The evaluation of bacteriocins and enzymes for biopreservation of wine

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

The winemaking process involves a number of microorganisms, each with its own role. Yeasts are responsible for the alcoholic fermentation, the lactic acid bacteria (LAB) are Gram-positive bacteria associated with must and wine and perform the malolactic fermentation (MLF), while the acetic acid bacteria (AAB) are Gram-negative bacteria converting ethanol to acetic acid. These microorganisms are present in the cellar and fermentation tanks and can be seen either as beneficial or as wine spoilage microorganisms because, under certain circumstances, they affect the wine quality if they should grow in the wine or must.

Strict measures need to be implemented in the cellar during the winemaking process to ensure microbiological stability. This can be achieved through good microbiological practices and, additionally, chemical preservatives. Sulphur dioxide (SO_2) is widely used as the primary preservative in winemaking. However, consumer resistance has been building up against the use of chemical preservatives, due to the possible health risks and a decrease in nutritional value and sensorial quality of certain foods and beverages.

Biopreservation as an alternative to the traditionally-used chemical preservation is a new approach and has been attracting much attention. This implies the use of the natural microflora and/or their antibacterial products, such as bacteriocins and bacteriolytic enzymes (e.g. lysozyme). Bacteriocins from LAB are proteins or protein complexes, produced by Gram-positive bacteria, with antibacterial activity against closely-related Gram-positive species. Lysozyme occurs in substances such as hen egg white and has lytic activity against Gram-positive bacteria.

The bacteriocins nisin, of the class I lantibiotics, and pediocin PA-1 and leucocin B-TA11a, of the class IIa *Listeria*-active bacteriocins, have been investigated for the biopreservation of wine. Nisin, however, is the only bacteriocin that has been approved for use as a preservative, while pediocin is likely to follow in approval. Lysozyme has been approved for use in winemaking by the *Office International de la Vigne et du Vin* (OIV).

The main objectives of this study were to determine whether these substances showed any antimicrobial action against wine-associated microorganisms, namely LAB, AAB and yeasts. The stability and suitability of the bacteriocins and lysozyme as antimicrobials in wine was researched, especially when used in combination. Possible synergistic or antagonistic interactions between the bacteriocins were also investigated by means of the microtitre broth dilution method and scanning electron microscopy, as well as at what concentration and combinations the bacteriocins were most effective against increasing LAB concentrations.

It was found that nisin, pediocin and leucocin were effective to varying degrees against a test panel of LAB type and reference strains, as well as wine isolates. Nisin repeatedly had the highest level of inhibition against all the LAB tested, followed by pediocin and leucocin. There was no inhibition of the wine-associated AAB and yeasts tested. Pediocin stability was evaluated in simulated wine must and proved to be stable for at least 20 days, without being affected by the sulphur or alcohol content. A low pH, however, led to a more rapid decrease in activity. The same was found for nisin and leucocin in other studies.

Combinations of bacteriocins at increasing concentrations were evaluated against increasing concentrations of a LAB wine isolate. When used in pairs (namely, nisin-leucocin, nisin-pediocin and pediocin-leucocin), the combinations were most effective against lower concentrations of bacteria, namely 10^2 and 10^4 cfu/ml. At lower concentrations, the pairs of bacteriocins were much less effective against the higher bacterial concentrations of 10^6 and 10^8 cfu/ml. Leucocin-pediocin combinations were the least effective, while nisin-leucocin combinations were marginally more effective than the nisin-pediocin combinations. The most pronounced effect was observed when all three the bacteriocins were used together. Combinations of bacteriocins had no inhibitory effect against AAB. Pediocin and lysozyme was used in combination against the same wine isolate, but no conclusive conclusions could be drawn in this experiment.

Scanning electron microscopy was used to investigate any disturbances in cell morphology when bacteriocins were added to LAB. The above-mentioned LAB was subjected to bacteriocins used singularly and also in combinations of equal amounts of bacteriocins. The action of the bacteriocins led to major disturbances in cell morphology. Once again, the combination of leucocin-pediocin was the least effective, even less so than when the single bacteriocins were used. The nisin-pediocin and nisin-leucocin combinations seemed to be more effective in causing cell disturbances and perturbations.

The microtitre broth dilution method was used to further characterise the nature of the interaction of the pairs of bacteriocins. This test showed clearly that the bacteriocins had definite interactions. By adding one bacteriocin to varying concentrations of another bacteriocin, the inhibitory action of the second bacteriocin was affected, either increasing or decreasing its effectiveness. The most important factor to consider seems to be the ratio at which the bacteriocins should be used together, leading either to synergism or antagonism, and this also implies a very complex interaction.

This project indicated that it is indeed possible to use both bacteriocins and lysozyme in wine preservation, both being stable in wine environments and effective against LAB without affecting the yeast fermentation. Bacteriocins could also be used in combination, to broaden the inhibition spectrum, as well as possibly increasing the inhibitory potential of the individual antimicrobials. The underlying interactions in such combinations should be carefully researched, however, when considering using combinations of antimicrobials in food and beverage products. Further attention can also be given to finding biopreservatives against the Gram-negative AAB, as well as to research the interaction of the pairs of bacteriocins over time. Another point to consider would be the engineering of yeasts or bacteria to produce these antibacterial substances *in situ* as part of their metabolism.

OPSOMMING

Daar is 'n verskeidenheid mikroörganismes in die wynmaakproses betrokke, elkeen met sy eie rol. Giste is vir die alkoholiese fermentasie verantwoordelik, die Gram-positiewe melksuurbakterieë (MSB) wat in mos en wyn voorkom, is vir die appelmelksuurgisting (AMG) verantwoordelik, terwyl die Gram-negatiewe asynsuurbakterieë (ASB) etanol in asynsuur omskakel. Hierdie mikroörganismes is in die wynkelder en fermentasietenke teenwoordig en kan as óf gunstig óf ongunstig beskou word, afhangende van die toestande waaronder hulle groei en hoe die wyn daardeur beïnvloed word.

Om mikrobiologiese stabiliteit in wyn te verseker, moet daar streng higiëniese maatreëls in die kelder toegepas word en word daar ook van addisionele chemiese preserveermiddels gebruik gemaak. Swaweldioksied (SO_2) word tans algemeen as primêre preserveermiddel in die wynbedryf gebruik. Weens die moontlike gesondheidsrisiko's wat SO_2 mag inhou en die moontlike verlaging van die voedingswaarde en sensoriese gehalte waarmee dit in sommige voedsel- en drankprodukte geassosieer word, bou daar tans verbruikersweerstand teen die gebruik daarvan as chemiese preserveermiddel op.

Biopreservering is 'n alternatief tot hedendaagse chemiese preservering en het reeds baie belangstelling ontlok. Hierdie metode impliseer die gebruik van die natuurlike mikroflora en/of die antimikrobiese produkte van hierdie mikroörganismes, soos bakteriosiene en bakteriolitiese ensieme (bv. lisosiem). Bakteriosiene van MSB is proteïene of proteïenkomplekse met antimikrobiese aktiwiteit teen naby-verwante Gram-positiewe spesies. Lisosiem kom in produkte soos hoendereierwit voor en het litiese aktiwiteit teen Gram-positiewe bakterieë.

Die bakteriosiene nisien, wat tot die klas I lantibiotiese bakteriosiene behoort, en pediosien PA-1 en leukosien B-TA11a, wat tot die klas IIa *Listeria*-aktiewe bakteriosiene behoort, is as moontlike biopreserveringsagense in wyn ondersoek. Nisien is egter tot op hede die enigste bakteriosien wat amptelik vir gebruik as 'n preserveermiddel in voedsel goedgekeur is, terwyl pediosien moontlik sal volg. Lisosiem is vir gebruik in wynmaak deur die *Office International de la Vigne et du Vin* (OIV) goedgekeur.

Die hoofdoelwitte van hierdie studie was om te bepaal of die bogenoemde stowwe antimikrobiese werking teen wyngeassosieerde mikroörganismes het, soos die ongewenste MSB, ASB en giste. Die stabiliteit en geskiktheid van dié bakteriosiene en lisosiem as antimikrobiese middels in wyn is ook ondersoek, veral wanneer hulle in kombinasie vir preservering gebruik is. 'n Mikrotiterverdunningsboeljon-metode en skanderingselektronmikroskopie is gebruik om moontlike sinergistiese en antagonistiese interaksies tussen bogenoemde bakteriosienpare te ondersoek. Terselfdertyd is die effektiefste konsentrasies en kombinasies van bakteriosiene teen stygende MSB-getalle bepaal.

Daar is bevind dat nisien, pediosien en leukosien in verskillende mates teen 'n toetspaneel van MSB tipe- en verwysingsrasse, asook MSB-wynisolate, effektief is. Nisien was herhaaldelik die effektiefste teen dié MSB, gevolg deur pediosien en dan leukosien. Die bakteriosiene was nie teen die wyngeassosieerde ASB of giste wat getoets is, effektief nie. Daar is ook bewys dat pediosien vir tot 20 dae stabiel in 'n gesimuleerde wyn-omgewing was, sonder dat die alkohol- of die swaweldioksiedkonsentrasie 'n invloed op

die aktiwiteit gehad het nie. 'n Lae pH het geblyk om die grootste invloed op die afname in aktiwiteit te hê. Hierdie bevindinge ten opsigte van pediosien het die resultate van nisien en leukosien in ander, soortgelyke ondersoeke, bevestig.

