carbon dioxide. The production of gas from glucose is, a characteristic feature of these bacteria. The most important obligate heterofementative *Lactobacillus* associated with food fermentations is *Lactobacillus sanfrancisco* which converts flavour compounds in sourdough bread (Stile and Holzapfel, 1997).

2.1.1.2

Streptococcus

The generic name *Streptococcus* was first used by Rosenbach (1884) to describe the chain-forming, coccus-shape bacteria associated with wound infections. The genus *Streptococcus* was originally describe based on morphological, serological, physiological and biochemical characteristics and it comprised a wide range of organisms including the highly pathogenic bacteria. Jones (1978) reviewed the composition and differentiation of the genus *Streptococcus* and proposed seven groups, including the strict anaerobes and pneumococci, based on artificial criteria of pathogenicity, habitat and oxygen tolerance that do not necessarily imply any close relationship between the streptococci included in any one group. *Streptococcus thermophilus* is an exception in this genus because it is an important starter organism for yogurt and cheese manufacture.

2.1.1.3

Vagococcus

Vagococcus are easily confused with lactococci, but the genera are clearly distinguished by the fatty acid composition. Some but not all strains of vagococci are motile (Collins *et al*, 1989; Pot *et al*, 1994). Genus-and species-specific oligonucleotide probes are available for vagococci (Williams and Colins, 1992), which make a reliable identification of these bacteria feasible.

2.1.1.4

Lactococcus

The genus *Lactococcus* includes several uncommon species: *Lactococcus gavieae* associated with mastitis in cows, *Lactococcus piscium* from salmonid fish, *Lactococcus plantarum* from frozen peas and *Lactococcus raffinolactis* from raw milk (Schleifer *et al*, 1985, Williams *et al*, 1990). The use of lactococci is widespread and has the longest tradition in industrial starter culture technology.

Strains of *Lactococcus lastis* produce a range of bacteriocins the most important of which is the lantibiotic and nisin which is a relatively broad spectrum bacteriocin that is active against gram-positive bacteria, including *Clostridium botulinum* and its spores. Nisin is licensed for use as a food preservative in over 45 countries (Delves-Broughton, 1990).

2.1.1.5

Enterococcus

The taxonomy of this group of bacteria has been vague. There are no phenotypic characteristics that separate the genus from the other genera of gram-positive, catalase-negative cocci; in fact, phenotypic identification is generally by reverse identification (Devriese *et al*, 1993). The enterococci were described by Sherman (1937) as those organisms that grow at 10 and 45 °C, in 6.5% NaCl and at pH 9.6, survive heating at 60 °C for 30 minutes and react with lancefield group D antisera. Several species and strains of the genus *Enterococcus* do not meet all of these criteria (Devriese *et al*, 1993). The importance of the enterococci for food and public health microbiologists is related to their enteral habitat, their use as indicators for food safety and their possible involvement in foodborne illness (Stile, 1989).

2.1.1.6

Pediococcus

As a result of their association with beer spoilage, the pediococci were among the first bacteria to be studied by Louis Pasteur. Tetrad formation and spherical shape served as key characteristics for their early recognition. They were the only LAB that divides in two planes to produce tetrads or pairs, but taxonomic changes have increased the number of tetrad forming genera to three. Pediococci are most likely to be confused with micrococci because of morphological similarities, pseudocatalase production and salt tolerance (Garvie, 1986b) and also with the aerococci. The pediococci are homofermentative and, with the exception of *Pediococcus dextrinicus* which produces L (+)-lactic acid, all species produce DL-

lactate from glucose (Raccach, 1987). The pediococci are important starter bacteria in fermented sausages of some regions. Some of the widely used starter strains such as *Pediococcus acidilactici* and *Pediococcus pentosaceus* produce bacteriocins. Although, they are of economic importance as starter culture, not all of the fermentation pathways utilised by pediococci are clear (Fukui *et al*, 1957).

2.1.1.7

Tetragenococcus

The genus *Tetragenococcus* contains strains previously regarded as *Pediococcus halophilus*. Only one species, *Tetragenococcus halophilus* is currently recognized, but one enterococcal species, *Enterococcus solitarius*, has been show to be related to *Tetragenococcus* phylogenetically (Williams *et al*, 1991). In addition to its extreme salt tolerance (> 18%) NaCl, which distinguishes it from other LAB, *T. halophilus* has a salt requirement for growth, generally 5 % NaCl (Garvie, 1986b). *T. halophilus* is an important species in lactic fermentations of high-salt-containing food, such as Soy sauce (Garvie, 1986b; Abe and Uchida, 1989).

2.1.1.8

Aerococcus

Aerococci constitute the tetrad-forming LAB. The genus *Aerococcus* currently contains two species *Aerococcus viridans* and *Aerococcus urinae*. Aerococci are of minor interest in food technology and will not be dealt with further. Information on the genus *Aerococcus* is given in a review by Weiss (1991).

2.1.1.9

Leuconostoc

Leuconostoc is a genus of Gram-positive bacteria, placed within the family of *Leuconostocaceae*. They are generally ovoid cocci often forming chains. The species of this genus are distinguished from those in the previously discussed

genus in that they are heterofermentative; that is, they produce levorotatory lactic acid, acetic acid, ethanol and carbon dioxide from glucose. Fructose is often reduced to mannitol in sucrose solutions, many strains grow with a characteristic slime or dextran formation. The cells are normally spherical and, like the streptococci, may occur slingly, in pairs, or in short chains. These are saprophytic organism which were first recognized as nuisance or spoilage organism in sugar factories where they produce the troublesome, typical dextran slime growth. They are very important in initiating fermentation of vegetables and are found in fruit juices, wines, mashes and other foods (Carl, 1978).

2.1.1.10
Oenococcus

The genus *Oenococcus* was proposed for these bacteria (Dicks *et al*, 1995). Thus, *Leuconostoc oenos* is now designated *Oenococcus oeni*. It is important to note that although these taxonomic revisions were necessary from a phylogenetic point of view, they do not make the practical classification of "leuconostoc-like" LAB simpler than before. Oenococci are (as was *Ln. oenos*) easy distinguishable by their extreme acid and ethanol tolerance, but separating *Weissella* from *Leuconostoc sensustricto* and from some heterofermentative lactobacilli is still problematic (Collin *et al*, 1993).

2.1.1.11
Weissella

The genus *Weissella* was suggested to comprise these "leuconostoc-like" bacteria (Collins *et al*, 1993). As mentioned, *Weissella* therefore includes both cocci and rods. The phylogenetic studies also revealed that the so-called wine *Leuconostocs*, allotted to the species *Ln. oenos*, were only distantly related to other *Leuconostocs* and that this species therefore warranted a separate genus (Yang and Woese, 1989; Martinez-Murcia and Collins, 1990). Many *Weissella* species seem to be associated with meat where they may proliferate at low temperature (Collins *et al*,

1993). *Weissella* are distinguished by characters such as carbohydrate fermentation patterns, dextran formation from sucrose hydrolysis of esculine, growth requirements, growth at different pH and temperatures, and dissimilation of citrate and/or malate, but classification is difficult (Garvie, 1986a; Collins *et al*, 1993; Dellaglio *et al*, 1995).

