CHAPTER 2

LITERRATURE REVIEW

2.1 General Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria united by a constellation of morphological, metabolic, and physiological characteristics. The general description of the bacteria included in the group is Gram-positive, non-sporing, non respiring cocci or rods, which produce lactic acid as the major end-product during the fermentation of carbohydrates. The boundaries of the group have been subject to some controversy, but historically the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Streptococcus* from the core of the group. (Wood and Hollzapfel, 1995)

Two main sugar fermentation pathways can be distinguished among lactic acid bacteria. Glycolysis (Embden-Meyerhof pathway) results in almost exclusively lactic acid as end-product under standard condition, and the metabolism is referred to as homolactic fermentation. The 6-phosphogluconate / phosphoketolase pathway results in significant amounts of other end-products such as ethanol, acetate, and CO_2 in addition to lactic acid, and the metabolism is referred to as heterolactic fermentation. Various growth conditions may significantly alter the end-product formation by some lactic acid bacteria. These changes can be attributed to an altered pyruvate metabolism and / or the use of external electron acceptors such as oxygen or organic compounds. (Axelsson, 1993)

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Orla-Jensen (1919) used a few characters as classification basis : morphology (cocci or rods, tetrad formation), mode of glucose fermentation (homo-or heterofermentation), growth at certain “cardinal” temperatures (e.g., 10^0C and 45^0C), and range of sugar utilization. As will be seen later in this chapter, these characters are still very important in current classification of LAB. After the work by Orla-Jensen, the view emerged that the core of LAB comprised four genera : *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus.* There has always been some controversy on what the boundaries of the group are (Ingram, 1975), but this will not be dealt with here. The classification section of this chapter will concentrate on what historically constituted these four genera. In the last decade, many new genera have been described, most of them comprising strains which were previously included in one of the four mentioned above.

2.2 Classification of Lactic Acid Bacteria

An unequivocal definition of the term lactic acid bacteria does not exist. Inevitably, most characteristics that would be used in such a definition are subject to qualification (Ingram, 1975). Meaning that they are accurate only under conditions that might be termed “normal” or “standard” and that exceptions to the definition can be found. Therefore, it is more appropriate to describe the typical lactic acid bacterium, which is Grampositive, nonsporing, catalase-negative, devoid of cytochromes, of nonaerobic habit but aerotolerant, fastidious, acid-tolerant, and strictly fermentative with lactic acid as the major end-product during sugar fermentation. LAB generally associated with habitats rich in nutrients, such as various food products (milk, meat, beverages, vegetables), but some are also members of the normal flora of the mouth, intestine, and vagina of mammals. Variation of this general theme are common. It is really only the Gram-positive character that cannot be argued with. For instance, catalase and cytochromes may be formed by some LAB on certain media, some streptococci (e.g., *Streptococcus bovis*) have quite limited nutritional requirements,

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sugar fermentation may result in very little lactic acid under certain conditions, etc. Furthermore, there are no strong scientific arguments for excluding spore-forming bacteria, which otherwise resemble LAB (i.e., *Sporolactobacillus*), since some of the genera we consider "genuine" LAB are not clearly separated from these phylogenetically. In this chapter, I will follow the historic tradition not to include spore formers in the LAB group. The above "definition", despite its limitations, is useful in being a core or center around which the actual descriptions of genera or species are formulated. A key feature of LAB that must be emphasized is the inability to synthesize porphyrin groups (e.g., heme). This is the actual physiological background for some of the characters mentioned above. This makes LAB devoid of a "ture" catalase and cytochromes when grown in laboratory growth media, which lack hematin or related compounds. Under these conditions, which are normal in most studies of these bacteria, LAB do not possess the mechanism of an electron transport chain and rely on fermentation, i.e., substrate level phosphorylation, for generating energy. Because catalase activity, mediated by a nonheme "pseudocatalase". Can occur in some LAB (Engesser and Hammes, 1994), the lack of cytochromes may be a more reliable character in preiiminnary diagnosing than the commonly used catalase test (Ingram, 1975). However, it is important to note that the situation may be totally different if hemation (or hemoglobin) is added to the growth medium. A true catalase and even cytochromes may be formed, in some case resulting in respiration with a functional electron transport chain (Whittenbury, 1964, 1978; Bryan-Jones and Whittenbury, 1969; Ritchey and Seeley, 1976; Wolf *et al*., 1991; Meisel *et al*., 1994).

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The genera that fit the general description of typical LAB in most respects are: *Aerococcus* (*A*.), *Lactobacillus* (*Lb*.), *Leuconostoc* (*Ln*.), *Pediococcus* (*P*.), and *Streptococcus* (*S*.), Some authors would also include *Gemella* and *Erysipelothrix* (Aguirre and Collins, 1993), but these will not be considered here. The genus *Bifidobacterium* is historically also considered to belong to the LAB group., The bifidobacteria were designated *Lb. bifidum.* Although *Bifidobacterium* species do fit

the general description above, they are phylogenetically more related to the Actinomycetaceae group of Gram-positive bacteria. In addition, they have a special pathway for sugar fermentation, unique to the genus, which clearly separates them from the LAB group. Bifidobacteria will therefore not be considered in this general overview of LAB. However, due to their significance in the gastrointestinal tract of animals and humans and possible probiotic action. (Hardie, 1986a; Schleifer, 1986). The former genus *Streptococcus* was first divided into three: *Enterococcus* (*E.*), *Lactococcus* (*Lc.*), and *Streptococcus sensu stricto* (Schleifer and Kilpper-Balz, 1984; Schleifer *et al*., 1985; Schleifer, 1986). Later, some motile LAB, otherwise resembling lactococci, were suggested to form a separate genus, *Vagococcus* (*V.*) (Collins *et al*., 1989). The general *Lactobacillus*, *Leuconostoc*, and *Pediococcus* have largely remained unchanged, but some rod-shaped LAB, previously included in *Lactobacillus*, are now forming the genus *Carnobacterium* (*C.*) (Collins *et al*., 1987) and the former species *Pediococcus halophilus* has been raised to genus level, forming the genus *Tetragenococcus* (*T.*) (Collins *et al*., 1990). A distinct phylogenetic cluster of heterofermentative LAB, including species previously assigned to either *Lactobacillus or Leuconostoc*, has been suggested to form a separate genus, *Weissella* (*W.*) (Collins *et al*., 1993). *Leuconostoc oenos*, the "wine ieuconostocs", has been proposed to from a genus of its own, *Oenococcus* (*O.*) (Dicks *et al*., 1995). New genera, e.g., *Alloiococcus*, *Dolosigranulum*, *Globicatella* and *Lactosphaera* have also been described to allocate some strains that were shown to be related to the LAB group, both physiologically and phylogenetically (Collins *et al*., 1992; Aguirre *et al*., 1993; Janssen *et al*., 1995).

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2.2.1 Classification at the Genus Level

As mentioned, the general basis for the classification of LAB in different genera has largely remained unchanged since the work of Oral-Jensen (1919).