Die werking van toenemende konsentrasies van bakteriosienkombinasies (as pare van nisien-leukosien, nisien-pediosien, leukosien-pediosien, en al drie saam as nisien-pediosien-leukosien) teen toenemende getalle van ´n wyngeïsoleerde MSB is geëvalueer. Wanneer die bakteriosiene in pare gebruik is, was die kombinasies die effektiefste teen laer MSB selgetalle (10^2 en 10^4 kfe/ml), terwyl dit baie minder effektief teen hoër selgetalle (10^6 en 10^8 kfe/ml) was, veral wanneer lae bakteriosienkonsentrasies gebruik is. Die nisien-leukosien kombinasie was tot 'n geringe mate meer effektief as die nisien-pediosien kombinasie. Die leukosien-pediosien kombinasie het die laagste effektiwiteit van al die pare bakteriosiene wat gebruik is, getoon. Die sterkste werking is waargeneem toe al drie die bakteriosiene saam teen bogenoemde MSB gebruik is. Die bakteriosien kombinasies het geen effek teen ASB gehad nie. Pediosien en lisosiem is ook in kombinasie teen dieselfde wynisolaat gebruik, maar geen oortuigende afleidings kon van hierdie eksperiment gemaak word nie.

Skanderingselektronmikroskopie is gebruik om enige morfologiese verandering in die MSB-wynisolaat waar te neem wanneer bakteriosiene daarby gevoeg is. Dieselfde wynisolaat is weer gebruik en bakteriosiene is by die bakterieë gevoeg, enkelvoudig asook in kombinasies (soos voorheen gebruik) teen gelyke hoeveelhede. Die werking van die bakteriosien het gelei na merkbare veranderinge in selmorfologie, en die kombinasie van pediosien-leukosien was weereens die minste effektief.

Die mikrotiterverdunningsboeljon-metode is gebruik om die aard van die bakteriosieninteraksies verder te karakteriseer. Die toetse het duidelik aangedui dat die bakteriosiene op mekaar reageer. Deur een bakteriosien tot variërende konsentrasies van ´n ander bakteriosien te voeg, is die inhibitoriese werking van die tweede bakteriosien geaffekteer deurdat die effektiwiteit daarvan toegeneem of afgeneem het. Dit het ook geblyk dat die belangrikste faktor wat hier in ag geneem moet word die verhouding is waarteen die bakteriosiene met mekaar gebruik word, aangesien dit tot òf sinergisme òf antagonisme kan lei. Dít dui op 'n baie komplekse interaksie.

Die resultate van hierdie projek het dus daarop gedui dat dit inderdaad moontlik is om beide bakteriosien en lisosiem in wynpreservering te gebruik, aangesien beide nie net stabiel in 'n wynomgewing is nie, maar ook effektief is teen MSB sonder dat die gisfermentasies geaffekteer word. Bakteriosiene kan ook in kombinasie gebruik word om die inhibisie spektrum te verbreed, en om ook moontlik die inhibisiepotensiaal van die individuele peptiede te verhoog. Onderliggende interaksies by sulke kombinasies moet egter sorgvuldig ondersoek word wanneer daar oorweeg word om kombinasies van hierdie antimikrobiese middels in voedsel- en drankprodukte te gebruik. Verder moet daar ook aandag geskenk word om biopreserveermiddels te vind wat ook teen die Gram-negatiewe ASB effektief is, asook aan die aard van die verloop van interaksies van pare van bakteriosiene oor tyd. Nog 'n punt om te oorweeg is die manipulasie van giste of bakterieë om die antimikrobiese peptiede *in situ*, as deel van hulle metabolisme, te produseer.

BIOGRAPHICAL SKETCH

Corina du Toit was born in Johannesburg on 27 April 1976. After matriculating with distinction at the DF Malan High School in 1994, she enrolled at Stellenbosch University and obtained a BScAgric degree in Food Science. In 1999 Corina enrolled for a MScAgric degree in Wine Biotechnology.

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PREFACE

This thesis is presented as a compilation of four chapters. Each chapter is introduced separately and is written according to the style of the journal, *International Journal of Food Microbiology*, to which Chapter 3 will be submitted for publication.

CHAPTER 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

CHAPTER 2 **LITERATURE REVIEW**
Biopreservation of fermented foods and beverages by antimicrobial peptides and enzymes

CHAPTER 3 **RESEARCH RESULTS**
The application of nisin, pediocin, leucocin and lysozyme as biopreservatives in winemaking

CHAPTER 4 **GENERAL DISCUSSION AND CONCLUSIONS**

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 BIOPRESERVATION OF WINE

The winemaking process can be seen as an ancient fermentation biotechnology, even more so than that of bread making. There are different microorganisms involved in vinification, namely the yeasts that perform the alcoholic fermentation, the Gram-positive lactic acid bacteria (LAB) responsible for the malolactic fermentation, and the Gram-negative acetic acid bacteria (AAB) (Vaughn, 1955; Kunkee, 1984; Davis et al., 1985; Ribéreau-Gayon, 1985; Boulton et al., 1996; Fleet, 1998). Traditionally, chemical preservatives, such as sulphur dioxide (SO_2), were used widely in the food and beverage industries to prevent the growth of spoilage microbial populations and oxidative reactions (Taylor et al., 1986; Britz and Tracey, 1990; Usseglio-Tomasset, 1992; Ribéreau-Gayon et al., 2000).

A number of health risks along with organoleptic or sensorial considerations are associated with the use of SO_2, however, and these are accompanied by some consumer resistance, as well as a call for products that are "clean and green". Biopreservation has attracted much attention in recent years and implies a novel, scientifically-based approach to improve the microbiological safety of foods. This preservation method refers to extended storage life and the enhanced safety of foods, using the natural microflora of the product and/or its antibacterial products to inhibit or destroy undesired microorganisms (Schillinger et al., 1996; Stiles, 1996).

An abundance of novel, gene-encoded antimicrobial peptides from animals, plants and bacteria have been described in the past decade (Sahl and Bierbaum, 1998). One such class of peptides is the bacteriocins, defined as proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producing organism (Tagg et al., 1976; Van Belkum and Stiles, 2000). The bacteriocins of Gram-positive bacteria exhibit activity towards a relatively wide range of Gram-positive species, but not Gram-negative bacteria, yeasts or fungi (De Vuyst and Vandamme, 1994). Bacteriocins act on the cytoplasmic membrane of sensitive organisms by dissipating the membrane potential and causing the collapse of the proton motive force (Bruno and Montville, 1993; Fimland et al., 1996, 1998; Ennahar et al., 2000). Four classes of bacteriocins have been defined, based mainly on observed common structural characteristics (Ennahar et al., 2000), but also taking into account biochemical and genetic studies (Venema et al., 1995) and mechanisms of action (Klaenhammer, 1993; Jack and Jung, 2000). This study focused mainly on the class I lantibiotic nisin, as well as pediocin PA-1 and leucocin B-Ta11a, both from the group IIa, non-lanthionine, pediocin-like, *Listeria*-active peptides.

There appears to be general consensus that nisin and other bacteriocins are non-toxic when eaten, as they are proteinaceous in nature and the digestive system degrades the peptides to their constitutive amino acids (Hansen, 1993). The use of nisin as a preservative was first approved about 35 years ago. Its use as a safe food preservative has since been allowed in at least 48 countries and it was granted approval by the American Food and Drug Administration (FDA) in 1998 (Ross et al., 1999). Nisin has been used successfully as a preservative in a number of foodstuffs, including dairy products, such as cheeses and heat-sterilised milk, canned foods and meat. Some experiments have also been performed on the application of nisin on alcoholic beverages, such as wine and beer (Hurst, 1981; Ogden, 1987, Ogden et

al., 1988; Radler, 1990a, b; Delves-Broughton, 1990a, b; Delves-Broughton et al., 1996). Pediocin is one of the most characterised bacteriocins and will probably be the second bacteriocin, after nisin, to find practical applications in the food industry (Chikindas et al., 1995; Venema et al., 1995). Pediocin has been shown to be useful in the preservation of grape juice and meat, to name a few (Cintas et al., 1995; Strasser de Saad et al., 1995; Montville and Chen, 1998). The same applications may be found for leucocin.

Lysozyme is a bacteriolytic enzyme commonly found in cow's milk, domestic egg white, nasal secretions and human tears (McKenzie and White, 1991) and is another proteinaceous substance that can be used as a biopreservative. This enzyme is effective only against certain bacteria, such as sensitive Gram-positive bacteria, and not those infectious to humans (Cunningham et al., 1991). This enzyme contributes to bacterial killing by degradation of the peptidoglycan component of the bacterial outer membrane (Ohno and Morrison, 1989). Lysozyme has also found several applications in food preservation, especially in wine, hard cheeses, products of animal origin and vegetable products, especially in Japan (Hughey and Johnson 1987; Proctor and Cunningham, 1988; Gerbaux et al., 1997).