2.1.1.12

Carnobacterium

The genus *Carnobacterium* were originally classified as group III lactobacilli under the designations *Lactobacillus divergens*, *Lactobacillus carnis* and *Lactobacillus piscicola* (Kandler and Weiss, 1986; Collins *et al*, 1987). Later studies showed that these bacteria were separate from lactobacilli and warranted a separate genus (Collins *et al*, 1987) and that the metabolism of glucose was predominantly homofermentative (De Bruyn *et al*, 1988). Generally, carnobacteria grow at relatively high pH (e.g., pH 9), whereas lactobacilli do not (Schillinger and Holzapfel, 1995). Furthermore, the fatty acid composition of carnobacteria differs from that of lactobacilli (Collins *et al*, 1987). Members of the genus *Carnobacterium* are heterofermentative, Gram-positive, rod-shaped organisms. Gas production is variable and frequently negative depending on the growth substrate. Carnobacteria produce L (+)-lactate from glucose, and they do not grow on acetate agar adjusted to pH 5.6 or in 18% NaCl. They grow at 15 °C and at temperatures as low as 0 °C, but not at 45 °C (Holzapfel and Gerber, 1983). The Carnobacteria are characteristically found in meat and meat products, where they are able to proliferate even at low temperatures (Collins *et al*, 1987). A simple identification key, confirmed by DNA-DNA hybridization, for distinguishing between *C. divergens*, *C. piscicola*, and typical meat-associated lactobacilli has been published (Montel *et al*, 1991). In addition, a genus-specific probe has been developed (Nissen *et al*, 1994).

A summary of the differentiation of the LAB genera with classical phenotypic teste is show in Table 2.4

Table 2.4 Differential charecteristics of lactic acid bacteria[a]

Character	Rods		Cocci							
	Carnob.	*Lactob.*	*Aeroc.*	*Enteroc.*	*Lactoc. Vagoc.*	*Leucon. Oenoc.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*	*Weissella*[b]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[c]	-[c]	±	-	-	-	+	-	-	-	+
Growth at 10°C	+	±	+	+	+	+	±	-	+	+
Growth at 45°C	-	±	-	+	-	-	±	±	-	-
Growth at 6.5% NaCl	ND[f]	±	+	+	-	±	±	-	+	±
Growth at 18% NaCl	-	-	-	-	-	-	-	-	+	-
Growth at pH 4.4	ND	±	-	+	±	±	±	-	+	-
Growth at pH 9.6	-	-	+	+	-	-	-	-	+	-
Lactic acid[d]	L	D, L, DL[g]	L	L	L	D	L, DL[g]	L	L	D, DL[g]

[a] +, positive; -, negative; ±, response varies between species; ND, not determined.
[b] *Weissella* strains may also be rod-shaped.
[c] Test for homo- or heterofermentation of glusecose; negative and positive denotes homofermentative and heterofermentative, respectively.
[d] Configuration of lactic acid produced from glucose.
[e] Small amounts of CO2 can be produced depending on media.
[f] No growth in 8% of NaCl has been report.
[g] Production of D-, L- or DL-lactic acid varies among species.

Source: Axelsson (1998)

2.1.2 Lactic acid bacteria in fermented meat products.

LAB provides the major preservative effects in food fermentation which mankind has practiced for thousands of years. LAB plays an essential role in the production of fermented meat products. In spontaneous meat fermentation, the LAB derived from the raw materials or the environments are responsible for both lactic acid production resulting from carbohydrate utilization and also a low pH value (5.9-4.6). As a consequence of this, the muscle protein coagulated, resulting in the sliceability, firmness and cohesiveness found in the final product. Ripening is also favoured when pH values decrease and approach the isoelectric point of proteins. The development of curing colour occurs also in acidic condition when nitric oxide is produce from nitrate and can then react with myoglobin (Hugas and Monfort, 1997). In meat fermentations, LAB have generally three different purposes; First, to improve safety by inactivating pathogens; Second, to improve the product stability and shelf life by inhibiting undesirable changes brought about by spoilage microorganism or antibiotic reactions; Third, to provide diversity by the modification of raw material to obtain new sensory properties (Lücke, 2000). The inhibition of pathogenic and spoilage bacteria is a consequence of the accumulation of lactic acid as well as acetic acid, formic acid, ethanol, ammonium, fatty acids, hydrogen peroxide, acetaldehyde, antibiotics and bacteriocins (Hugas and Monfort, 1997).

Nowdays, the consumer pays a lot of attention to the relation between food and health (Leroy and De vuyst, 2004). Therefore, the used of LAB as starter culture or protective coculture in the *in situ* control of food pathogens is one of the possible way to imporve food safety (Kim, 1993; Holzapfel *et al*, 1995; Stiles, 1996; Caplice and Fitzgerald, 1999; Hugas, 1999). LAB originating from fermented meats is particularly well adapted to the ecology of meat fermentation (Hugas and Monfort, 1997). The first stage in the starter culture designing process is to characterize LAB isolated from the given meat products, in order to select the best strains (Ammor *et al*, 2005). Thus, many studies have focused on isolated natural antimicrobial substances to inhibit undesirable microoganism. Garriga *et al*. (1993) studied the bacteriocinogenic activity of the strains isolated by Hugas *et al*. (1993)

from fermented sausages and determined that *Lb. plantalum* CTC305 produce a bacteriocin-like compound (plantaricin D) active against different LAB and other Gram-positive pathogens, like *Listeria monocytogenes*. *Lactobacillus sake* CTC372 produced a bacteriocin (sakarin T) not secreted in to the media and active against *L. monocytogenes* and *Staphylococcus aureus*. Hugas *et al*. (1995) isolated another heat stable proteinaceous antagonistic compound active against *L. monocytogenes* from *Lb. sake* CTC494 called sakacin K.

2.2 Antimicrobial compounds produced by lactic acid bacteria

LAB is a group of phylogenetically diverse Gram-positive bacteria characterized by some common morphological, metabolic and physiological traits. This group includes cocci or rods, nonsporing, microaerophilic or facultatively anaerobic, lacking of cytochroms and catalase (Cintas *et al*, 2001).

LAB is involved in the fermentation of a range of milk, meat, cereal and vegetable foods (McKay and Baldwin, 1990). The antimicrobial compounds produced by LAB can inhibit the growth of pathogenic bacteria of possible contaminants in the fermented products (Raccah *et al*, 1979; Smith and Palumbo, 1983; Cintas *et al*, 1998).