However, with the description of new genera and species, it is becoming increasingly difficult to use these classical tests for reliable genus identification. Still, these phenotypic characters are useful as a starting point for more sophisticated tests. Although morphology is regarded as questionable as a key character in bacterial taxonomy (Woese, 1987), it is still important in the current descriptions of the LAB genera. Thus, LAB can be divided into rods (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). One exception is the newly described genus *Weissella,* which is the first genus in the LAB group that by definition can include both cocci and rods (Collins *et al*., 1993). Furthermore, cell division in two perpendicular directions in a single plane previously incorrectly described as "division in two planes" (Simpson, 1994), leading to tetrad-forming genera are *Aerococcus, Pediococcus* and *Tetragenococcus*. An important character used in the differentiation of the LAB genera is the mode of glucose fermentation under standard conditions, i.e., nonlimiting concentrations of glucose and growth factors (amino acids, vitamins, and nucleic acid precursors) and limited oxygen availability. Under these conditions, LAB can be divided into two groups : the homofermentative, converting glucose to lactic acid, ethanol / acetic acid, and CO_2. In practice, a test for gas production from glucose will distinguish between the group (Sharpe, 1979). (For a more detailed discussion concerning the metabolic pathways, see next section.) *Leuconostocs*, *Oenococci*, *Weissellas* and a subgroup of lactobacilli are heterofermentative ; all other LAB are homofermemtative.

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Growth at certain temperatures is mainly used to distinguish between some of the cocci. The "classical" enterococci grow at both 10^0C and 45^0C, lactococci and vagococci at 10^0C, but not at 45^0C. Streptococci generally do not grow at 10^0C, whereas growth at 45^0C is dependent on the species. Salt tolerance (6.5% NaCl) may also be used to distinguish between enterococci, lactococci / vagococci, and streptococci, although variable reaction can be found amoung streptococci (Mundt,

1986). Extreme salt tolerance (1.8% NaCl) is confined to the genus *Tetragenococcus*. Tolerance to acid and/or alkaline conditions may also be a useful characteristic. Aerococci, carnobacteria, enterococci, tetragenococci, and vagococci are characterized by growth at relatively hight pH, although not all can grow at the test pH of 9.6. The formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to distinguish between leuconostocs and most heterofermentative lactobacilli, as the former produce only D-lactic acid and the latter a racemate (DL-lactic acid), but *Weissella* strains may cause confusion in this regard.

A summary of the differentiation of LAB genera with classical phenotypic tests in shown in Table 2.1 : The genus *Carnobacterium* is indistinguishable from *Lactobacillus* with these terts, as is *Vagococcus* from *Lactococcus*. *Vagococcus* and *Carnobacterium* have a unique fatty acid composition, which separates these genera from most other LAB (Collins *et al*., 1987, 1989). In general, carnobacteria can be distinguished from lactobacilli by their ability to grow at pH 9.0 and inability to grow on acetate media, selective for lactobacilli. Pediococci can be confused with aerococci, since the morphologies are similar. However, pediococci are more acid-tolerant than aerococci and grow well anaerobically, contrary to the more microaerophilic nature of aerococci (Evans, 1986). *Weissella* species can easily be confused with leuconstocs or heterofermentative lactobacilli. Oenococci fall into the *Leuconostoc* group with the classical tests but are easily distinguished by their extreme acid and ethanol tolerance (Dicks *et al*., 1995). It should be noted that there are phenotypic overlaps between genera and exceptions to the general rules outlined in table 2.1 can be found. For example, the genus *Enterococcus*, as it is currently circumscribed, contains many species that do not conform to the classical tests (Devriese *et al*., 1993). Classification of LAB is becoming dependent on more sophisticated methods, of which rRNA sequencing probably is the most accurate at the genus level. Known rRNA sequences are also being used to develop genus-specific probes (Collins *et al*., 1993 ; Nissen *et al*., 1994)

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Table 2.1 Differential Characteristics of Lactic Acid Bacteria[a]

	Rods		Cocci							
Character	*Carnob.*	*Lactob.*	*Aeroc.*	*Enteroc.*	*Lactoc.* *Vagoc.*	*Leucon.* *Oenoc.*	*Pedioc.*	*Streptoc.*	*Tetragenoc.*	*Weissella*[b]
Tetrad formation	-	-	+	-	-	-	+	-	+	-
CO_2 from glucose[c]	-[e]	±	-	-	-	+	-	-	-	+
Growth at 10^0C	+	±	+	+	+	+	±	-	+	+
Growth at 45^0C	-	±	-	+	-	-	±	±	-	-
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 glucose; negative and positive denotes homofermentative and heterofermentative, respectively.

[d]Configuration of lactic acid produced from glucose., [e]Small amounts of CO_2 can be produced, depending on media.

[f]No growth in 8% of NaCl has been reported., [g]Production of D-, L- or DL-lactic acid varies among species., Source**;** Axelsson (1993)

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2.2.2 Classification at the Species Level

It is impossible to describe the classification of all species of LAB in the scope of this chapter. For instance, only the genus *Lactobacillus* comprises about 50 recognized species (Collins *et al*., 1991). Therefore, the following section will be a summary, concentrating on the means by which classification within a genus can be done and mentioning some of the most interesting species from a food technology point of view. For a recent, comprehensive review on the taxonomy of LAB, the reader is referred to Volume 2 in the series Lactic Acid Bacteria edited by B.J.B. Wood (Wood and Holzapfel, 1995). Which also includes descriptions of the individual species.

As indicated previously, proper classification of LAB is beginning to rely on molecular biology methods, although some of Oral-Jensen's concepts are still viable. This is perhaps truer regarding Classification at the species level than at the genus level. In some cases, only an analysis at the nucleic acid level will resolve classification problems. Still, the classical phenotypic/biochemical characterization is important for a preliminary classification as well learning about the properties of the strains. Some of the characters listed in Table 1 are also useful in the classification at the species level, e.g., salt and pH tolerance, growth at certain temperatures, and configuration of the lactic acid produced. Other characters used in the phenotypic/biochemical characterization of strains are range of carbohydrates fermented, arginine hydrolysis, acetoin formation (Vogues-Proskauer test), bile tolerance, type of hemolysis, production of extracellular polysaccharides, growth factor requirements, presence of certain enzymes (e.g., β-galactosidase and β-glucoronidase), growth characteristics in milk, and serological typing. Further characterization includes more molecular/chemotaxonomic approaches, including type of diamino acid in the peptidoglycan, presence and type of teichoic acid, presence and

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type of menaquinones, guanine + cytosine (G+C) ratio of the DNA, fatty acid composition and electrophoretic mobility of the lactate dehydrogenase (LDH)

1. *Enterococcus, Lactococcus, Streptococcus* and *Vagococcus*

As mentioned, the genera *Enterococcus*, *Lactococcus, Streptococcus,* and *Vagococcus* were earlier included in one genus, *Streptococcus*. For details regarding the major taxonomic revision of the "streptococci" which was in effect about a decade ago, the reader is referred to a review by Schleifer and Kilpper-Balz (1987), which summarizes the phenotypical, biochemical, and molecular characteristics of the genera.

Historically, serological typing with the Lancefield grouping (Lancefield, 1993) has been very important in the classification of streptococci. The method is now considered to be less important in classification but still very useful in the rapid identification of major pathoges (Sharpe, 1979; Hardie, 1986a; Schleifer, 1987). However, there is undoubtedly some correlation between presence of the group D antigen and enterococci (previously designated "group D streptococci" or "fecal streptococci"). Similary, the group N antigen is correlated with lactococci (previously :group N streptococci" or "lactic streptococci"), but note that the vagococci also possess the group N antigen (Collins *et al*., 1989)

2. *Aerococcus, Pediococcus* and *Tetragenococcus*

Aerococcus, Pediococcus, and *Tetragenococcus* constitute the tetrad-forming LAB. The genus *Aerococcus* currently contains two species (A. viridans and A. 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).