The antimicrobial efficiency of a bacteriocin may be enhanced or broadened by combining it with other bacteriocins, antimicrobial substances or sterilisation techniques. Leistner (1992) called this "hurdle technology" for the preservation of foods. A synergistic effect, where combinations of the antimicrobials are more effective than either one alone in growth inhibition, has been observed with a number of bacteriocin combinations (Schillinger et al., 1996; Mulet-Powell et al., 1998; Parente et al., 1998). Combinations of lysozyme and nisin have proved to be effective in the inhibition of sensitive organisms (Chung and Hancock, 2000).

The improvement of existing microbial strains or the development of novel strains by genetic engineering is an active research area worldwide. As more knowledge about the LAB accumulates, it becomes possible to construct genetically useful LAB strains with characteristics customised for specific purposes. The quality and preservation of fermented foods and beverages can be enhanced by LAB that have been improved through genetic methods (McKay and Baldwin, 1990). LAB strains that are adapted to a specific kind of food can be constructed to co-produce multiple bacteriocins, or higher levels than are found naturally (Chikindas et al., 1995; Horn et al., 1998, 1999; Ennahar et al., 2000). The yeast *Saccharomyces cerevisiae* has been used as a model eukaryotic organism for the heterologous expression of genes encoding for bacteriocins or lysozyme. A modified version of lysozyme was also created with increased stability or extending its inhibition spectra to Gram-negative bacteria (Kato et al., 1992, 1994; Ibrahim et al., 1993; Nakamura et al., 1993; Schoeman et al., 1999).

The challenge in the food and alcoholic beverage industries for the future therefore will be to find alternatives to chemical preservatives. In preliminary results, antimicrobial peptides and enzymes have been shown to have great potential as biopreservatives. The application of these compounds in the food and beverage industries will be dependent, however, on their cost-effectiveness. Means such as genetic engineering of starter cultures could be utilised to overcome these considerations, customising these bacteria or yeasts to produce an array of natural antimicrobial substances.

1.2 PROJECT AIMS

This study forms part of an important research project on the biopreservation of wine within the Institute for Wine Biotechnology. This biopreservation can be achieved with bacteriocins produced by the native producing lactic acid bacteria or bacteriolytic enzymes, such as lysozyme, or with yeast strains engineered to produce bacteriocins or lysozyme. The possibility of controlling wine spoilage bacteria with the respective antimicrobials was investigated, as well as their stability in a wine environment. The specific aims of this study were as follows:

(i) to screen nisin, pediocin PA-1 and leucocin Ta33a against wine-associated lactic acid bacteria, yeasts and acetic acid bacteria to determine the inhibition range of the bacteriocins;
(ii) to evaluate the stability of pediocin and leucocin under simulated wine conditions over an extended period of time;
(iii) to test for inhibition of Gram-negative acetic acid bacteria by combinations of nisin, pediocin and leucocin, with the bacteriolytic enzyme, lysozyme;
(iv) to examine the action of pediocin and lysozyme used in combination;
(v) to establish the efficiency of combinations of nisin, pediocin and leucocin at varying concentrations against increasing cell concentrations of a sensitive LAB, to examine the possibility of the occurrence of synergism or antagonism; and
(vi) to confirm and characterise the synergism or antagonism of combinations of the bacteriocins by:
    (a) scanning electron microscopy, to observe the cell wall damage and possible changes in cell wall morphology;
    (b) the microtitre broth dilution method for comparative inhibition.

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CHAPTER 2

LITERATURE REVIEW

Biopreservation of fermented foods and beverages by antimicrobial peptides and enzymes

2. LITERATURE REVIEW

Biopreservation of fermented foods and beverages by antimicrobial peptides and enzymes

Historically, food fermentations were empirical and happened spontaneously (more by default than design due to the activities of the natural flora contained in the raw material) (Stiles, 1996). The role of these organisms has primarily been to preserve the foodstuffs, but they are also used to provide variety in the food consumed, by altering the flavour, texture and appearance of the raw commodities in a favourable way (Davidson and Hoover, 1993; Nettles and Barefoot, 1993). Popular fermented food and beverages include products such as cheese, yoghurt, fermented sausages and meat, beer and wine, as well as vegetable fermentations. The biopreservation of these foods will be the main subject matter of this literature review, with special attention given to wine and wine-associated microorganisms.

2.1 MICROORGANISMS ASSOCIATED WITH WINEMAKING

In modern times, the winemaking process consists of a yeast-based primary alcoholic fermentation and an optional secondary fermentation, the **m**alo**l**actic **f**ermentation (MLF), which is performed by **l**actic **a**cid **b**acteria (LAB). The microorganisms involved in the vinification process, namely yeast, LAB and **a**cetic **a**cid **b**acteria (AAB), whether as inoculated starter cultures or part of the natural microflora, have the potential to affect the quality of the end product. The control and management of these microorganisms is of the utmost importance to ensure consistent, high-quality alcoholic beverages.

2.1.1 Yeasts

Yeasts are primarily responsible for the alcoholic fermentation, converting the sugar in the grape must to alcohol. A number of wild yeasts occur naturally in varying numbers on grapes, in the subsequent grape must, and in the cellar environment. Yeasts associated with the winemaking environment have adapted to survive in this hostile environment and include strains of the non-*Saccharomyces* genera *Brettanomyces* and its non-sporulating form *Dekkera*, *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Saccharomycodes, Schizosaccharomyces, Torulopsis, Torulaspora,* and *Zygosaccharomyces* (Ribéreau-Gayon, 1985; Boulton et al., 1996; Fleet, 1998; Pretorius et al., 1999). These non-*Saccharomyces* yeasts are often associated with wine spoilage and include faults such as turbidity, film formation and off-flavours (Du Toit and Pretorius, 2000). *Saccharomyces cerevisiae,* the principal wine yeast responsible for the alcoholic fermentation, occurs in very low numbers on grapes and at the beginning of the fermentation. However, it will gradually dominate the fermentation due to its higher ethanol tolerance (Boulton et al., 1996; Fleet, 1998). *S. cerevisiae* therefore is used mainly in commercial starter cultures to ensure a safer and faster fermentation, producing wines with a better sensorial quality and hygienic status (Lonvaud-Funel, 2001).

2.1.2 Lactic acid bacteria

Lactic acid bacteria also occur in must and wine and perform the secondary fermentation, known as the MLF. The MLF includes a reduction in acidity, resulting from the degradation of L-malic acid to L-lactic acid with concomitant CO_2 release (Lafon-Lafourcade et al., 1983; Wibowo et al., 1985; Van Vuuren and Dicks, 1993; Lonvaud-Funel, 1995). These LAB can be bacilli, cocci-bacilli or cocci, and are facultatively anaerobic (Kunkee, 1984; Davis et al., 1985). LAB isolated from grapes and wines are from the taxonomic genera *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus*. *Oenococcus oeni*, previously known as *Leuconostoc oenos* (Dicks et al., 1995), is used commercially in malolactic starter cultures, as they are the best adapted to wine conditions. The MLF is primarily performed to reduce wine acidity, especially in cooler climate regions, and is mostly considered beneficial to the wine's sensory quality due to flavour modification. The MLF also provides microbial stability, since malic acid, which can serve as a carbon source to support the growth of potential spoilage LAB, is degraded. Wine made in cooler climatic regions has a naturally high acid content due to the higher levels of malic and tartaric acid occurring in the grapes, and will benefit from a reduction in acidity and an increase in the pH (Wibowo et al., 1985). In contrast, wines made in a warm to hot climate have low acidity and a further loss of acidity is usually not desirable (Goswell, 1986).

LAB can also be perceived as spoilage bacteria occurring during the vinification process when the MLF is unwanted, or when these bacteria produce defects in the wines (Edwards et al., 1998). These bacteria are usually found in low numbers (less than 10^3 cfu/ml) on grapes and the must, since they have high nutritional requirements. Only a small number of LAB can grow in the presence of SO_2 and in the nutrient-poor must. Should the LAB be able to tolerate the stress conditions found in wine and the inhibitors produced during the fermentation, they would be able to proliferate and occur in numbers of up to 10^6-10^8 cfu/ml (Du Toit and Pretorius, 2000).

2.1.3 Acetic acid bacteria

Acetic acid bacteria are Gram-negative, catalase-positive aerobic organisms and are able to oxidise ethanol to acetic acid (Drysdale and Fleet, 1988; Boulton et al., 1996, Ribéreau-Gayon et al., 2000). The two genera associated with wine are known as *Acetobacter* and *Gluconobacter*. The genus *Acetobacter* contains four species – *A. aceti*, *A. hansenii, A. pasteurianus* and *A. liquefaciens*, which are reported to play a role in the winemaking process, while *Gluconobacter* has only one species, *G. oxydans,* which is associated with winemaking (Boulton et al., 1996). Acetic acid constitutes the major volatile acid in wine and is generally considered unfavourable at levels above 1.2 –1.4 g/L. The concentration of this acid in wine can be increased significantly by the action of spoilage yeasts and LAB, but especially by AAB, which also forms other compounds affecting wine quality (Drysdale and Fleet, 1988).