2.2.1 Organic acids

Fermentation by LAB is characterized by the accumulation of organic acids and the accompanying reduction in pH. The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules (Gould, 1991; Podolak *et al*, 1996). It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane (Kashket, 1987). The undissociated acid acts by collapsing the electrochemical proton

gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Smulders *et al*, 1986; Earnshaw, 1992).

2.2.1.1 Lactic acid

Lactic acid is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid was inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds (Woolford, 1975). All lactic acid bacteria produce lactic acid from hexoses and since they lack functional heme linked electron transport chains and a functional Krebs cycle, they obtain energy via substrate level phosphorylation. The lactic acid produced may be L (+) or, less frequently, D (-) or a mixture of both. It should be noted that D (-) lactic acid is not metabolized by humans and is not recommended for infants and young children. And L (+) lactic acid being more inhibitory than the D (-) isomer (Benthin and Villadsen, 1995).

2.2.1.2 Acetic and propionic acids

Acetic and propionic acids produced by LAB strains through heterofermentative pathways, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al*, 1986). They are more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of undissociated acids than lactic acid at a given pH. Acetic acid was more inhibitory than lactic and citric acids toward *Listeria monocytogenes* (Ahmad and Marth, 1989; Richards *et al*, 1995), and toward the growth and germination of *Bacillus cereus* (Wong and Chen, 1988). And they able to inhibits yeast and mould too (Blom and Mortvedt, 1991). Acetic acid also acted synergistically with lactic acid; lactic acid decreases the pH of the medium, thereby increasing the toxicity of acetic acid (Adams and Hall, 1988).

Propionic acid inhibits fungi and bacteria and is present in Microguard [R] as described above and also in another commercial product, Bioprofit[R] Where the use of a *Propionibacterium freudenreichii* strain along with *Lactobacillus rhamnosus* increases inhibitory activity against fungi and some gram positive bacteria (Mäyrä-Mäkinen and Suomalainen, 1995).

2.2.2 Hydrogen peroxide and carbon dioxide

2.2.2.1 Hydrogen peroxide

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine hydroxy dinucleotide (NADH) peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes and from the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davison, 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O^{2-}) and hydroxyl (OH^-) radicals which can damage DNA (Byczkowski and Gessner, 1988). It has been reported that the production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotrophic microorganisms in foods (Davidson *et al*, 1983; Cords and Dychdala, 1993). In raw milk, H_2O_2 activates the lactoperoxidase system, producing hypothiocyanate ($OSCN^-$), higher oxyacids (O_2SCN^- and O_3SCN^-) and intermediate oxidation products that are inhibitory to a wide spectrum of Gram-positive and Gram-negative bacteria (Reiter and Härnulv, 1984; Conner, 1993).

2.2.2.2 Carbon dioxide

Carbon dioxide is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may

cause a disfunction in permeability (Eklund, 1984). CO_2 can effectively inhibit the growth of many food spoilage microorganisms. Especially, Gram-negative psychrotrophic bacteria (Farber, 1991; Hotchkiss, 1999). The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% could lower the total bacterial counts by 50% (Wagner and Moberg, 1989) and at 20-50% it had a strong antifungal activity (Lindgren and Dobrogosz, 1990).

2.2.3 Aroma components

2.2.3.1 Diacetyl

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. The antimicrobial effect of diacetyl has been known since the 1930s (Jay, 1982). It inhibits the growth of Gram-negative bacteria by reacting with the arginine binding protein, thus affecting the arginine utilization (Jay, 1986).

Jay (1982) showed that Gram-negative bacteria were more sensitive to diacetyl than Gram positive bacteria; the former was inhibited by diacetyl at 200 μg/ml and the latter at 300 g/ml. Diacetyl at 344 g/ml inhibited strains of *Listeria*, *Salmonella*, *Yersinia*, *Escherichia coli*, and *Aeromonas*. Since the production of diacetyl during lactic fermentation is low, e.g. 4 g/ml produced by *Lc. lactis* ssp. diacetylactis (Cogan, 1980), and the acceptable sensory levels of diacetyl are at 2-7 g/ml (Earnshaw, 1992), its practical use as a food preservative is limited. However, diacetyl may act synergistically with other antimicrobial factors (Jay, 1982) and contribute to combined preservation systems in fermented foods.

2.2.3.2 Acetaldehyde

Acetaldehyde is produced by *Lb. delbrueckii* ssp. *bulgaricus* by the action of a threonine aldolase, which cleaves threonine into acetaldehyde and glycine. Since *Lb.delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in yoghurt can not metabolize acetaldehyde, it accumulates in the product at a concentration of about 25

ppm acetaldehyde at 10-100 ppm inhibits the growth of *S. aureus, Salmonella typhimurium* and *E. coli* in dairy products (Piard and Desmazeaud, 1991).

2.2.4 Fatty acids

Under certain conditions, some lactobacilli and lactococci possessing lipolytic activities may produce significant amounts of fatty acids, e.g. in dry fermented sausage (Sanz et *al,* 1988) and fermented milk (Rao and Reddy, 1984). The antimicrobial activity of fatty acids has been recognized for many years. The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on chain length, concentration, and pH of the medium (Gould, 1991). The antimicrobial action of fatty acids has been thought to be due to the undissociated molecule, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at lower pH (Kabara, 1993).

2.2.5 Reuterin

Reuterin is produced by *Lb. reuteri,* a heterofermentative species inhabiting the gastrointestinal tract of humans and animals (Axelsson *et al,* 1987). It is formed during the anaerobic growth of *Lb. reuteri* by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin (Talarico *et al,* 1988). Reuterin has been chemically identified to be 3-hydroxypropanal (hydroxypropionaldehyde), a highly soluble pH neutral compound which is in equilibrium with its hydrated monomeric and cyclic dimeric forms (Axelsson *et al,* 1989; Talarico and Dobrogosz, 1989). The biosynthesis pathway from glycerol to the three forms of reuterin is shown below (Figure 2.1). Reuterin exhibits a broad spectrum of antimicrobial activity against certain Gram-positive and Gram-negative bacteria, yeast, fungi and protozoa. Spoilage organisms sensitive to reuterin include species of *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida,* and *Trypanosoma* (Axelsson *et a1,* 1989).

2.2.6 Bacteriocins

A recent definition of bacteriocins produce by LAB suggests that they should be regarded as extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity. They should include at least some strain of the same species as the producer bacterium and against which the producer strain has some mechanism(s) of specific self protection (Jack *et al*, 1995), see also text edited by De vuyst and Vandamme (1994). the possibility of exploiting bacteriocins in food fermentations arises where the inhibitory spectrum includes food spoilage and/or pathogenic microorganisms or gives the producing strain a competitive advantage in the food milieu.