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With the recent transfer of *P. urinae-equi* to *Aerococcus* and *P. halophilus* to *Tetragenococcus*, the genus *Pediococcus* can be described as "the only acidophilic, homofermentative, lactic acid bacteria that divide alternately in two perpendicular directions to form tetrads" (Simpson and Taguchi, 1995). Pediococci are important in food technology, both in a negative and positive sense *P. damnosus* is a major spoilage organism in beer manufacture because growth may lead to diacetyl/acetoin formation, resulting in a buttery taste (Garvie, 1986b).

3. *Leuconostoc, Oenococcus* and *Weissella*

The genus Leuconostoc was previously defined as being heterofermentative, coccoid LAB producing only D-lactic acid from glucose and not producing ammonia from arginine. The leuconostocs were thus separated from other cocci of the LAB by their heterofermentative metabolism and from heterofermentative lactobacilli by morphology and some key traits. It was, however, easy to confuse leuconostocs with some "coccoid rods" of the heterofermentative lactobacilli. Phylogenetic analysis of the leuconostocs revealed considerable heterogeneity of the genus (Yang and Woese, 1989; Martinez-Murcia and Collins, 1990). It was anticipated that *Ln. paramesenteroides* together with some heterofermentative lactobacilli (e.g., *Lb. confuses* and *Lb. viridescens*) could reprecent the nucleus of a new genus, since this group was separated from both other leuconostocs and lactobacilli. Later, other heterofermentative LAB falling into this group were isolated from meat sources and the genus *Weissella* was suggested to comprise these "leuconostoc-like" bacteria (Collins *et al*., 1993).

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4. *Lactobacillus* and *Camobacterium*

The genus *Lactobacillus* is by far the targest of the genera included in LAB. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. The heterogeneity is reflected by the rang of mol % G+C of the DNA of species included in the genus. This range is 32-53%, which is twice the span usually accepted for a single genus (Schleifer and Stackebrandt, 1983). The heterogeneity and the large number of species are due to definition of the genus, which essentially is : rod-shaped lactic acid bacteria. Such a definition is comparable to an arrangement whereby all of the coccoid LAB were included in one genus. However, among the cocci, phenotypic traits were early recognized, which made differentiation into several genera possible. Even if the situation was more difficult for the rod-shaped LAB, Oral-Jensen (1919) essentially tried to divide this group in a similar way as with the cocci. Thus, the subgenera of *Lactobacillus* were created: *Thermoba*cterium, *Streptobacterium*, and *Betabacterium*. Remarkably, this division is still valid to a considerable degree, although the designations have been dropped and some modifications of the definitions of the subgroups have been made (Kandler and Weiss, 1986; Hammes and Vogel, 1995). Table 2.2 shows a summary of the characters used to distinguish between the three groups and some of the more well-known species included in each group. The physiological basis for the division is (generally) the presence or absence of the key enzyme of homo-and heterofermentative sugar metabolism, fructose-1,6-diphosphate aldolase and phosphoketolase, respectively (Kandler; 1983, 1984; Kandler and Weiss, 1986).

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Table 2.2 Arrangement of the Genus *Lactobacillus*

Character	Group I obligately homofermentative	Group II obligately heterofermentative	Group III facultatively 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. delbruckii	*Lb. curvatus*	*Lb. buchneri*
	Lb. helveticus	*Lb. plantarum*	*Lb. fermentum*
	Lb. salivarius	*Lb. sake*	*Lb. reuteri*

[a]When fermented.

[b]Inducible by pentoses.

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

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2.3 Phylogeny of the Lactic Acid Bacteria

Comparisons of the sequence of rRNAs is now regarded to be the optimal measure for determining true phylogenetic relations among bacteria (Woese, 1987). Initially, these comparisons were made by DNA-rRNA hybridizations or oligonucleotide cataloging (i,e., sequencing of cleavage products of rRNA). Advances in molecular genetic techniques have lead to methods for sequencing long stretches of rRNA, first by the use of reverse transcriptase (Lane *et al*., 1985, 1988), but now replaced by direct PCR sequencing of the rRNA genes. The computerized methods now available for handling large amounts of sequence data have made it possible to construct meaningful phylogenetic trees of the entire bacterial kingdom as well as details of certain parts of it (Woese, 1987).

From the data obtained, both from oligonucleotide cataloging and rRNA sequencing, if has been shown that the Gram-positive bacteria cluster in one of 11 major eubacterial phyta (however, not all bacteria in this phylum have a Gram-positive cell wall; Woese, 1987). The Gram-positive bacteria can be further divided into two main groups or clusters. It is common to designate them the high-G+C and the low-G+C subdivision, which reflects the mol % G+C in the DNA. The "split point" is often set at 50% but is rather an interval around 53-55%, since some species (e.g., *Lb. fermentum* and *Lb. pontis*) clearly belonging to the low-G+C subdivision have a G+C content in that range. The hight-G+C or *Actinomycetes* subdivision encompasses genera such as *Bifidobacterium*, *Arthrobacter*, *Micrococcus*, *Propionibacterium*, *Microbacterium*, *Corynebacterium*, *Actinomyces*, and *Streptomyces* (Woese, 1987; Stackebradt and Teuber, 1988). The low-G+C or the *Clostridium* subdivition includes all LAB, together with aerobes and facultative anaerobes such as *Bacillus*, *Staphylococcus*, *Listera*, and anaerobes such as *Clostridium*, *Peptococcus*, and *Ruminococcus* (Woeses, 1987; Stacke-brandt and Teuber, 1988).

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2.4 New Tools for classification and Identification

The classification of LAB described above is largely based on phenotypical and biochemical characters. In practice, meaning the routine identification of isolates, these characters may not be enough to definitely assign a strain to a part icular species. In fact, DNA-DNA homology studies have, in some cases, been the only way to resolve identification problems (Kandler and Weiss, 1986).

A much more attractive way of identifying strains than hybridizations with total DNA is the use of specific DNA probes, directed at nucleic acid targets of the cells. The main advantage with this is that once the probe has been designed the time-consuming and laborious DNA preparations can be avoided. Instead, colonies on an agar medium can be tested directly with common colony hybridization methods (Sambrook *et al*., 1989). However, a specific probe will only detect one specific species. This means that the probing technique must be used in combination with other identification criteria to narrow the number of possible species to which a strain can belong. The probing technique is perhaps more useful in being the optimal tool for answering questions like, how many bacteria of species X contains this sample? and so on. Strain-specific probes may be of importance of the study of LAB starter cultures in unsterilized food or feed. One fundamental problem with DNA problem is to find a DNA (or RNA) stretch that is specific for one species (or maybe one particular strain). Two approaches to this problem can be distinguished. The first is an empirical method of trial-and-error character. A DNA library from a particular species is screened for DNA fragments that, when tested as probes, show specificity to that species. The method has been used for developing DNA probes for *Lb. curvatus* (Petrick *et al*.,1988), *Lb. delbruckii* (Delley *et al*., 1990), and *Lb. helveticus* (Pillound and Mollet, 1990). The second method starts from known nucleic acid sequences and oligonucleotide probes are designed and synthesized after examinations of the sequences. The nucleic acid of choice is rRNA. The rRNA molecules, in particular 16S and 23S rRNA, contain alternating sequences of more or less conserved regions.