Strict measurements need to be implemented in the cellar during the winemaking process to ensure microbiological stability. As can be seen in the above section, wine spoilage microorganisms are present in the cellar and fermentation tanks, affecting the wine quality negatively if they should grow in the wine or must. The control of the proliferation of these microorganisms can be achieved through good

microbiological practices and, additionally, chemical preservatives are used widely in the wine industry. Biopreservation is an alternative to chemical preservation and holds promise for future use. These two preservation methods will be discussed in the following sections, with the emphasis on biopreservation.

2.2 CHEMICAL PRESERVATION

When considering the microbiological preservation of a product, in this case wine, one must take a number of aspects into consideration. These include the properties of the food system, such as the pH, lipid content, protein content and the natural inhibitors in the food. The organisms targeted should also be considered – are they bacteria (prokaryotes), or yeasts or moulds (eukaryotes)? Are the bacteria Gram-positive or Gram-negative? How effective is the preservative in food systems in comparison to model systems? What concentration of the preservative is needed? Another important point is whether the preservative is natural or synthetic and whether it has received GRAS-status (Generally Regarded As Safe), as well as what levels are allowed. The next point of interest is the mode of action of the preservative – are the cells killed (bactericidal) or just inhibited (bacteriostatic) – also taking into consideration the specificity towards the organisms to be targeted. In the wine industry, sulphur dioxide is the most widely used chemical preservative to inhibit the growth of unwanted yeasts and bacteria. Products like sorbic acid, octanoic and decanoic acid, ascorbic acid and ethyl pyrocarbonate can also be used to complement the effect of sulphur dioxide (Ribéreau-Gayon et al., 2000).

2.2.1 Properties and use of sulphur dioxide (SO_2)

The general use of sulphur dioxide seems to date back to the late 18th century, when sulphur fumes were used to clean wine containers. The many properties of SO_2 make it an indispensable aid in the winemaking process (Ribéreau-Gayon et al., 2000). Sulphiting agents are added to foods for many important technical purposes, and serve more than one purpose in many products (Taylor et al., 1986).

The use of SO_2 in the winemaking process involves hygienic and technological risks, also influencing the organoleptic qualities of wine. The functions of SO_2 involve a number of properties (Usseglio-Tomasset, 1992). The principal properties are: (i) antiseptic – it inhibits the development of microorganisms; (ii) antioxidant – binding with dissolved O_2, protecting the wine from chemical (but not enzymatic) oxidation; (iii) antioxidasic – immediately inhibiting the action of oxidation enzymes, and can ensure their destruction over time; (iv) binding of acetaldehyde and other similar products, thus protecting the wine aromas and causing a flat character to disappear (Ribéreau-Gayon et al., 2000). The oenological importance of SO_2 applies to white wine especially, in which case the wines would lose some of their important characteristics if they were not protected by SO_2. Red wines can be affected negatively by the use of SO_2, in which case the reaction products of SO_2 and anthocyanins worsen the organoleptic qualities (Usseglio-Tomasset, 1992).

Although SO_2 is not a natural product of the must, it is a natural compound of wine, as it is a product of the sulphur metabolism of yeast (Usseglio-Tomasset, 1992). When SO_2 is dissolved in an aqueous solution, it is present in three different pH-dependent forms that are in equilibrium with each other. The different forms are

collectively named sulphiting agents (Rose, 1987; Zoecklein et al., 1995), and are: molecular SO_2, bisulphite, (HSO_3^-) and sulphite (SO_3^{2-}) (Usseglio-Tomasset, 1992):

$$SO_2 \leftrightarrow H^+ + HSO_3^- \leftrightarrow H^+ + SO_3^{2-}$$

Active SO_2 or sulphurous acid in the free acid state represents free sulphur dioxide as it is defined in oenology. Free and undissociated SO_2 has the most effective action (Britz and Tracey, 1990). SO_2 binds reversibly to some of the wine components with carbonyl groups and is called bound SO_2. The sum of the free SO_2 plus the bound SO_2 equals the total SO_2 in the wine (Ribéreau-Gayon et al., 2000). **Fig. 2.1** depicts the different fractions of sulphur that exist in wine.

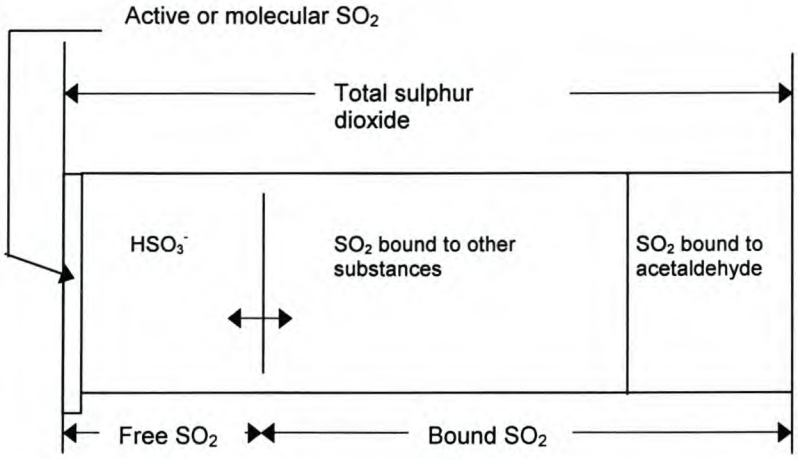

Figure 2.1 The different states of sulphur dioxide in wine (Ribéreau-Gayon et al., 2000).

The antimicrobial activity of free SO_2 at a given concentration against microorganisms varies in function according to the pH. The amount of molecular SO_2, however, depends on the pH of the wine; for example, the same amount of free SO_2 at pH 3,0 corresponds to double the amount at pH 3,4 - a normal wine pH with the bisulfite form is the most prevalent at this specific pH (Usseglio-Tomasset, 1992). SO_2 reacts with compounds such as acetaldehyde, anthocyanins, sugars, keto-acids (such as gluconic and pyruvic acid) and dihydroxyacetone, losing its antimicrobial as well as antioxidant properties (Usseglio-Tomasset, 1992).

SO_2 is an effective antimicrobial agent active against the LAB associated with must and wine, whereas wine yeasts are less sensitive. LAB strains vary in their sensitivity to SO_2. The lactobacilli and pediococci are more resistant than the oenococci (Davis et al., 1985; Wibowo et al., 1985). A lower pH and higher ethanol levels can also enhance the inhibitory activity of SO_2 (Britz and Tracey, 1990). The growth of AAB should be inhibited with the correct use of SO_2. However, it was found that AAB could proliferate under conditions in which the must contained more than 100 mg/L SO_2. This might offer an explanation why AAB are found to such a great extent under modern winemaking conditions, as less SO_2 is used today.

Due to the alleged role played by sulphites in the initiation of asthmatic and other reactions in certain sensitive individuals, the safety of its usage has been questioned (Taylor et al., 1986). There are a number of advantages and disadvantages involved in the use of SO_2. The advantages, as mentioned above, are numerous, but SO_2 use also presents some disadvantages. The wine taste is

compromised when used in very high concentrations, giving the wine a disagreeable odour and bad taste; also, when stored too long on the lees, the taste of hydrogen sulphite and mercaptans can appear in young wines. Incorrect sulphiting can also lead to a slowing or inhibition of malolactic fermentation in red wines (Ribéreau-Gayon et al., 2000). In recent years, there has been a call for the lowering of SO_2 levels in food and the oenological community has joined this trend. Techniques such as filter sterilisation and mild heat treatments have been employed, lowering the need for the use of high levels of SO_2 (Usseglio-Tomasset, 1992). Alternatively, a more consumer-friendly means of wine preservation is being researched for possible use in the wine industry, for example the use of biopreservatives.

2.3 BIOPRESERVATION

Throughout history, food spoilage has plagued man, and early preservation attempts included readily-available processes or substances such as wood smoke, sugars, salts and spices. Preservation today utilises factors such as temperature, water activity, pH, salts, irradiation or combinations thereof (Wagner and Moberg, 1989). A new approach, biopreservation, has attracted much attention in recent years, since food safety has become an increasingly important international concern. Biopreservation implies a novel, scientifically-based approach to improve the microbiological safety of foods. This preservation method refers to extended storage life and the enhanced safety of foods, using the natural microflora of the product and/or their antibacterial substances to inhibit or destroy undesired microorganisms. Biopreservation may consist of (i) adding bacterial strains that grow rapidly and/or produce antagonistic substances; (ii) adding purified antagonistic substances; (iii) adding the fermentation liquor or concentrate from an antagonistic organism; or (iv) adding mesophilic LAB as "fail-safe" protection against temperature abuse (Schillinger et al., 1996; Stiles, 1996).