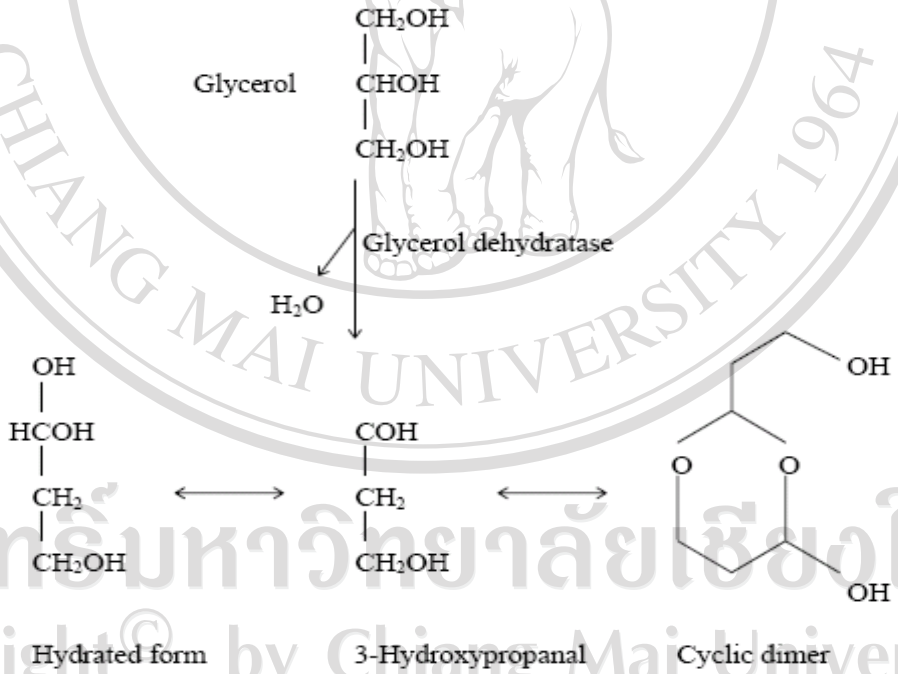

Fig 2.1 The biosynthesis pathway from glycerol to the three forms of reuterin. (Axelsson *et a1*, 1989)

The target of bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the LPS of the outer membrane of Gram-negative bacteria, they are generally only active against Gram-positive cells (Ray, 1993; Abee *et al*, 1995; Sahl *et al*, 1995; Venema *et al*, 1995). In the context of fermentation, important targets include spoilers such as species of *Clostridium* and heterofermentative lactobacilli and foodborne pathogens including *Listeria monocytogenes*, *Staphylococcus* spp., *Clostridium*, *Enterococcus* and *Bacillus* spp. The permeability of Gram-negative bacteria can be increased by sublethal injury including that which can occur when using ultrahigh hydrostatic pressure (UHP) and pulsed electric filed (PEF) as nonthermal methods of preservation (Kalchayanand *et al*, 1992). In addition, disruption of the integrity of the outer membrane (Kordel and Sahl, 1986; Kalchayanand *et al*, 1992) through the use of food grade chelating agents such as EDTA and citrate which bind magnesium ions in the LPS layer can increase the effectiveness of bacteriocins against Gram-negative bacteria (Stevens *et al*, 1992).

Many bacteriocins are most active at low pH (Mortvedt-Abildgaard *et al*, 1995) and there is evidence that bacteriocinogenic strains can be readily isolated from fresh and fermented foods (Schillinger and Lucke, 1989; Vaughan *et al*, 1994; Cintas *et al*; Kelly *et al*, 1998). Strains may naturally produce more than one bacteriocin (van Belkum *et al*, 1992; Quadri *et al*, 1994; Worobo *et al*, 1995) and heterologous expression of bacteriocins has been demonstrated in constructed strains (Allison *et al*, 1994). Protein engineering has led to the development of nisin derivative with altered antimicrobial activities or greater solubility at pH 6 than the wild-type nisin (Kuipers *et al*, 1992; Rauch *et al*, 1994). An advantage of bacteriocins over classical antibiotics is that digestive enzymes destroy them. Bacteriocin producing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality.

2.3 Classification of bacteriocins from LAB

In 1992 Klaenhammer and cowokers defined three classes of bacteriocins produced by LAB 1) Lantibiotics; 2) small hydrophobic heat-stable

peptide (<13,000 D); 3) large heat-labile proteins (>13,000 D). A year later, the classification was refined and expanded with one class (Klaenhammer, 1993; see Table 2.5). The majority of those produced by bacteria associated with food belong to classes I and II (Caplice and Fitzgerald, 1999).

In the past two decades, there have been many reports on the bacteriocins produced by LAB. These bacteriocins are of a proteinaceous nature and they have been grouped into class I, lantibiotics which are small peptides (e.g. nisin), class II, small heat-stable peptides, class III, large heat-labile proteins, and class IV, complex bacteriocins which are not well defined (Klaenhammer, 1993). In the following text, the LMM (low- molecular- mass) antimicrobial compounds produced by LAB will be discussed. Some bacteriocins may be used in the future in practical application. They can either be added as preservative (nisin is the only bacteriocin so far to be used in this way) or they can be produced *in situ*, i.e., in the product in the case of starter culture or in the gastrointestinal tract in the case of probiotic strains. Some bacteriocins appear to be produced in a product (Blom and Mörtvedt, 1991; Winkowski *et al*, 1993; Ryan *et al*, 1996), but in vivo production is still an open question. What argues in favor of *in vivo* production of bacteriocins is the wide distribution of strains being capable of doing so, suggesting some evolutionary advantage. In nature one-third of the *E. coli* population is able to produce bacteriocins (van der Wal *et al*, 1995). On the other hand, Jiménez-Díaz *et al*. (1993) found that only 4 out of 26 *L. plantalum* strains showed some bacteriocin-like activity. *In situ* production of antimicrobial substances in the intestine might be enhanced by increasing the adhesion of the probiotic strains to the intestinal mucosa. The following paragraphs will deal with the four classes of bacteriocins in greater detail, including examples of bacteriocins in each class (producer strain, spectrum of activity, molecular mass), their molecular mechanism of activity and protection against it (immunity).