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Thus, probes can be designed for different levels of phylogenetic groups, from kingdom to species (Giovannoni *et al*., 1988; Betzl *et al*., 1990). Another advantage of rRNA is that these molecules are present in several copies (Up to 10^4) in each cell. Therefore, a method employing a probe with an rRNA target will be more sensitive than if a DNA (plasmid or chromosome) directed probe is used.

Identification of LAB with the use of 16S or 23S rRNA-targeted probe has been used for lactococci and enterococci (Betzl *et al*., 1990; Klijn *et al*., 1991), lactobacilli from different niches (Hertel *et al*., 1991; Hensiek *et al*., 1992; Vogel *et al*., 1994), carnobacteria from meat (Brooks *et al*., 1992), distinguishing vagococci from other LAB (Williams and Collins, 1992), *S.thermophiius* (Ehrmann *et al*., 1992), and even for distinguishing between the subspecies *lactis* and *cremoris* of *Lc. Lactis* (Salama *et al*., 1991). 16S rRNA sequence data from LAB have been accumulated during recent years and the list of available probes is growing (Pot *et al*., 1994b). For certain applications (e.g., analysis of food samples) it may also be interesting to determine the occurrence of specific groups of LAB. Genus-and group-specific probes have been developed for such purposes (Willliams and Collins, 1992; Collins *et al*., 1993; Nissen *et al*., 1994)

Bacteria typically have five to seven copies of each rRNA gene in the chromosome. This has been exploited in a restriction fragment length polymorphism (RFLP) molecular typing method, more commonly known as ribotyping (Grimont and Grimont, 1992). When the chromosomal DNA of a strain is digested with a restriction enzyme, separated in agarose gels, blotted onto a filter, and hybridized with an rRNA-specific probe, a banding pattern is obtained. Depending on the complexity of the patterns, they can be analyzed manually or by computer techniques. Similar to the actual rRNA sequences, which are more conserved than many other genes, the organization of the rRNA genes is also conserved to a certain degree in related species and this is reflected in the banding patterns or “fingerprints”. It appears that the method is useful for species and subspecies recognition in some cases, e.g., lactococci (Rodrigues *et al*., 1991) and *Lb. plantarum* (Johansson *et al*., 1995a), but not in others,

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e.g., *Lb. reuteri* (Stahl et al., 1994). It is interesting to note that *Lb. reuteri*, a quite homogeneous species phenotypically, showed heterogeneous ribopatterns while *Lb. plantarum*, phenotypically heterogeneous, had more homogeneous ribopatterns (Johansson *et al*., 1995a). Therefore, this method has to be evaluated from case to case as to its applicability in strain or species recognition. Automated instruments that generate large databases of reprodudible ribopatterns are now available (e.g., "Riboprinter" from DuPont), possibly setting the standard for interlaboratory work with this identification method.

2.5 Antimicrobial components from Lactic Acid Bacteria

Lactic acid bacteria (LAB) have long been used in fermentations to preserve the nutritive qualities of various foods. The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Daeschel, 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high - molecular-mass (HMM) compounds like bacteriocins (Jay,1982; Klaenhammer, 1988 ; Piard and Desmazeaud, 1991, 1992). All of which can antagonize the growth of some spoilage and pathogenic bacteria in foods.

2.5.1 Organic Acids

Upon fermentation of hexoses, lactic acid is produced by homofermentation or equimolar amounts of lactic acid, acetic acid / ethanol, and carbon dioxide are produced by heterofermentation.

It has long been observed that weak acids have a more powerful antimicrobial activity at low pH that at neutral pH (Simon and Blackman, 1949). Of the two acids, acetic acid is the strongest inhibitor and

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has a wide range of inhibitory activity, inhibiting yeasts, molds, and bacteria (Blom and Mortvedt, 1991).

LAB. produce also other inhibitory substance although in much smaller amounts. These include hydrogen peroxide, diacetyl, bacteriocins and secondary reaction products such as hypothiocyanate generated by action of lactoperoxidase on hydrogen peroxide and thiocyanate.

2.5.2 Hydrogen peroxide

In the presence of oxygen, lactic acid bacteria are able to generate hydrogen peroxide (H_2O_2) through the action of flavoprotein-containing oxidases, NADH oxidases, and and superoxide dismutase. In the absence of the source of heme, lactic acid bacteria will not produce catalase. Other systems that eliminate hydrogen peroxide are less active than the ones producing it. This allows hydrogen peroxide to accumulate (Condon, 1987). However, Fontaine and coworkers (1996) argue that hydrogen peroxide does not accumulate to significant amounts in vivo because it is decomposed by peroxidases, flavoproteins, and pseudocatalase. The bactericidal effect of hydrogen peroxide has been attributed to its strong oxidizing effect on the bacterial cell; sulfhydryl groups of cell proteins and membrane lipids can be oxidized (Morris, 1976; Schlegel, 1985; Lindgren and Dobrogosz, 1990). Also, some of the hydrogen peroxide-producing reactions scavenge oxygen, thereby creating an anerobic environment that is unfavorable for certain organisms. Some workers, however, question whether in vivo hydrogen peroxide per se has significant bactericidal activity (Nagy *et al*., 1991; Fontaine *et al*., 1996).

Under natural conditions, the antimicrobial effects of hydrogen peroxide may be enhanced because of the presence of lactoperoxidase and thiocyanate (SCN^-). The glycoprotein lactoperoxidase is found in saliva, tears, and milk. It catalyzes the oxidation of thiocyanate by hydrogen peroxide, generating hypothyanite ($OSCN^-$). and in the presence of an excess of hydrogen peroxide, also O_2SCN^-

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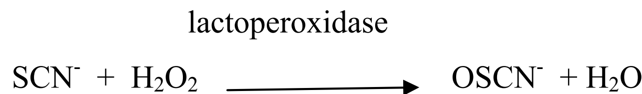

$$SCN^- + H_2O_2 \xrightarrow{\text{lactoperoxidase}} OSCN^- + H_2O$$

Structural damage and changes in bacterial membranes due to exposure to $OSCN^-$ have been reported (Kamau et al., 1990). However, the main antimicrobial effect is contributed to blocking of the glycolysis. It is proposed that it inhibits glucose transport, hexokinase activity, and glyceraldehydes-3-phosphate dehydrogenase activity due to the oxidation of sulfhydryl groups in these metabolic enzymes. The latter enzyme, glyceraldehyde-3-phosphate dehydrogenase, appears to be the primary target (Carlsson *et al*, 1983). The activity toward Gram-possitive bacteria, including lactic acid bacteria, is generally bacteriostatic, whereas many Gram-negative bacteria are rapidly killed (Condon, 1987; Lindgren and Dobrogosz, 1990; Blom and Mortvedt, 1991).