There are a number of requirements for the use of naturally-occurring antimicrobial substances for use as food preservatives. These include:

(i) the toxicology must be acceptable by recognised authorities (FDA, WHO etc.);
(ii) it must not have any negative effect on the organoleptic properties of the foods in which it is used;
(iii) the cost must be economical and acceptable to the industry;
(iv) it must be stable during storage before use, and be sufficiently stable for the shelf-life of the food;
(v) it should be effective at relatively low concentrations; and
(vi) it should have no medical use (Daeschel, 1989).

Consumer resistance to conventional preservatives, especially chemical preservatives, and the ever-increasing resistance of bacteria to conventional antimicrobial substances, has encouraged the search for new antimicrobial agents that have alternative targets (Yan et al., 2000). This literature review will focus on the application of the bacteriocins nisin, pediocin and leucocin, and the bacteriolytic enzyme, lysozyme, as biopreservatives.

2.3.1 Lactic acid bacteria starter cultures

LAB, especially the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus*, have traditionally been used as starter cultures in the fermentation of

foods and beverages. Research and characterisation of the starter culture microorganisms have, however, only recently been given attention, even though their use dates back to antiquity (De Vuyst and Vandamme, 1994b). Historically, food fermentations happened spontaneously, due to the activities of the natural flora contained in the raw material (Stiles, 1996). These organisms primarily preserve the foodstuffs, but they also provide variety in the food consumed by altering the flavour, texture and appearance of the raw commodities in a favourable way (Davidson and Hoover, 1993; Nettles and Barefoot, 1993). The starter cultures present in food contribute to its organoleptic properties and to its preservation by the production of antimicrobial substances (Nettles and Barefoot, 1993). In many cases, the precise mechanism of antimicrobial action cannot be defined, due to the complex interaction of several phenomena in combination with each other (Earnshaw and Gidley, 1992). Bacteriocin production by LAB starter cultures can give them a competitive advantage, by interfering with the growth of the other LAB present (De Vuyst and Vandamme, 1994a).

2.3.1.1 Metabolites

The preservation effect of LAB during the manufacture and subsequent storage of fermented foods and beverages is due mainly to the acidic conditions that are created during their development. This acidification is due largely to the fermentative conversion of carbohydrates to organic acids (mainly lactic and acetic acid), with a subsequent lowering of the pH. This leads to an increased shelf life and safer final product (Buckenhüskes, 1993; Davidson and Hoover 1993; De Vuyst and Vandamme, 1994b). LAB, however, have a far more complex antagonistic system and produce a number of inhibitory metabolites in addition to the above-mentioned acids. These substances include formic acid, free fatty acids, ammonia, ethanol, hydrogen peroxide, diacetyl, acetoin, 2,3-butanediol, acetaldehyde, benzoic acid and antibiotics, as well as several less well-defined or completely unidentified inhibitory substances (Klaenhammer, 1988; Daeschel, 1989; Schillinger, 1990; Vandenbergh, 1993; De Vuyst and Vandamme, 1994b; Stiles, 1994).

2.3.1.2 Peptides

Bacteriocins produced by the LAB may also contribute to the preservative effect discussed above. A major focal point of research has involved the characterisation and production of bacteriocins from the LAB isolated from food and beverages, in the light of the potential of these bacteria to improve food safety and preservation. A number of these food grade microorganisms are in use in the food industry and now offer the possibility to improve food preservation (Jack et al., 1995). Chemical preservatives are being employed currently, but consumer awareness of the potential health risks associated with these substances is on the increase and has led researchers to examine the use of bacteriocins as biopreservatives for food (Abee et al., 1995). These compounds will be discussed in full later.

Bacterial-derived antimicrobial polypeptides exhibit a large degree of structural and chemical diversity. It has been known for some time that bacteria produce vast amounts of peptide antibiotics (Jack and Jung, 2000). The application of bacteriocins, however, has been researched extensively in a number of foods and many applications have been discovered. Many bacteriocins are heat stable and can be spray-dried to powder form and used as an ingredient in foods (Ross et al., 1999).

Bacteriocin-producing organisms have been used to great effect in preventing the growth of foodborne pathogens in meat (McMullen and Stiles, 1996), in the dairy industry (Barefoot and Nettles, 1993; Davidson and Hoover 1993), in canned food, where *Clostridium botulinum* can be problematic (Stiles, 1996), and vegetable fermentations have also received attention (Choi and Beuchat, 1994; Choi and Park, 2000).

2.3.2 Enzymes

Many enzymes are already used in food processing and it would not be surprising if enzymes are used in food preservation in the future as well (Fuglsang et al., 1995). Once again a number of points should be considered before an enzyme is considered for use, such as the legal aspects, the potential health considerations, possible side effects due to the activity of the enzyme in the foods and beverages and the stability of the enzymes in these systems. The enzyme must not affect the organoleptic properties of the foodstuff (Meyer and Isaksen, 1995).

Enzymes can easily be applied in the food and beverage industry, specifically as biopreservatives, since enzymatic cell lysis can be highly specific in terms of the target microorganism. The cell walls of yeast and bacteria are distinctly different and, in general, lytic systems are specific for particular groups of microorganisms (Andrews and Asenjo, 1987).

Lysozyme has received considerable attention for this application, as it can lyse several pathogens found in food. However, its possibilities as a food preservative are still somewhat limited. The possibility does exist to use it in combination with other food preservatives, such as sorbate, ethanol, temperature and low pH, possibly increasing the microbial safety of food products. Other bacteriolytic enzymes, such as β-lytic protease, β-N-acetylglucosaminidase and β-N-acetylmuramidase, have been reported, but little has been done to find applications for these enzymes in food systems. Microorganisms that produce these enzymes will usually produce them as a complex of several cell wall-degrading enzymes specific for different substrates, which will then act synergistically to degrade the cell wall of a given target organism (Andrews and Asenjo, 1987).

2.4 BACTERIOCINS

2.4.1 General

An abundance of novel, gene-encoded antimicrobial peptides from animals, plants and bacteria have been described in the past decade. Scientific reports on the production of antibacterial molecules by Gram-positive bacteria, categorised as bacteriocins, are abundant (Jack et al., 1995; Sahl and Bierbaum, 1998; Ennahar et al., 2000b; Jack and Jung, 2000)

Tagg et al. (1976) defined bacteriocins as antagonistic substances of proteinaceous nature, with bactericidal acitivity against species that are usually closely related to the producing organism. Four classes of bacteriocins have been defined, based on observed common structural, biochemical and genetic characteristics (Venema et al., 1995a; Ennahar et al., 2000b), and mechanisms of action (Klaenhammer, 1993; Jack and Jung, 2000). The four classes are:

(I) Lantibiotics: small membrane active peptides (<5 kDa) containing the unusual amino acids lanthionine and β-methyl lanthionine and dehydrated residues, e.g. nisin, lacticin 481, carnocin U149, lactocin S.

(II) Small heat stable, non-lanthionine containing membrane active peptides (<10 kDa), characterised by a Gly-Gly^{-1**+1}Xaa processing site in the bacteriocin precursor. The mature bacteriocins are predicted to form amphiphilic helices with varying amounts of hydrophobicity, β-sheet structure and moderate (100°C) to high (121°C) heat stability. Further subgroups can by defined within the class II bacteriocins:

(IIa) *Listeria*-active peptides with a consensus sequence in the N-terminal of –Tyr-Gly-Asn-Gly-Val-Xaa-Cys-; this group includes pediocin PA-1, curvacin A, sakacin A, leucocin A;

(IIb) Poration complexes consisting of two proteinaceous peptides for activity, e.g. lactococcin G, lactococcin M, lactacin F;

(IIc) Thiol-activated peptides, requiring cysteine residues for activity, e.g. lactococcin B;

(IId) A small group of bacteriocins that do not contain the YGNGVXC motif and contain only one or no cysteine residues, for example lactococcin A;

(IIe) These bacteriocins consist of two separate peptides that are either type E (enhancing), in which case one of the peptides that has antibacterial activity is enhanced by the other, e.g. thermophilin 13 and plantaricin S, or type S (synergistic), in which the peptides have little or no activity alone, e.g. lactococcin G and brochocin-C;

(IIf) Atypical class II bacteriocins: These are cyclic peptides and leaderless peptides, e.g. cidocin B and enterocin L50A.

(III) Large, heat-labile proteins (>30 kDa), e.g. helviticin V-1829, acidophilucin A, lactacin A.

(IV) Complex bacteriocins, composed of protein plus one or more chemical moieties (lipids, carbohydrates) required for activity, e.g. leuconocin S, lactocin 27, pediocin SJ-1 (Klaenhammer, 1993; De Vuyst and Vandamme, 1994a; Nes et al., 1996; Van Belkum and Stiles, 2000).