2.3.1 Class I

As mentioned in Table 2.5, this class consists of so-call lantibiotics. These are small peptides containing the unusual dehydroamino acids and thioether amino acids lanthionin and 3-methyllanthionine. These amino acids are synthesized

by posttranslational modifications. The lantibiotic produce by LAB all belong to type A. The type lantibiotic are elongated screw-shaped peptides whereas type B lantibiotics are mainly globular (Sahl *et al*, 1995). Type a lantibiotics can be subdivided into two classes. Class AI contains lantibiotics of slightly positive or

Table 2.5 Classes of bacteriocins produced by lactic acid bacteria

Class	Subclass	Description
I	-	Lantibiotics
II	-	Small (<10 kDa), moderate (100 °C) to high (121°C) heat-stable, non-lanthionine-containing membrane-active peptide
	IIa	Listeria-active peptide with Y-G-N-G-V-X-C near the amino terminus
	IIb	Two peptide bacteriocins
	IIc	Thiol-activated peptides
	IId	other bacteriocins
III	-	Large (> 30 kDa) heat-labile proteins
IV	-	Complex bacteiocins: protein with lipid and/or carbohydrate

Sources: (de Vos *et al*, 1995; Nes *et al*, 1996 and Moll *et al*, 1999b)

charge. Class AII consists of highly negatively charged lantibiotic (de Vos *et al*, 1995). The model-type lantibiotic nisin is discussed as an example for the models of action of the lantibbiotic. Nisin has a broad spectrum of activity against Gram-positive bacteria. *E. coli* and other Gram-negative bacteria are only affected whentheir outer membranes are sublethally damaged. Under these conditions other bacteriocins also show antimicrobial activity against Gram-negative bacteria (Ray, 1993; Sahl *et al*, 1995; Venema *et al*, 1995). Over the years, many diferent

mechanisms for the antimicrobial action of nisin have been described. The primary target is believed to be the cell membrane. For its interaction with the cell membrane, nisin dose not need any receptor, unlike some other antimicrobial peptides. However, it dose need the presence of a membrane potential (Sahl *et al*, 1987; Bruno and Montville, 1993). The dehydroamino acids have been suggested to interact with sulfhydryl group of enzymes (Gross and Morell, 1971). Reuterin has been suggested to interfere with cell wall biosynthesis (Linnett and Strominger, 1973; Reisinger *et al*, 1980) and has been suggested to inhibit biosynthesis of DNA, RNA, proteins and polysaccharides. This has led to the suggestion that nisin interferes with the energy supply of the cell. Pores are thought to be created in the cell membrane, allowing dissipation of the membrane potential (Jack *et al*, 1995; de Vos *et al*, 1995; Sahl *et al*, 1995). Cell lysis has been explained by a cation exchange-like process, where the strongly cationic lantibiotics displace autolytic enzyme. The enzymes weaken the cell wall. The lantibiotics interfere with the cell's energy supply, inhibiting cell wall repair. The pores formed by the lantibiotics do not allow passage of high molecular weight compounds, resulting in net influx of water increasing the osmotic pressure and causing cell lysis (Sahl *et al*, 1995).

The bacteriocin producing strain needs to protect itself from the antimicrobial activity of its bacteriocin. Also target strains can develop resistance to bacteriocin. Klaenhammer (1993) defined three categories of nisin resistance, immulity, resistance not genetically linked to production and nisin-resistance mutation.

1. An immunity lipoprotein is formed by the producer strain and is anchored in the outside of the cell membrane (Kuipers *et al*, 1993). The so-called ABC exporter that is involved in the translocation of (precursors of) the lantibiotics is also thought to be involved in immunity by expelling lantibiotics from the cell (Venema *et al*, 1995). A hydrophobic protein, encoded for the nisin operon, has been suggested to work analogous to coliscin immunity protein by interaction with the bacteriocins and closing the pores formed (Siegers and Entian, 1995; Venema *et al*, 1995).

2. Many non-nisin-producing Gram-positive strains have been found to have natural resistance against nisin by destroying the nisin activity (Harris *et al*, 1992) *B. cereus* has been found to in activate nisin by reducing the dehydroamino acid (Jarvis and Farr, 1971; Venema *et al,* 1995).

3. Nisin resistance may be acquired in the presence of sublethal nisin concentrations. The mechanism of resistance may differ from strain to strain (Harris *et al,* 1992). Klaenhammer (1993) suggested mutational changes in cell components directly or indirectly involved with nisin adsorption or membrane insertion. As mentioned above, however, nisin dose not appear to need any receptor (Sahl *et al,* 1987), posing difficuties for the mentioned hypothesis.

2.3.2 Class II

As shown in Table 2.5, class II contains a wide variety of bacteriocin and has been subdivided in to three subclass (Klaenhammer, 1993). Their general description, however, is small heat stable membrane active peptides.

A number of class II bacteriocins have been shown to be membrane-active peptide. They destroy the integrity of the membrane by the formation of pores. As an example, lactococcins are discussed in more detail. In contrast to nisin, lactococcins act on the target cells regardless of their energization (Jack *et al,* 1995). Because lactococcin A was only active against membrane vesicles derived from sensitive strains, it was concluded that it needs a specific receptor protein (ven Belkum *et al,* 1991; 1992; Venema *et al,* 1995). It has been suggested that lactoccocin A could from a membrane-spanning α helix. The helix would have amphiphilic properties. The molecules would aggregate like barrel staves around a central water-filled pore (Klaenhammer, 1993; Kok *et al,* 1993; Venema *et al,* 1995). The pores formed are thought to exist in different sizes depending on the bacteriocin concentration (Venema *et al,* 1995). This increases membrane per meability and explains the observed influx of small molecules, efflux of UV-absorbing materials

(Jack *et al,* 1995) and dissipation of the proton motive force (Bruno and Montville, 1993). For other class II bacteriocin it has been observed that RNA, DNA, and protein synthesis are inhibited. Inhibiton of transport of precursors and leakage out of the cell of essential small molecules has also been observed (Venema *et al,* 1995).

Many of these findings can be linked to dissipation of the proton motive force, which has been observed for many class IIb bacteriocin (Christensen and Hutkins, 1992; Bruno and Montville, 1993; Gonzáles *et al,* 1996). The production of lactacin B has been found to be induced by a dimeric protein from a target strain (Barefoot *et al,* 1994). As mentioned in section VI, *Lreuteri* is stimulated in its reuterin production in the presence of a target strain. It may be anticipated that production of other bacteriocins could also be induced or enhanced in the presence of a targat strain. Bacteriocin production has been observed in coculture and would also give the producing strain a competitive advantage under natural condition.

As with lantibiotics, producer strains protect themselves against the bacteriocin with the help of immunity protein. The lactococcin a immunity protein has been identified as an 11 kDa protein containing an amphiphilic α helix (Nissen-Meyer *et al,* 1993; Venema *et al,* 1995). The immunity protein is thought to interact with the lactococcin A receptor protein, preventing the insertion of bacteriocin in to the membrane (Nissen-Meyer *et al,* 1993; Venema *et al,* 1995). The immunity protein of carnobacteriocin B2 was identifiled as 12.7 kDa protein and was proposed to interfere with the formation of a functional pore or to block the function pore (Quadri *et al,* 1995).

2.3.3 Class III

Class III bacteriocins are defined as large heat-labile protein. This class may therefore include bacteriolytic extracellular enzyme (hemolysins and muramidases) that may mimic the physiological activities of bacteriocins (Jack *et al,* 1995).