2.5.3 Carbon dioxide

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

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2.5.4 Diacetyl

Diacetyl (2,3-butanedione) was identified by van Niel *et al*. (1929) as the aroma and flavor component, is produced by strains within all genera of LAB by citrate fermentation. It inhibits the growth of Gram-negative bacteria by reacting with arginine utilization (Jay, 1986). Jay (1982) observed that diacetyl was progressively more effective at pH<7. It was also observed that the antimicrobial activity was antagonized by the presence of glucose, acetate, and tween 80. Diacetyl was found to be more active against Gram-negative bacteria, yeasts, and molds than against Gram-positive bacteria; lactic acid bacteria were the least sensitive. Diacetyl is though or react with the arginine binding protein of Gram-negative bacteria and thereby interfering with the utilization of arginine (Jay, 1986).

2.5.5 Low molecular weight antimicrobial substances

There are several reports on the production of low molecular weight components with antimicrobial activity by lactic acid bacteria (Reddy and Shahani, 1971 ; Reddy *et al*., 1984 ; Silva *et al*.,1987). Except for a low molecular weight, these components also share other properties ; 1) active at low pH 2) thermostable 3) broad spectrum of activity and 4) soluble in acetone (Axelsson, 1990). Detailed information not been able to reproduce the findings (Spillman *et al*., 1978).

2.5.5.1 Reuterin

Reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde. It has a broad spectrum of activity and inhibits fungi, protozoa and a wide range of bacteria including both Gram-positive and Gram-negative microorganisms. This compound is produced by stationary phase cultures during anaerobic growth on a mixture of glucose and glycerol or glyceraldehyde. Consequently, in order to use reuterin-producing L.reuteri for biopreservation in a food product, it would be beneficial to include glycerol with the strain. This approach was used to extend the shelf-life of

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herring fillets stored at 5 ^{o}C and involved dipping the fish in a solution containing $1{\times}10^{9}$ cfu/ml of *L. reuteri* and 250 mM glycerol (Lindgren and Dobrogosz,1990). Results demonstrated that after 6-day of storage, there were approximately 100–fold-less Gram-negative bacteria in the *L. reuteri* samples than in the untreated control.

2.5.5.2 2-Pyrorolidone-5-Carboxylic Acid (PCA)

Pyroglutamic acid, or PCA was found to be produced by *Lactobacillus casei* spp. *casei*, *L. casei* ssp. pseudoplantarum and Streptococcus bovis (Chen and Russell, 1989; Huttunen *et al*., 1995). It was found to be inhibitory to *Bacillus subtilis*, *Enterobacter cloacae* and *Pseudomonas putida.*

2.6 Bacteriocins

The term bacteriocin was introduced by Jacob and coworker (Jacob *et al*., 1953). It was defined as protein antibiotics of relative high molecular weight mainly working against the same, or closelyrelated species by adsorption to receptors on the target cells. In 1992, Klaenhammer and coworkers defined three classes of bacteriocins produced by lactic acid bacteria: 1) Lantibiotics; 2) small hydrophobic heat-stable peptides (<13,000 D); 3) large heat-labile proteins (>30,000 D). A year later, the classification was refined and expanded with one class (Klaenhammer, 1993; see table 2.3)

Some bacteriocins may be used in the future in practical applications. 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 cultures or in the gastrointestinal tract in the case of probiotic strains. Some bacteriocins appear to be produced in a product (Blom and Mortvedt, 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

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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, Jimenze-Diaz and coworker (1993) found that only 4 out of 26 *L. plantarum* 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. We have observed in our laboratory that bovine colostrums contains substances that cause a threefold increase in the adhesion of *Lactobacillus casei* ssp. *rhamnosus* GG to enterocyte-like Caco-2 tissue culture cells (Saarinen *et al*., manuscript). However, a potential risk with in situ production of bacteriocins in the intestine is that beneficial members of the normal microflora are aifected (Sanders, 1993).

It has been observed that bacteriocin molecules adsorb to the cell producing it, especially close to pH 6.0. Adsorption is lowest at pH 1.5-2.0. This has been suggested as a method for the production of large quantities of pure bacteriocins (Yang *et al*., 1992).

The following paragraphs will deal with the four classes of bacteriocins in greater detail, including examples of bacteriocins in each class (producer strains, spectrum of activity, molecular mass), their molecular mechanism of activity and protection against it (immunity).

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Table 2.3 Classes of bacteriocins Produced by Lactic Acid Bacteria

Class	Subclass	Description
Class I		Lantibiotics
Class II		Small (<10 kDa), moderate (100^{0}C) to hight (121^{0}C) heat-stable, non-lanthionine-containing membrane-active peptides
	IIa	Listeria-active peptides with –Y-G-N-G-V-X-C - near the amino terminus
	IIb	Two-peptide bacteriocins
	IIc	Thiol-activated peptides
Class III		Large (>30 kDa) heat-labile proteins
Class IV		Complex bacteriocins: protein with lipid and/or Carbohydrate

Modified after Nes *et al*. (1996).

A. Class I

AS mentioned in Table 2.4, this class consists of so-called lantibiotics. These are small peptides containing the unusual dehydroamino acid and thioether amino acids lanthionin and 3-methyllanthionine. These amino acid are synthesized by posttranslational modifications. Examples of lantibiotics produced by lactic acid bacteria are shown in Table 2.4. The lantibiotics produced by lactic acid bacteria all belong to type A. The type A lantibiotics 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 AII consists of highly negatively charged lantibiotics (de Vos *et al*., 1995).

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Table 2.4 Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Class I)

Lantibiotic	Producer strain	Molecular mass	Antimicrobial activity
Carnocin UI149	*Carnobacterium piscicola* UI149	4635 Da	*Carnobacterium, Lactobacillus Pediococcus, and Lactobacillus*
Cytolysin L1	*Enterococcus faecalis*	4164 Da	
Cytolysin L2	*E. faecalis*	2613 Da	
Lacticin 481	*Lc. lactis* 481	2901 Da	Lactic acid bacteria and *Clostridium tyrobutyricum*
Lactocin S	Lb. sake 145	3769 Da	*Lactobacillus, Pediococcus, Leuconostoc*
Lactococcin	*Lc. lactis* ADRI85L030	2300 Da	*C. tyrobutyricum, Lb. helveticus, and Streptoccus thermophilus*
Mutacin	*Sc. mutans*	3245 Da	
Nisin A	*Lc. lactis* ssp. *lactis*	3488 Da	*Lactococcus, Streptococcus, Staphylococcus, Micrococcus Pediococcus, Lactobacillus, Listeria, Mycobacterium, Clostridium* (+ spores), and *Bacillus* (+ spores)

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Table 2.4 Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Class I), (Continued)

Lantibiotic	Producer strain	Molecular mass	Antimicrobial activity
Nisin Z	*Lc. lactis* ssp. *lactis* NIZO 22186	3453 Da	As above
Salivaricin A	*Sc. salivarius* 20P3	2315 Da	*Micrococcus luteus*
Streptococcin A-FF22	*Sc. pyogenes*	2795 Da	

Compiled from Klaenhammer (1993); Jack *et al*. (1994); Sahl *et al*. (1995).

The model-type lantibiotic nisin is discussed as an example for the modes of action of the lantibiotics. Nisin has a broad spectrum of activity against Gram-positive bacteria. *Escherichia coli* and other Gram-negative bacteria are only affected when their 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 different mechanisims for antimicrobial action of nisin have been described. The primary target is belived to be the cell membrane. For its interaction with the cell membrane; nisin does not need any receptor, unlike some other antimicrobial peptides. However, it does need the presence of a membrane potential (Sahl *et al*., 1987; Bruno and Montville, 1993). The dehydroamino acids been suggested to interact with sulfhydryl groups of enzymes (Gross and Morell, 1971).