This study will focus mainly on the lantibiotic nisin and its natural variants, as well as pediocin PA-1 and leucocin B-Ta11a, both from the group IIa, non-lanthionine, pediocin-like, *Listeria*-active peptides. **Table 2.1** summarises the main characteristics of these bacteriocins. It has been shown that the sequence of pediocin PA-1 is identical to pediocin AcH (Klaenhammer, 1993; Abee et al., 1995; Miller et al., 1998) and that of leucocin A-Ta11a is identical to leucocin B-Ta11a and A-UAL 187 (Ennahar et al., 2000b).

Table 2.1 Producing organisms and properties of the bacteriocins nisin, pediocin PA-1 and leucocin A-Ta33a.

Producing Organism	***Inhibitory Spectrum***	***Inactivating Enzymes***	***pH Range (most active)***	***No. Amino Acids***
LANTIBIOTICS				
Nisin A				
Lactococcus lactis subsp. *lactis* ATCC 11454, 6F3	Gram-positive bacteria	α-chymo-trypsin Nisinase	2.0-7.0 (2)	34
NON-LANTIBIOTIC, CLASS IIA, SMALL, HEAT-STABLE BACTERIOCINS				
Pediocin PA-1				
Pediococcus acidilactici PAC-1.0	Pediococci Lactobacilli Leucoconstocs *List. monocytogenes*	Protease Papain α-chymo-trypsin	2.0-10.0 (4.0-7.0)	44
Leucocin A-UAL (identical to leucocin B-TA11a, A-TA33a)				
Leuconostoc gelidum UAL 187	Pediococci Lactobacilli Leucoconstocs *List. monocytogenes*	Protease Trypsin	2.3-3.0	37
Lc. carnosum TA11a				
Lc. mesenteroides TA33a				

2.4.2 Mode of action

The LAB bacteriocins share a common mechanism of action, and act primarily by making the membranes of susceptible microorganisms permeable and dissipating the proton motive force (PMF) (Bruno and Montville, 1993; Ennahar et al., 2000b). An important factor in determining susceptibility is presumably the lipid composition of the target cell membrane (Nissen-Meyer and Nes, 1997). The killing mechanism of bacteriocins is widely believed to involve an interaction with the cytoplasmic membrane (Friedrich et al., 2000). The cytoplasmic membrane forms the border between the cytoplasm and the external environment in both Gram-negative and Gram-positive bacteria. A layer of peptidoglycan surrounds the cytoplasm and is significantly thinner in Gram-negative bacteria than in Gram-positive bacteria. Gram-negative bacteria possess an additional layer, the outer membrane, which is composed of phospholipids, proteins and lipopolysaccharides (LPS) (**Fig. 2.2**). This layer is impermeable to most molecules; however, the presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da. Most bacteriocins larger than 3 kDa are too large to reach the target cytoplasm membrane (Stiles and Hastings, 1991; Klaenhammer, 1993; Abee et al., 1995).

No inhibition of Gram-negative bacteria has been demonstrated, unless the permeability barrier properties of the outer membrane have been changed by adding detergents (Abee et al., 1995). The outer membranes can be weakened or disrupted by treatment with ethylene diaminetetra-acetic acid disodium salt (EDTA) or by

osmotic shock (Stevens et al., 1992; Hansen, 1993; Ray 1994; Venema et al., 1995a; Parente et al., 1998; Boziaris and Adams, 1999). Cutter and Siragusa (1995) demonstrated that chelating agents, such as EDTA, EGTA, citrate and phosphate, made Gram-negative bacteria susceptible to the action of the bacteriocin nisin. Kalchayanand et al. (1992) showed that Gram-negative cells subjected to sub-lethal injury were also sensitive to bacteriocins, possibly by impairing the cell wall function. This observation of sensitivity can be explained by analysis and comparison of the composition of the Gram-positive and Gram-negative bacterial cell walls (Abee et al., 1995).

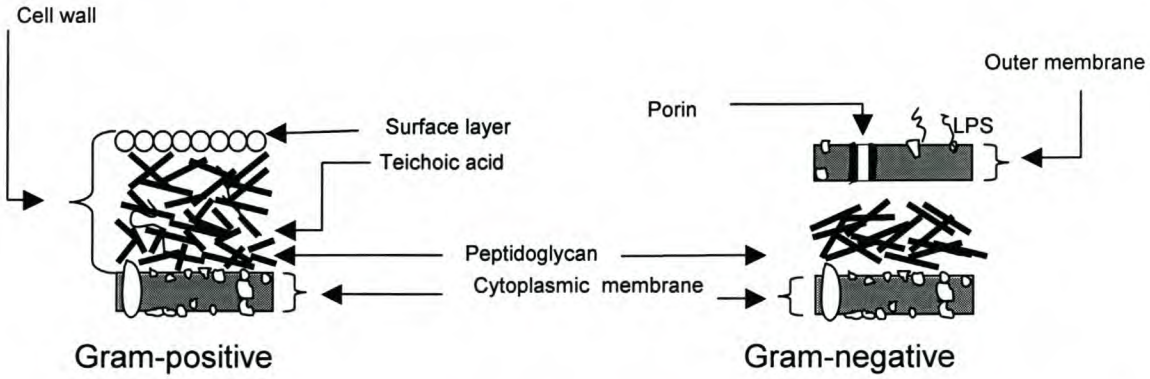

Figure 2.2 Schematic representation of the cell wall envelope of Gram-positive and Gram-negative bacteria. LPS=Lypopolysaccharide (Abee et al., 1995).

Dielbandhoesing et al. (1998) found that the cell wall of a yeast forms a barrier to various proteinaceous and non-proteinaceous molecules, and that nisin was inactive against intact cells.

2.4.2.1 Lantibiotics

The best-studied lantibiotic in this group is nisin and numerous studies have been done on the mode of action of nisin on both susceptible vegetative cells and spores. Nisin has been shown to act on energised membrane vesicles in Gram-positive bacteria. The primary antimicrobial activity of nisin is thought to result from the formation of pores in the target outer membrane (Driessen et al., 1995).

The "barrel-stave", "wedge" and "carpet" models provide mechanisms for pore formation. Research drawn largely from nisin and performed with highly-defined *in vitro* systems on target membranes, focuses on the interaction of the bacteriocin with the target membranes (Montville and Chen, 1998), and the evidence indicates that nisin, like other lantibiotics, forms unstable pores (Moll et al., 1999).

Class I lantibiotics may form pores according to a wedge-like model, while class II bacteriocins may cause membrane permeability through the formation of barrel-stave pores or by means of the carpet mechanism:

Wedge model Pore formation is caused by the disturbance of the local lipid bilayer that occurs when the nisin molecule binds. Nisin is then pulled into the membrane by a PMF component, either by the membrane potential ($\Delta\psi$, inside negative) or the pH gradient (ΔpH, alkaline inside). The orientation of the nisin molecule relative to the lipid head groups is not changed. The peptide has no contact with the hydrophobic core of the membrane. Instead, the hydrophobic residues of the nisin are inserted shallowly into the outer leaflet of the lipid bilayer (Montville and Chen, 1998, Moll et al., 1999).

Barrel-stave model Generally, the bacteriocins bind as monomers and insert into the lipid bilayer; the monomers then aggregate laterally to form pores. Studies show that the C-terminus of the molecule inserts into the membrane. Alternative studies suggest that the N-terminal inserts into the lipid phase, while the C-terminal part is responsible for binding to the target membrane (Montville and Chen, 1998, Moll et al., 1999).

Carpet mechanism Single peptide molecules might be oriented parallel to the membrane surface and interfere with the membrane bilayer organisation without forming a peptide aggregate. The membrane will collapse temporarily due to a strong phospholipid mobilising activity that co-operatively results in a local and transient permeability, once there are sufficient peptides in each other's vicinity (Moll et al., 1999).

The evidence supporting each of the models seems to contradict the others and reflects the complex nature of the nisin-membrane interactions, which are far from being understood (Montville and Chen, 1998).

The adsorption of nisin to the bacterial cell does not take place at specific bacteriocin receptors, but at anionic constituents of the negatively charged Gram-positive cell wall, most probably as a result of electrostatic or hydrophobic interactions (De Vuyst and Vandamme, 1994c; Abee et al., 1995). The PMF is the driving force for many vital, energy-demanding processes in the cytoplasmic membrane, notably the accumulation of ions and metabolites and the synthesis of ATP (Bruno and Montville, 1993; Montville and Chen, 1998). Membrane insertion, pore formation and simultaneous depolarisation induce a rapid and aspecific efflux of cytoplasm cell constituents of low molecular mass, thereby causing an ionic imbalance. These would include cations, such as potassium and hydrogen, amino acids, and nucleotides such as ATP and ADP (De Vuyst and Vandamme, 1994c). A further inhibition of the uptake of amino acids will also take place. Such disruptions lead to the dissipation of the PMF, which involves the partial or total dissipation of the $\Delta\psi$ and the ΔpH (Ennahar et al., 2000b; Friedrich et al., 2000). Nisin can also functionally insert into membranes in the presence of a pH gradient (alkaline inside). In severe cases, nisin has been shown to cause the lysis of susceptible cells (Delves-Broughton, 1990a, b; Jung, 1991b; Bruno et al., 1992; De Vuyst and Vandamme, 1994c; Abee et al., 1995; Montville and Chen, 1998; Ennahar et al., 2000b).