Venema (1995) also included lactacins A and B in class III. However they clarly belong to class II because of their molecular weight. Class III bacteriocins have so far only been isolated from members of the genus *Lactobacillus* (Klaenhammer, 1993). The mechanisms of action and immunity of these larger bacteriocins remain largely unknown.

2.3.4 Class IV

This class contains complex bacteriocins. Lipid or carbohydrate moieties appear to be necessary for activity. The exisstence of class IV is not generally accepted as it may include regular peptide bacteriocins that have not been properly purified (Venema *et al*, 1995; Nes *et al*, 1996). Also Klaenhammer (1993) urges caution with this class. It is important to assure that the lipase and amylase preparations used to show the necessity of lipid and/or carbohydrate moieties for activity are free from protease activity (Klaenhammer, 1993). As with Class III, the mechanism of action and immunity of these complex bacteriocins remains to be investigated.

An overview of biochemically and genetically characterized bacteriocins is given in Table 2.6

Table 2.6 Overview of biocemically and genetically characterized bacteriocins

Bacteriocin	producer Organism	Reference(s)
Class I (lantibiotic)		
Nisin A	*L. lactis*	de Vuyst and Vandamme (1994b)
Nisin Z	*L. lactis*	de Vuyst and Vandamme (1994b)
Lactococcén DR(*)	*L.$lactis* ALRIA 85L0s0	Dufour *et al.*"(1991)
Laticin 481(*)	*L. lactis* CNRZ481	Piard *et al.* (1¹92)
(Lacticin 3147 (LtnA1 and LtnA2)	*L.`lactis* DPC3147	Dougherty *et al*. (1998)
Esstreptococcin A-FF22	*S. pyogenes* FF22	Hynes *et al.* (1993)
Salivaricin A	*S. salivarius* 20P3	Ross *et al.* (1993)

Sources: (Cintas *et al*, 2001)

Table 2.6 Overview of biocemically and genetically characterized bacteriocins(Continue)

Bacteriocin	producer Organism	Reference(s)
Cytolysin (cylL1 and CylL2)	*E. faecalis*	Gilmor *et al.* (1994)
Carnocin Ul49	*C. piscicola* U149	Stoffels *et al.* (1992b)
Lactocin S	*Lb. sakei* L45	Mortvedt *et al.* (1991)
Class II		
Class IIa (pediocin-like bacteriocins)		
Pediocin PA1	*P. acidilactici* PAC-1.0	Henderson *et al.* (1992)
Pediocin AcH	*P. acidilactici* H	Bhunia *et al.* (1988)
Mesentericin Y105	*Lc. mesenteroides* Y105	Héchard *et al.* (1992)
Mesentericin 52B	*Lc. mesenteroides* FR52	Héchard et al. (1999)
Mesentericin B105	*Lc. mesenteroides* Y105	Revol-Junelles *et al.* (1996)
Acidocin A	*Lb. acidophilus* TK9201	Kanatani *et al.* (1995)
Bavaricin A	*Lb. bavaricus* Ml401	Larsen *et al.* (1993)
Curvacin A	*Lb. cuvatus* LTH1174	Tichaczek *et al.* (1992)
Sakacin A	*Lb. sakei* LB706	Holck *et al.* (1992)
Sakacin P	*Lb. sakei* LTH673	Tichaczek *et al.* (1992)
Sakacin 674	*Lb. sakei* LB674	Holck *et al.* (1994a)
Carnobacteriocin BM1	*C. piscicola* LB17B	Quadri *et al.* (1994)
Carnobacteriocin B2	*C. piscicola* LV17B	Quadri *et al.* (1994)
Divercin V41	*C. divergens* V41	Métivier *et al.* (1998)
Enterocin A	*E. faecium* CTC492	Aymerich *et al.* (1996)
Class IIa (two-peptide bacteriocins)		
Lactacin F (LafA and LafX)	*Lb. johnsonii* VPI11088	Alison *et al.* (1994)
Plantaricin S (PIsα and PIsβ)	*Lb. plantarum* LCPO10	Jimenez-Diaz *et al.* (1995)
Plantaricin EF (PinE and PinF)	*Lb. plantarum* C11	Diep *et al.* (1996)
Plantaricin JK(PinJ and PinK)	*Lb. plantarum* C11	Diep *et al.* (1996)
Leucocin H (α and β)	*Leuconostoc* sp. MF215B	Blom *et al.* (1999)
Termophilin 13 (ThmA/ThmB)	*S. thermophilus* SPi13	Marciset *et* al. (1997)
Class IIc (sac-dependent bacteriocins)		
Acidocin B	*Lb. acidophilus* M46	Leer *et al.* (1995)
Divergicin A	*C. divergens* LV13	Worobo *et al.* (1995)
Bacteriocin 31[a]	*E. faecalis* YI17	Tomita *et al.*(1996)
Enterocin[a]	*E. faecium* P13	Cintas *et al.* (1997), Casaus (1998)

Sources: (Cintas *et al*, 2001)

Table 2.6 Overview of biocemically and genetically characterized bacteriocins (Continue)

Bacteriocin	producer Organism	Reference(s)
Lactococcin 972	*L. lactis* IPLA972	Martinez *et al.* (1999)
Class IId (other bacteriocins)		
Lacococcins A and B	*L. cremoris* 9B4	Van Belkum *et al*. (1991a,b)
	L. lactis WM4	Stoddard *et al*. (1992)
	L. cremoris LMG2130	Holo *et al.* (1991)
Acidocin 8912	*Lb. acidophilus* TK8192	Kanatani *et al.* (1995)
Peptide A	*Lb. acidophilus* LF221	Bogovic-Matijasic *et al.* (1998)
Peptide B	*Lb. acidophilus* LF221	Bogovic-Matijasic *et al.* (1998)
Lactobin 705	*Lb. casei* CRL 705	Palacios *et al.* (1999)
Plantaricin 1.25α	*Lb. plantarum* TMW1.25	Ehrmann *et al.* (2000), Remiger *et al.* (1999)
Plantaricin 1.25β	*Lb. plantarum* TMW1.25	Ehrmann *et al*. (2000), Remiger *et al.* (1999)
Divergicin 750	*C. divergens* 750	Holck *et al.*(1996)
Carnobacteriocn a	*C. piscicola* LV17A	Worobo *et al.* (1994)
Piscicolin 61	*C. piscicola* LV61	Holck *et al.*(1994b)
Leucocin B-TA33a	*Lc. mesenteroides* TA33a	Papthanasopoulos *et al*. (1998)
Enterocin B	*E. faecium* T136	Casaus *et al.* (1997)
Enterocin L50 (EntL5oA and EntL5oA)	*E. faecium* L50	Cintas *et al.* (1998b)
Enterocin Q	*E. faecium* T136	Casaus *et al.* (2000)
Class III		
Helveticin	*Lb. helveticus* 481	Joerger and Klaenhammer (1986)
Caseeicin 80	*Lb. casei* B80	Rammelsberg *et al.* (1990)

Bacteriocins with the same symbol in brackest have identical amino acid sequences.