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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*., 1994; de Vos *et al*., 1995; Sahl *et al*., 1995). Cell iysis has been explained by a cation exchange-like process, where the strongly cationic lantibiotics displace autolytic enzymes. 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 bacteriocins. Klaenhammer (1993) defined three categories of nisin resistance: 1) immunity; 2) resistance not genetically linked to production and 3) 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 novolved in the translocation of (precursors of) the lantibiotics is also thought to be involved in immunity by expelling lantibiotics from the cell (Siegers and Entian, 1995; Venema *et al*., 1995). A hydrophobic protein. Encoded for the nisin operon, has been suggested to work analogous to colicin immunity proteins by interaction with the bacteriocins and closing the pores formed (Siegers and Entain, 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). *Bacillus cereus* has been found to inactive nisin by reducing the dehydroamino acids (Jarvis and Farr, 1971; Venema *et al*., 1995).

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3. Nisin resistance may be acquired in the presence of sublethal nisin concentrations. The mechanisms of resistance may differ from 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 does not appear to need any receptor (Sahl *et al*., 1987), posing difficulties for the mentioned hypothesis.

B. Class II

As shown in Table 2.5, class II contains a wide variety of bacteriocins and has therefore been subdivided into there subclasses (Klaenhammer, 1993). Their general description, however, is small heat stable membrane active peptides. Example of this class of bacteriocins are shown in Table 5.

A number of class II bacteriocins have been shown to be membrane-active peptides. 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*., 1994). Because lactococcin A was only active against membrane vesicles derived from sensitive strains, it was concluded that it needs a specific receptor protein (van Belkum et al., 1991, 1992; Venema *et al*., 1995). It has been suggested that lactococcin A could from a membrane-spanning α helix. The helix would have amphiphilic properties. The molecules would aggregate like barrel staves around acentral water-filled pore (Klaenhammer, 1993; Kok *et al*., 1993; Venema *et al*., 1995). The pores formed are

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Table 2.5 Class II Bacteriocins: Small Non-Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria

Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
Acidocin 8912	*Lactobacillus acidophilus*		*Lb. acidophilus, Lb. casei, Lb. plantarum*, and *Lactococcus lactis*
Brevicin 37	*Lb. brevis*		*Pediococcus damnosus, Lb. brevis, Leuconostoc oenos*
Carnobacteriocin A	*Carnobacterium piscicola* LV17A	5100 Da	“medium”
Carnobacteriocin BM1	*Cb. piscicola* LV17B	4524.6 Da	*Listeria sp., Enterococcus sp., Carnobacterium sp., Lb. plantarum, P. parvulus*
Carnobacteriocin B2	*Cb. piscicola* LV17B	4969.9 Da	As above
Curvacin A	Lb. curvatus LTH 1174		*Lb. curvatus, Lb. sake, Lb. fructivorans, Carnobacterium Enterococcus faecalis, Listeria monocytogenes, L. ivanovii*

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Table 2.5 Class II Bacteriocins: Small Non-Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Continued)

Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
Gassericin A	*Lb. gasseri*		*Lb. acidophilus, Lb. delbrueckii, Lb. helveticus, Lb. casei, Lb. brevis*
Hiraecin S	*E. hirae* C311		*Listeria sp.*
Lactacin B	*Lb. acidophilus*	6300 Da	*Lb. delbrueckii, Lb. Helveticus*
Lactacin F	*Lb. johnsonii* 11088	6300 Da	*Lb. fermentum, E. faecalis, Lb. delbrueckii, Lb. helveticus, Aeromonas hydrophilia, Staphylococcus aureus*
Lacticin 3147	Lc. lactis DPC3143		*Clostridium sp., Enterococcus sp., Lactobacillus sp., Lactococcus sp., Leuconostoc sp., Pediococcus sp., Staphylococcus aureus, Streptococcus sp.*
Lactococcin A	*Lc. lactis ssp. cremoris* 9B4		"Narrow"
Lactococcin B	*Lc. lactis ssp. cremoris* 9B4		"Narrow"

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Table 2.5 Class II Bacteriocins: Small Non-Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Continued)

Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
Lactococcin G	*Lc. lactis ssp. lactis* LMG 2081	α_1: 4376 and β: 4109 Da	"Narrow"
Lactococcin M	*Lc. lactis ssp. cremoris* 9B4		"Narrow"
Leucocin A	*Ln. gelidum*	3900 Da	"Wide"
Mesentericin Y105	*Ln. mesenteroides* Y 105	3700 Da	"Wide"
Pediocin AcH (Pediocin PA-1)	*P. acidilactici* H	4629 Da	"Wide"
Pediocin JD	*P. acidilactici*		*L. monocytogenes*
Piscicolin 126	*Cb. piscicola*	4417 Da	

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Table 2.5 Class II Bacteriocins: Small Non-Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Continued)

Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
Plantaricin A	*Lb. plantarum*	>8 kDa	*Lb. plantarum, Lactobacillus sp., Leuconostoc sp., Pediococcus sp., Lc. lactis, E. faecalis*
Plantaricin C	*Lb. plantarum* 11441	3500 Da	*Lb. fermentum, Lb. sake*
Plantaricin S	*Lb. plantarum*	Two proteins α and β	*Leuconostoc, Clostridium tyrobytiricum, Lb. helveticus, Lb. plantarum, Lb. curvatus, Lb. reuteri, Lb. delbrueckii, Lb. fermentum, Enterococcus, Pediococcus, Lactococcus. Streptococcus, Micrococcus, Propionibacterium.*
Plantaricin T	*Lb. plantarum*	<2500 Da	*Lb. fermentum, Lb. helveticus, Lb. plantarum, Lb. sake, P. pentosaceus, Lc. cremoris, Propionibacterium sp.*
Reutericin 6	*Lb. reuteri*	200 kDa[a]	*Lb. acidophilus, Lb. delbrueckii ssp. lactis and bulgaricu*

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Table 2.5 Class II Bacteriocins: Small Non-Lanthionine-Containing Bacteriocins Produced by Lactic Acid Bacteria (Continued)

Bacteriocin	Producer strain	Molecular mass	Antimicrobial activity
Sakacin A	*Lb. sake* LB 706	4308 Da	*Cb. piscicola, Enterococcus sp., Lb. sake, Lb. curvatus, Lb. brevis, Ln. paramesenteroides, L. monocytogenes, A. hydrophilia, S. aureus, Lc. lactis*
Sakacin P	*Lb. sake* LTH 673		*Lb. curvatus, Lb. delbrueckii, Lb. sake, Lb. pentosus, Lb. plantarum, Lb. reuteri, Lb. fructivorans, E. faecalis, Livanovii, Carnobacterium sp. ssp. cremoris*

[a]Molecular mass estimated by ultra filtration. The retentate was not tested for the absence of low molecular mass compounds with e.g., SDS- PAGE(Toba *et al*., 1991). Compiled from Christensen and Hutkins (1992); Siragusa (1992); Jimenez-Diaz *et al*. (1993); Klaenhammer (1993); Jack *et al*. (1994); Quadri *et al*. (1994); Jimenez-Diaz *et al*. (1995); Gonzales *et al*. (1996).