Membrane insertion and pore formation by the highly charged lantibiotics requires the existence of a transmembrane potential. The lantibiotic killing is less dramatic with de-energised cells, as well as with stationary-phase cells compared to log-phase cells, and no insertion takes place with non-energised liposomes (De Vuyst and Vandamme, 1994a; Abee et al., 1995). The potential must have the proper trans-negative orientation (negative inside), with sufficient magnitude. The insertion of cationic peptides is voltage-dependent, and thus is the rate-limiting step.

There is less information on the action of bacteriocins against spores than for vegetative cells; most studies deal with nisin, which is sporostatic, rather than sporocidal. Nisin inhibits the step between botulinal spore germination and pre-emergent swelling. *Bacillus* spore coats that are opened by mechanical pressure rather than lysis are more sensitive to nisin action. Nisin modifies the sulphydril groups in the envelopes of the germinated spores (Montville et al., 1995). *Clostridium botulinum* is relatively nisin insensitive (Hurst, 1972), but the sensitivity

between strains varies notably (Montville et al., 1995). It has been shown that spores damaged by heat are more sensitive to nisin (Delves-Broughton, 1990a, b). The heat-shock temperature and duration, pH of the medium and spore load all have profound effects on the effectivity of nisin (De Vuyst and Vandamme, 1994a).

2.4.2.2 Class IIa bacteriocins

The pediocin-like bacteriocins are characterised by a YGNGV motif and a disulphide bridge in a highly conserved N-terminal region, by high antilisterial activity and by their membrane permeabilisation mode of action (Eijinsk et al., 1998; Ennahar et al., 1999). **Figure 2.3** gives a schematic representation of the structure of a model class IIa bacteriocin. Some bacteriocins, such as pediocin PA-1, also contain a disulphide bridge in the C-terminal region, while others do not (Johnsen et al., 2000). The highly conserved N-terminal region is hydrophilic and cationic. This region possibly mediates the initial binding, through the electrostatic interactions of the bacteriocins, to the target cells (Chen et al., 1997). The C-terminal is somewhat less conserved and is hydrophobic and/or amphiphilic. It is thought that this section penetrates into the hydrophobic part of the target cell membrane, thereby mediating membrane leakage (Fimland et al., 1998; Johnsen et al., 2000).

Like other LAB bacteriocins, class IIa act primarily by permeabilising the membranes of susceptible organisms, probably by the formation of poration complexes, causing an ionic imbalance and leakage of inorganic phosphates (Bruno and Montville, 1993; Chikindas et al., 1993; Klaenhammer, 1993; Bhunia et al., 1994; Abee, 1995; Jack et al., 1995). This has been demonstrated convincingly for pediocin PA-1 (Chikindas et al., 1993), mesentericin Y105 and bavaricin MN (Ennahar et al., 2000a). The best-studied bacteriocin in this group is pediocin PA-1, which is identical to pediocin AcH (Klaenhammer, 1993; Abee et al., 1995; Miller et al., 1998).

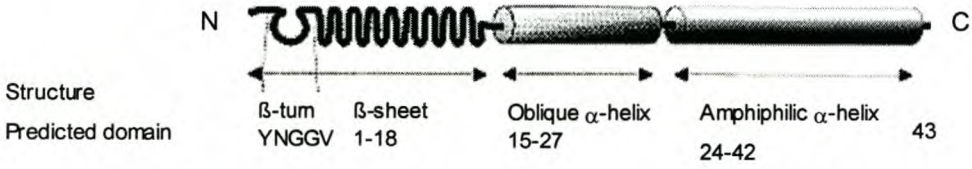

Figure 2.3 Schematic presentation of the structure of a model class IIa bacteriocin and the predicted location of its domains with respect to the target cell membrane (Ennahar et al., 2000b).

The above-mentioned disruptions lead to the dissipation of the PMF, with the partial or total dissipation of either or both the $\Delta\psi$ and the ΔpH. This is unlike the lantibiotics, in which a total dissipation of both the $\Delta\psi$ and ΔpH takes place (Montville and Chen, 1998). Class IIa bacteriocins readily provoke a total dissipation of ΔpH, but only a partial dissipation of $\Delta\psi$ (Bhunia et al., 1991, Bruno and Montville, 1993; Chikindas et al., 1993). The newly-discovered mundticin is the only bacteriocin that causes a complete dissipation of $\Delta\psi$ (Bennik et al., 1997).

The class IIa bacteriocins may form poration complexes following the barrel-stave model, as a result of the amphiphilic segments that are putative transmembrane helices, their water solubility and membrane-binding ability (Ennahar et al., 2000b). The initial step of the interaction with the membrane is generally

believed to be by electrostatic binding, mediated by a putative membrane-bound, receptor-type molecule (Chikindas et al., 1993; Abee et al., 1995; Venema et al, 1995b). Earlier findings, however, suggested a protein receptor-mediated activity (Bruno and Montville, 1993; Chikindas et al., 1993). Recent studies investigating the effect of class IIa bacteriocins indicated that protein "receptors" may not be an absolute requirement for pore formation. Another possibility is that the functional binding of the positively charged and polar residues occurs primarily in conjunction with anionic phospholipid head groups in the membrane. It is possible that all class IIa bacteriocins rely in part on the same type of functional binding, due to their cationic nature and the high structural similarity of the hydrophilic N-terminal half (Ennahar et al., 2000b).

The lethal activity of class IIa bacteriocins therefore is ascribed mainly to the dissipation of the PMF (Abee, 1995; Jack et al., 1995; Venema et al., 1995b). Bruno and Montville (1993) demonstrated that the bacteriocin, pediocin PA-1, acts in an energy-dependent fashion. In particular, the ATP is depleted and the uptake of amino acids mediated by active transport is blocked. A leakage of pre-accumulated amino acids furthermore has been reported for pediocin PA-1 and mesentericin (Ennahar et al., 2000b). The efflux of amino acids may occur through diffusion through the pores, probably in combination with reflux via the PMF transport system (Montville and Chen, 1998). The very rapid efflux of amino acids in the case of mesentericin Y105 suggests that it also may occur through simple leakage (Chikindas et al., 1993). No leakage of ATP seems to occur with class IIa bacteriocins, unlike the lantibiotics, possibly due to the smaller pore sizes formed in the case of the class IIa bacteriocins. The intracellular depletion of ATP in the cells might stem from an accelerated consumption of ATP to maintain or restore the PMF and/or the inability of the cell to produce ATP, due to an efflux of phosphate (Ennahar et al., 2000b). While *in vitro* models for the insertion into the lipid bilayer are known for the lantibiotic nisin (Montville and Chen, 1998), it was only recently that a number of models were introduced for the possible orientation of class IIa bacteriocins into lipid bilayers (Bhugaloo-Vial et al., 1996; Chen et al., 1997). Class IIa bacteriocins are believed to insert into the target membrane via their hydrophobic and/or amphiphilic C-terminal domain, and aggregate to form water-filled pores (Bhugaloo-Vial et al., 1996; Chen et al., 1997). Refer to **Figure 2.4** for a schematic representation of the mode of action.

Bacteriocin-induced cell death has been shown to occur in a concentration and time-dependent fashion and is further influenced by other factors, related either to the target cell or to the medium it functions in.

Nisin does not require a protein receptor for pore formation, since the bacteriocin dissipates the PMF and causes efflux from the lipid vessels, which lack membrane proteins. The necessity for a protein receptor for class IIa bacteriocin action seems unlikely to be an absolute requirement (Montville and Chen, 1998).

There seems to be no universal case for the influence of pH within the spectrum of bacteriocins researched. This may be an advantage in food preservation, for example, during which pediocin PA-1 and an acidic pH may work synergistically against target microorganisms (Montville and Chen, 1998).

The interactions of the cationic nisin molecules with anionic phospholipids contribute to the binding of nisin to the membranes (Abee, 1995). The activity of nisin can be reduced significantly by di- and trivalent cations, and can even be prevented

by gadolinium (Gd^{3+}), a lanthanide that is known to inhibit various channels in eukaryotic and prokaryotic cells (Abee et al., 1994). Altering the charge properties of either the bacteriocin, by changing the medium pH, or the membrane, by changing its lipid composition, influences this adhesion by affecting the dissociation of peptide-lipid interactions (Ennahar et al., 2000b).

Nisin has a lower activity at temperatures below 7°C (Abee et al., 1994). This is presumably because increased ordering of the lipid hydrocarbon chains in the cytoplasm membrane inhibits nisin insertion (Venema et al., 1995a).