[a]These bacteriocins can also be included in class IIa.

[b]These bacteriocins resemble the two-peptide bacteriocins (IIa).

Sources: (Cintas *et al*, 2001)

2.4 Bacteriocins and antibiotics

From very genesis, bacteriocin has been referred to as “antibiotics.” Reeves (1965) contrasted bacteriocins to other antibiotics by the fact that bacteriocins are proteinaceous. Since many articles refer to bacteriocins as antibiotics (Buchman *et al,* 1988) and it is illegal to use antibiotics as food preservatives, the question should be asked, “Are bacteriocins antibiotics?”

The discussion is, of course, a matter of semantics. It should be framed in the context of nisin’s discovery in 1924, before penicillin and colicins, early in the age of antibiotics. Because of the tremendous increase in knowledge about antibotics during this period, It was reason able to consider that the newly discovered protein antimicrobials were antibiotics. Technically, antibiotics are made by a restricted group of organisms though the enzymatic packaging of primary metabolites into structurally related secondary matabolites that have no apparent function in the growth of producing cell and are easilly secreted from the cell. There are many peptide antibiotics made by fungi and yeast that meet this definition. For example, gramacidin S, bacitracin A, and polymixin are all peptide antibiotics. All are made by enzymatic condensation reactions of amino acids to package free amino acids into larger compounds. When the amino acid composition of nisin was discovered to contain several unusual amino acids such as dehydroalanine, dehydrobuterine and single sulfur lanthione bridges. It was thought that nisin could not be a protein because ribosomes do not process these unusual amino acids. The discovery of the nisin analogues, subtilin and epidermin, further suggested families of structurally related compounds. Only recently has Hansen’s elegant work (Hansen *et al,* 1990) proved that nisin is, in fact, made ribosomally. The dehydration of the amino acids and the lanthione ring formation occur post translationally. The presence of discrete genes for bacteriocin synthesis confirms that many other bacteriocins are ture proteins. An alternate approach to the semantic argument is to abandon the semantic issue and examine the reason for prohibiting the use of antibiotics as food preservative. This prohibition is rooted in the well-justified concern that widespread use of antibiotics in the food supply might compromise the clinical efficacy of antibiotics. The dictionary

includes “used to inhibit or treat infections diseases” in its definition of “antibiotic.” Bacteriocins slated for use in food are not used to inhibit nor treat the clinical progression of an infections disease. Indeed, this was a major consideration in 1964 when the World Health Organization approved the use of nisin in foods. Chromatography (RP-HPLC) using two C18 columns in series with water as the mobile phase. The LMM antimicrobial compound (hydrogen peroxide, carbon dioxide), acidolin, produced by *Lb. acidophilus* 2181, was separated by methanol and acetone precipitation, and further purified by gel filtration on Sephadex G-25, and by thin-layer chromatography on silica gel to remove lactic acid (Hamdan and Mikolajcik, 1974). The crude extract of a LMM antimicrobial compound from *Lb. delbrueckii* ssp. *bulgaricus* 7994 was separated by ethanol precipitation, and purified by RP-HPLC using an ODS-5 column to yield a single strong peak. However, rechromatography of the active fraction under the same conditions revealed two peaks, one of them being active (Abdel-Bar, 1987). Pulusani *et al*. (1979) reported the partial purification of LMM antimicrobial compounds produced by a *S. thermophilus* strain by methanolacetone extraction and gel filtration. Although there are some reports of the production of LMM antimicrobial compounds by *Lb. delbrueckii* ssp. *bulgaricus* DDS14 (Reddy *et al*, 1984) and *Lb. rhamnosus* GG (Silva *et al*, 1987), these compounds have not been separated and further purified.

2.5 Screening method for detecting bacteriocin activity

Among many methods available for evaluation of bacteriocin, the methods described below have been used for determining the bacteriocin produced by LAB. Hoover and Halander (1993) describes the Screening methods for detecting bacteriocin activity as follow.

2.5.1 Agar diffusion techniques

2.5.1.1 Plating methods

1. Deferred methods

The primary mean for detecting bacteriocin activity of the LAB is the agar plate diffusion assay. Agar plate assay are popular for screening of antagonistic activity as well as for monitoring expression, optimization and inactivation of bacteriocins for their characterization. While these plating methods are straightforward. They are not without certain problems and limitations, especially since LAB can produce other antagonistic compounds and create conditions that mimic bacteriocin activity.

In the screening of bacteriocin activity of Gram-positive bacteria, Tagg *et al*. (1976) classified the methods of detection as deferred or simultaneous. Fredericq (1948) first used deferred antagonism in study of the colicins. Using solid media in this approach. The producing culture is first grown under optimum conditions. This strain can then be killed by chloroform or heat and subsequently overlaid with indicator-seeded molten agar. The conditions for the second incubation can be optimum for the indicator strain. Killing the producing culture has been used less frequently since Brock *et al*. (1993) found that chlorofrom and heat can inactivate bacteriocins. The smearing associated with not killing the producing culture can be minimized by careful application of the solf agar overlay and comforts to control condensation; however, use of chloroform is not uncommon. In their study of diplococcin from *Streptococcus cremoris* 346, Davey and Richardson (1981) swobed M17 agar plates with *S. cremoris* and incubate for 48 hours at 22 °C; surface growth was then killed by exposure to chloroform vapors. The plates were overlaid with 2.5 ml of soft agar inoculated with 0.1 ml of a 24 h growth of indicator culture and incubated at 22 °C with examination for zones of inhibition at 24 and 48 h. Chloroform was used by Hastings and Stiles (1991) in their adaptation of the “spot-on-lawn” assay that included adjustment of the bacteriocin-producing culture of bacteria (*Leuconostoc gelidum*) to pH 6.5 with 10M NaOH before centrifugation at 6000 g for 5 min. Any remaining cells in the supernatant were then inactivated by mixing 1 part chloroform to 4 parts supernatant. After 5 minutes, the aqueous portion was aseptically removed and 20 ml spotted on the surface of an All-Purpose Tween (APT) agar plate overlaid with 6 ml soft APT agar (0.75 %) inoculated with 1% of

an overnight grown indicator culture. Controls with sterile APT broth were done to assure that no chloroform residues were present.

An alternative approach that omits chloroform treatment is a "slamtransfer" deferred plating method introduced by Kekessy and Piguet (1970). After growth of the producing strain the agar is aseptically dislodged from the bottom half of the petri dish, the dish is closed, and by striking the dish forcefully on the bench upside-down the agar layer will flip onto the top lid. This can then be overlaid with indicator-seeded solf agar and incubated. In this method, the producing cells and indicator cells are separated by a layer of agar. Antagonistic effects by phage and acid are minimized but the bacteriocin must be able to freely diffuse through the solid medium in concentrations adequate for zones of inhibition to form.