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Mutacin	*Sc. mutans*	3245 Da	
Nisin A	*Lc. lactis* ssp. *lactis*	3488 Da	*Lactococcus, Streptococcus, Staphylococcus, Micrococcus Pediococcus, Lactobacillus, Listeria, Mycobacterium, Clostridium* (+ spores), and *Bacillus* (+ spores)

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B. Class II

As shown in Table 2.5, class II contains a wide variety of bacteriocins and has therefore been subdivided into there subclasses (Klaenhammer, 1993). Their general description, however, is small heat stable membrane active peptides. Example of this class of bacteriocins are shown in Table 5.

A number of class II bacteriocins have been shown to be membrane-active peptides. 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*., 1994). Because lactococcin A was only active against membrane vesicles derived from sensitive strains, it was concluded that it needs a specific receptor protein (van Belkum et al., 1991, 1992; Venema *et al*., 1995). It has been suggested that lactococcin A could from a membrane-spanning α helix. The helix would have amphiphilic properties. The molecules would aggregate like barrel staves around acentral water-filled pore (Klaenhammer, 1993; Kok *et al*., 1993; Venema *et al*., 1995). The pores formed are

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thought to exist in differenct sizes depending on the bacteriocin concentration (Venema *et al*., 1995). This increases membrane permeability and explains the observed influx of small molecules, efflux of UV-absorbing materials (Jack *et al*., 1994), and dissipation of the proton motive force (Bruno and Montville, 1993). For other class II bacteriocins it has been observed that RNA, DNA, and protein synthesis are inhibited; inhibition 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 II bacteriocins (Christensen and Hutkins, 1992; Bruno and Montville, 1993; Gonzales *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, *L. reuteri* 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 target strain. Bacteriocin production has been observed in coculture and would also give the producing strain a competitive advantage under natural conditions.

As with lantibiotics, producer strains protect themelves against the bacteriocins with the help of immunity proteins. 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 of carnobacteriocin B2 was identified as 12.7 kDa protein and was proposed to interfere with the formation of a functional pore or to block the functional pore (Quadri *et al*., 1995)

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C. Class III

Class III bacteriocins are defined as large heat-labile proteins. This class may therefore include bacteriolytic extracellular enzymes (hemolysins and muramidases) that may mimic the physiological activities of bacteriocins (Jack *et al*., 1994). Examples of this class are shown in Table 2.6

D. Class IV

This class contains complex bacteriocins.Lipid or carbohydrate moieties appear to be necessary for activity. The existence 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 protrase activity (Klaenhammer, 1993). Examples of bacteriocins included in class IV are given in Table 2.7

As with class III, the mechanism of action and immunity of these complex bacteriocins remains to be investigated.

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Table 2.6 Class III Bacteriocins: High Molecular Weight Bacteriocins Produced by Lactic Acid Bacteria

Bacteriocin	Producer Strain	Molecular mass	Antimicrobial activity
Acidophilucin A	*Lactobacillus Acidophilus*		*Lb. delbrueckii, Lb. helveticus*
Caseicin 80	*Lb. casei* B80	40 kDa	*Lb. casei*
Helveticin J	*Lb. helveticus*	37 kDa	*Lb. helveticus, Lb. delbrueckii ssp. bulgaricus* and *lactis*
Helveticin V-1829	*Lb. helveticus*	>10 kDa	*Lb.helveticus, Lb. delbrueckii* ssp. bulgaricus
Lacticin A	*Lb. delbrueckii*		*Lb. delbrueckii ssp. lactis*
Lacticin B	*Lb. delbrueckii*		*Lb. delbrueckii ssp. bulgaricus* and delbrueckii

Complied from Klaenhammer (1993); Venema (1995).

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Table 2.7 Complex Bacteriocins, Consisting of protein, Lipid, and/or Carbohydrate, Produced by Lactic Acid Bacteria (Class IV)

Bacteriocin	Producer Strain	Characteristics	Antimicrobial activity
Bacteriocin	*Lb. fernenti*	Protein-lipocarbohydrate complex	*Lb. fermenti*
Lactocin 27	*Lb. helveticus*	Protein-lipopolysaccharide complex>300 kDa, protein 12,400 Da	*Lb. acidophilus, Lb. helveticus*
Leuconocin S	*Leuconostoc paramesenteroides*	Two glycoproteins 20,000 Da and 10,000 Da	*Listeria sp.,* lactic acid bacteria
Pediocin SJ-1	*Pediococcus acidilactici*	Glycoprotein 40,000 Da	

Complied from Klaenhammer (1993); Venema (1993); Jimenez-Diaz *et al*. (1995).

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2.7 Nisin—structure, function and genetics

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Nisin is undoubtedly the most well known and characterized bacteriocin and the only one to have realized widespread commercial use. This bacteriocin has consequently been the subject of a wide variety of fundamental studies as to its structure and genetics, and the reader is directed to a number of review articles and references therein (De Vuyst and Vandamme,1994b; Dutton *et al*., 2002). Nisin is

composed of 34 amino acids and has a pentacyclic structure (Gross and Morell, 1971; Shiba *et al*., 1991) with one lanthionine residue (ring A) and four β-methyllanthionine residues (rings B, C, D and E). A natural variant of nisin, nisin Z exists in which the histidine at position 27 is replaced by asparagines (Kuipers *et al*., 1991; Mulders *et al*., 1991). This variant has been reported to have improved solubility over nisin at higher pH values. An interesting feature of nisin is its unusually high specific activity when compared to eukaryotic-derived peptides such as meganin. Nisin can be effective at nanomolar concentrations depending on the target strain under investigation, while the eukaryotic peptide meganin is generally up to 1000-fold less active. Earlier studies with nisin demonstrated that it inhibited peptidoglycan biosynthesis (Linnet and Strominger, 1973) and that it interacted with either lipid I or lipid II (Reisinger *et al*., 1980). It was later found that nisin caused pore formation in the membranes of sensitive bacteria (Ruhr and Sahl, 1985; Sahl *et al*., 1987; Benz *et al*., 1991). More recently, it was shown that nisin interacts with a docking molecule, lipid II, which is a membrane-bound precursor for cell wall biosynthesis (Fig. 2.1). Indeed, in the absence of this precursor, significantly higher concentrations of nisin are required to form pores (Breukink *et al*., 1999; Wiedemann *et al*., 2001). Significantly, mutations in the N-terminal rings of nisin indicated that these are involved in lipid II binding, whereas mutations in the flexible hinge region severely affected the ability of the bacteriocin to form pores. Such experiments have revealed the dual functionality of the nisin molecule involving initial binding to lipid II followed by pore formation resulting in rapid killing of the target cell.

A number of genes are involved in the production and export of nisin as well as immunity (Rodriguez and Dodd, 1996). These genes are tightly linked together in the nisin cluster made up of a total of 11 genes of which nisA encodes the nisin precursor itself. Interestingly, the genes responsible for nisin A production and immunity are carried on a 70-kb conjugative transposon called Tn5301 from L. lactis NCFB894 (Dodd *et al*., 1990; Horn *et al*., 1991) or Tn5276 from L. lactis NIZO R5 (Rauch and De Vos,1992), while the genetic determinants for nisin Z are on the

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transposon Tn5278 (Immonen *et al.*, 1995). These transposons also encode sucrose utilization genes sacA, sacB and sacR. Nisin synthesis is regulated by a two-component regulatory system made up of the membrane-bound histidine kinase sensor protein NisK and the regulator NisR. This regulatory system responds to extracellular nisin, which leads to the expression of genes involved in immunity and synthesis/posttranslational modification (Kuipers *et al.*, 1995). Indeed, this regulatory system is the basis for the nisin-induced controlled expression system (NICE) which is a very useful overexpression system for heterologous expression of proteins in many Gram-positive bacteria (De Vos, 1999).

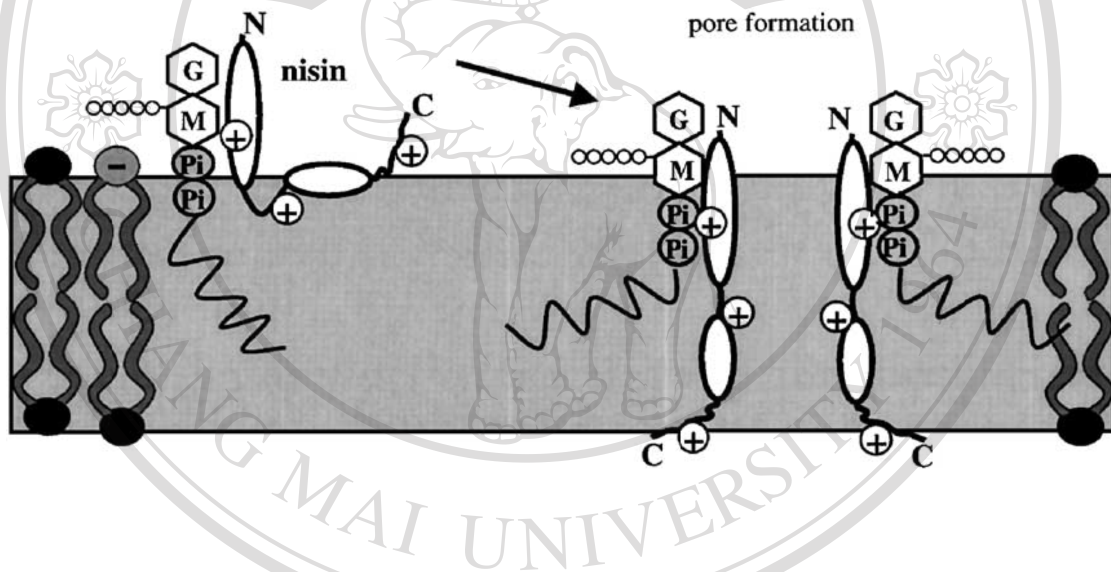

Fig. 2.1 A schematic representation of the mode of action of nisin (reproduced with kind permission of Wiedemann *et al.*, 2001).

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2.8 Using bacteriocins to improve food safety

Bacteriocins have often been mooted as potentially valuable biological tools to improve the food safety and reduce the prevalence of foodborne illnesses. It is usually suggested that bacteriocins should not be used as the primary processing step or barrier to prevent the growth or survival of pathogens, but rather that they could provide an additional hurdle to reduce the likelihood of foodborne disease. There

already exist many control measures within the food industry to prevent or minimise bacterial contamination, including good manufacturing practices, effective sanitation and hygiene measures with respect to raw materials, the food plant, the food products, the food processing personnel (Moberg, 1989) and other basic fundamentals of an effective Hazard Analysis Critical Control Point (HACCP) programme. These measures facilitate the identification, evaluation and control of food safety hazards (National Advisory Committee on Microbiological Criteria for Foods (NACMCF), 1998). However, despite these precautions, foodborne outbreaks do occur alarmingly frequently. *L. monocytogenes* is of particular concern to the food industry and susceptible consumers that include pregnant women, infants, immunocompromised individuals and the elderly. *L. monocytogenes* is ubiquitous in the environment and is extremely resilient, surviving refrigeration temperatures and high salt concentrations.Statistics for Wales and England show 194 hospital cases of listeriosis in 2000, of which 68 resulted in death (Adak, Long, and O'Brian, 2002).Within the United States, the microorganism is estimated to be responsible for 2500 cases, resulting in 500 deaths annually. A policy of zero tolerance in ready to eat foods has been implemented in the United States for *L. monocytogenes,* but outbreaks continue to occur. A recent outbreak associated with contamination of hot dogs resulted in 101 cases and 21 deaths and the recall of 500; 000 lbs of contaminated hot dogs and meats (Donnelly, 2001). This highlights the considerable burden this food pathogen can place on consumers and the food industry. It would seem particularly important to provide an additional hurdle in food to prevent such outbreaks, and bacteriocins would be an economically feasible option. *L. monocytogenes* is not the only concern, there exists a substantial list of food pathogens that result in foodborne illness every year, including many Gram-negative pathogens such as Escherichia coli VTEC 0157, Campylobacter and Salmonella among others (Adak *et al*., 2002). Although the nature of the Gram-negative cell wall restricts the activity of LAB bacteriocins, bacteriocins may be used in combination with other treatments, such as high hydrostatic pressure (HHP), to increase their effectiveness. Thus, bacteriocins may be best applied when

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providing an extra obstacle to prevent the growth of pathogenic and spoilage bacteria, especially in situations where contaminationcould occur post-production.

There are at least three ways in which bacteriocins can be incorporated into a food to improve its safety (Fig. 1), i.e., using a purified/semi-purified bacteriocin preparation as an ingredient in food, by incorporating an ingredient previously fermented with a bacteriocin-producing strain, or by using a bacteriocin-producing culture to replace all or part of a starter culture in fermented foods to produce the bacteriocin in situ. The use of purified bacteriocins is not always attractive to the food industry, as in this form they may have to be labelled as additives and require regulatory approval. Nisin is utilised as an additive and was assigned the number E234 (EEC, 1983 EEC commission directive 83/463/EEC). The two other alternatives (fermented ingredient/starter culture) do not require regulatory approval or preservative label declarations. These options are frequently regarded as more attractive routes through which bacteriocins can be incorporated into a food. These two options are described in greater detail below and a number of representative examples, primarily relating to the use of nisin, pediocin PA-1/AcH and lacticin 3147, are described.

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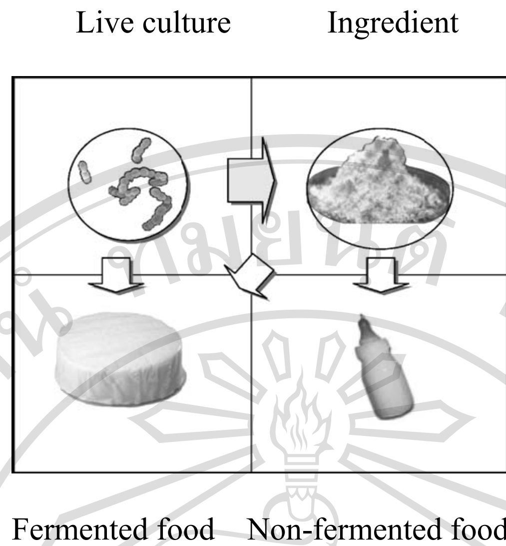

Fig. 2.2 Bacteriocins can be incorporated directly into fermented foods by using a bacteriocin producer as a starter or adjunct culture. Alternatively, the producer can be used to make a food-grade fermentate, which can be dried to make a powdered ingredient. This powder can be then incorporated into either fermented or non-fermented foods.

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