Agar methods can be adapted to visualize antagonism against an indicator strain from a mixed population sample. The sample can be diluted in molten agar spread over a suitable nutrient agar plate and then overlaid with indicator-seeded soft agar. Bacteriocin-producing colonies can be differentiated from non-producing clones in this manner. This is applicable for conjugation experiments using bacteriocin production as a plasmid marker where by bacteriocin-positive transconjugants can be discerned from bacteriocin-negative cells.

Bacteriophage can present an antagonistic effect similar to that of bacteriocins. In fact, it was not uncommon for early studies on lysogeny to be redirected into investigations on colicins when the search for one element uncovered action of another. Dropping dilution of cell-free supernatant on to an indicator-seeded agar plate with subsequent incubations will distinguish phage from bacteriocins, as phage will continue to cause formation of distinct plaques at high dilutions while the bacteriocin solution will demonstrate gradual decrease in the zone of inhibittion with dilution. In addition, bacteriopage can be differentiated from low molecular weight bacteriocins by ultracentrifugation. In extracts, bacteriophage can be eliminated by a 2 hour exposure to ultraviolet light; bacteriocins do not appear to lose any activity from this treatment (Mayr-Harting *et al*, 1972).

2. Simultaneous methods

Sumultaneous or direct assays are the simplest to do and can be done in a shorter amount of time then deferred assay. Gratia (1946) first published the “spot-on-lawn” procedure. In this appoach the producer and indicator culture are grown concurrently on the same solid media under the same conditions of incubation. The indicator is spread onto the surface of the agar medium and the producing culture is spottted on top of this. While a deferred test works well when antagonistic protein is synthesised and released late in the growth cycle of the producing cell, simultaneous methods require release of the bacteriocin early in the growth cycle or indicatororganism will enter log phase and be established before a diffusible inhibitor can be effective. Adaptations to the spot-on-lawn assay employ dual inoculations in close proximity to one another or overlapping drops on the same agar plate (Barrow, 1963). Wells cut into freshly seeded agars can contain either acell suspension of the producing strain or a growth extract (Sabine, 1963). A variation by Gagliano and Hinsdill (1970) for facultative anaerobes is stab-inoculation with a producing strain into freshly indicatorseeded pour plates or surface-streaked agar. The slightly diminished oxygen tension enhanced growth of microaerophiles and other facultative types.

2.5.1.2 Liquid media

Liquid methods are not as commonly used for the screening of bacteriocin activity. While plate plating methods are simpler and quicker to do, the use of liquid methods usually presents greater option for closer examination of bacteriocin activity. One approach is to prepare an extract of the growth medium of a bacteriocin-producing culture. Normally the cells are removed by centrifugation and filtration to present a crude cell-free preparation of the bacteriocin. Dialysis is common to remove organic acids and other dialyzable compounds from the preparation, or the acidity can be neutralized with sodium hydroxide. This extract can be added to a well cut into an agar plate and examined against an indicator strain; or the extract can be inoculated with, the indicator strain, with subsequent growth of the

indicator monitored by a spectrophotometer or by plate counts. The growth kinetics of the indicator can be followed in this way. Also, the crude bacteriocin extract can be pH adjusted, diluted, amended with supplements or additives, or manipulatedin other ways to measure different variables. Two factors to be addressed in such an approach are the appropriateness of the medium for growth of the indicator strain and the depletion of nutrients from the extract by the bacteriocin-producing culture. To correct the latter case, the extract can be dialyzed against sterile growth medium to replenish dialyzable nutrients or additional sterile medium can be added to the extract, although this would dilute the concentration of the bacteriocin, and often the bacteriocin is produced in such low amounts that further dilution is undessirable. Concentration of the bacteriocin in the growth extract is sometimes necessary. For example, the use of a dry filter paper disc method, whereby the paper is wetted with growth extract and then placed on the surface of indicator-seeded agar, may not be effective given the low amounts of bacteriocin produced by many lactic acid bacteria.

2.5.1.3 Titration of bacteriocin: Critical dilution method

The need frequently occurs for a means of quantitatively estimating the activity of a bacteriocin. Arbitrary units (AU) have been used that are commonly based on the critical dilution of antagonistic activity caused by a bacterial culture. The critical dilution method was developed for the assay of colicins and pyocins. In turn, these assays were derived from earlier methods developed for the titration of bacteriophage. The usual fundamentals are (Mayr-Harting *et al*, 1972):

1. preparation of a two-fold series of dilutions of the bacteriocin sample
2. use of a standardized plating method for evaluating the antagonistic effect of uniform aliquots from each dilution; and
3. after incubation, selection of an arbitrary endpoint, which is often the last dilution exhibiting total inhibition.

Normally the AU is the reciprocal of the dilution endpoint, that is, 100 AU/ml represents the 10^{-2} dilution that last displayed total inhibition.

The critical dilution method is well suited to preliminary study of a bacteriocin when the protein has not been adequately characterized or purified. The method can become comparative if a known reference sample is used, as would be the case with nisin, where its activity can be standardized.

Joerger and Klaenhammer (1986) adapted the critical dilution assay of Mayr-Harting *et al*. (1972) to titer helveticin J activity of *Lactobacillus helveticus* 481 against *Lactobacillus bulgaricus* 1489. To measure bactericidal action of helveticin J, growth extract was dialyzed against 0.1 M sodium acetate buffer (pH 5.3), filter sterilized, and diluted to give 0, 3.2 and 160 AU/ml. Ten ml of each preparation was added to a sterile cuvatte that also recived buffer-washed cells of *L. bulgaricus* to yield 10^7 cfu/ml. Optical density at 590 nm and cfu/ml were determined immediately after the indicator was added and after 3 h at 37°C.

Disadvantages of this method are the subjective judgment of the observer in determining the enpoint, and the vulnerability the method has to inconsistent procedural procedural anomalies existing among laboratories. Variation in techniques when using a critial dilution method will have some effect on the comparability of these data, day-to-day variation may also be a concern, as will loss in potency of a bacteriocin extract with storag.

2.5.1.4 Survival counts

Assessment of the efficary of a bacteriocin or bacteriocin-producing culture in a model food system is normally conducted by challenge with an indicator organism in the food and subsequent monitoring of the viability of the indicator using plate count method. Proper coutrols must be applied to determine the extent to which antibisis is due to bacteriocins and not to organic acids and other inhibitive compounds and conditions. For example, maintenance of anaerobic conditions for growth of any bacteriocin-producing LAB will preclude formation of hydrogen peroxide, as oxygen will be unavailable for the production of this toxic compound.

As with any enumerative method for microorganisms in foods, the sampling, media, and conditions of incubation should be optimal for the indicator strain to insure colony formation by all viable cells including injured ones. Also, the cells should not form chains or chains or clumps to ensure precision of colony counting.