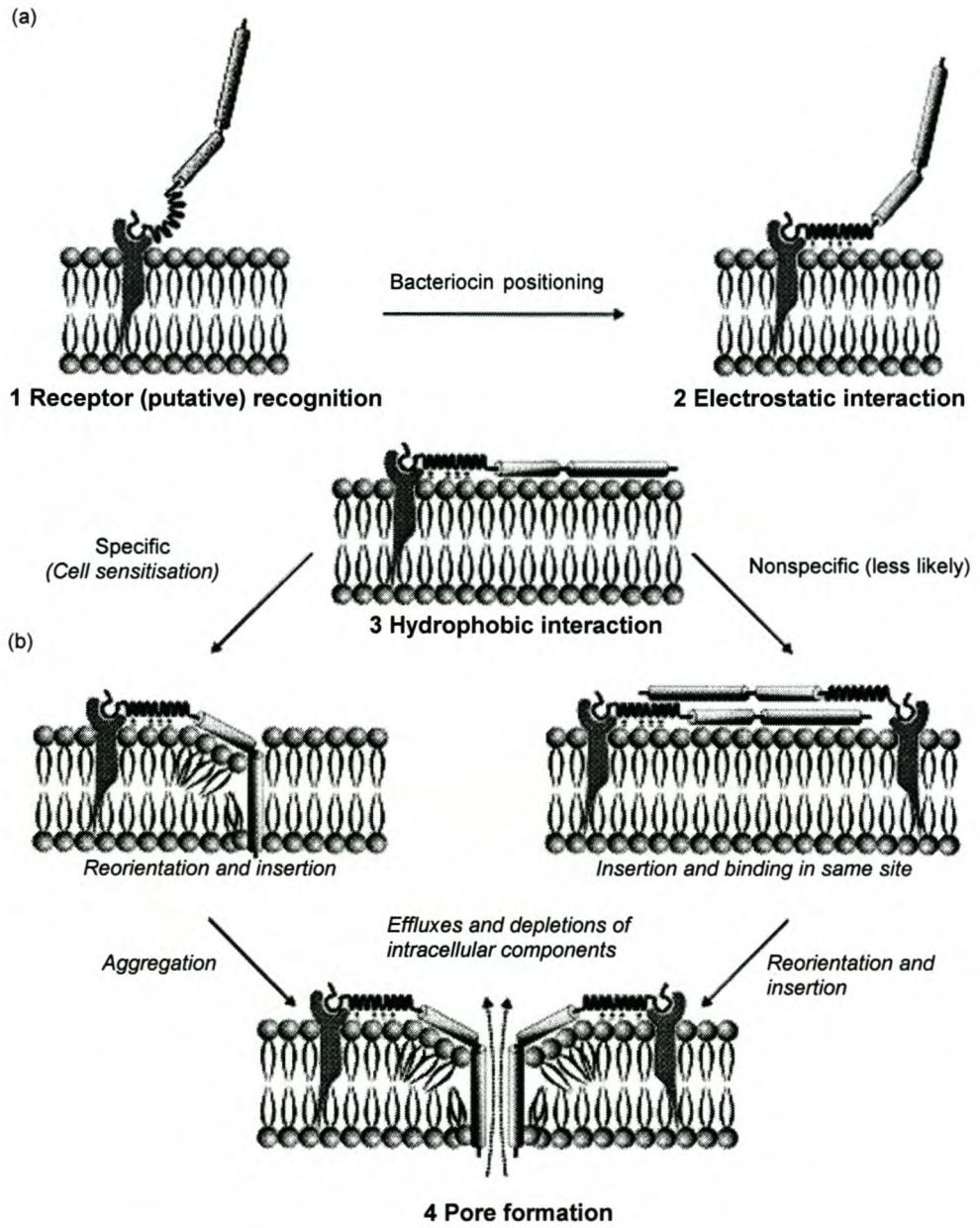

Figure 2.4 (a) Possible interactions of each domain with the membrane surface; (b) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and the hydrophilic face is shaded light (Ennahar et al., 2000b).

2.4.3 Immunity and resistance

The cells of a producer strain are immune to their own bacteriocin due to the presence of a specific immunity gene and the production of an immunity protein. Non-producing variants can be insensitive to a bacteriocin due to non-specific reasons (Noerlis and Ray, 1994). This resistance could be due to the inability of a bacteriocin to pass through the cell wall, which is a requirement in the case of bacteriocins such as pediocin (Ray, 1992b). In addition, *P. acidilactici* cells have intracellular proteases that can hydrolyse and inactivate pediocin. The mechanisms by which many other Gram-positive bacterial strains are resistant to this bacteriocin are unknown (Noerlis and Ray, 1994). It has been shown that the genes for both production and immunity are located close to each other in several strains of lactic acid bacteria (Hastings and Stiles, 1991). Resistant variants can be developed when grown in the presence of bacteriocins (Modi et al., 2000), but can revert to sensitivity when grown in the absence of the bacteriocin. This observation has important implications when bacteriocins are considered for use in food preservation (Noerlis and Ray, 1994).

2.4.4 Purification of bacteriocins

The purification of bacteriocins has proven to be a difficult task – especially those produced by lactic acid bacteria. Usually such problems are related to the tendency of the molecules to associate with other molecular substances (such as lipids, glucose and so forth), their hydrophobicity, as well as their being a very heterogeneous group of substances. This then usually calls for the design of a protocol for a specific bacteriocin (De Vuyst and Vandamme, 1994b).

2.4.4.1 Factors influencing bacteriocin purification

There are a number of factors that influence the production of bacteriocins by LAB and these should be considered before the initiation of the purification process:

2.4.4.1.1 *Growth and bacteriocin production*

The production of bacteriocins by lactic acid bacteria is dependent on the growth and physiological activity of the producing species, as the amount of the bacteriocin produced correlates with the quantity of biomass produced. Almost all bacteriocins produced by lactic acid bacteria display primary metabolite kinetics (De Vuyst and Vandamme, 1994a).

2.4.4.1.2 *Media*

The media used also plays a major role and production is affected greatly by the type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and inhibitors. Bacteriocins can be produced from media containing different carbohydrate sources, such as glucose, sucrose, galactose and xylose. Organic nitrogen sources often limit the growth and bacteriocin production. It has been found that nisin concentrations increased with an increase in organic nitrogen content (Parente and Ricciardi, 1999). The type of nitrogen, for example casamino acids, phytone peptone, soy peptone, casein hydrolysate and fish meal, also affects bacteriocin production (De Vuyst and Vandamme, 1993, 1994a). Anions (phosphate) and cations (Mg^{2+} and Ca^{2+}) affect bacteriocin production positively or negatively, but the effect is restricted to specific strains. Tween 80 has been found to stimulate the

production of some bacteriocins (Daba et al., 1993). However, this could be ascribed to the fact that Tween 80 prevents the adsorption of these bacteriocins onto polypropylene and glass surfaces, increasing the apparent bacteriocin titres (Joosten and Nuñez, 1995). Tween 80 could possibly interfere with the production of some bacteriocins, as well as affect the purification process (Carolissen-Mackay et al., 1997). Complex growth media, such as MRS (de **M**an, **R**ogosa, **S**harpe) broth, contain significant amounts of peptides in the molecular weight range of most bacteriocins and these may interfere with the purification process (Muriana and Klaenhammer, 1991). De Vuyst et al. (1996a) found that by preparing MRS from basic components, but autoclaving the glucose separately and adding it to the remaining media after it had cooled down, led to increased bacteriocin production.

2.4.4.1.3 *Fermentation condition effects*

The fermentation conditions should also be monitored. pH control improves the growth of LAB, resulting in improved bacteriocin production (Yang and Ray, 1994), with the optimal pH usually being from 5.5-6.0. This, however, is not the optimum growth pH for many LAB (Parente and Ricciardi, 1999). Pediocin AcH production was found to be minimal when the pH of the growth medium was maintained at 5.0 or above, even in the case of high biomass (Biswas et al., 1991). Optimal temperature conditions will usually result in optimal bacteriocin production (Daba et al., 1993). Aeration and agitation will also affect production levels, depending on the bacteriocin-producing LAB (De Vuyst et al., 1996a; Parente and Ricciardi, 1999).

2.4.4.1.4 *Fermentors*

Continuous fermentations allow high growth rates to be reached, and production can be improved using this method. One of the methods used was the immobilisation of cells in calcium alginate beads for continuous bacteriocin production, as described by Wan et al. (1995), Bhugaloo-Vial et al. (1997) and Scannell et al. (2000). Cho et al. (1996) investigated the continuous production of pediocin by immobilised *P. acidilactici* PO2 in a packed-bed bioreactor. The optimum conditions for production were researched and implemented, and the bioreactor operated efficiently for a number of months without major upkeep, indicating an easy, efficient way of producing large quantities of bacteriocin. Huang et al. (1996) investigated the use of free cell and immobilised cell fermentations. Although the free cell fermentations' production decreased with time, the production from immobilised cell fermentations stabilised with time. These methods all describe stable bacteriocin-producing systems, the set-up allowing long-term means of producing suitable bacteriocins.

2.4.4.2 Affinity purification

The cationic and hydrophobic nature of bacteriocins is used for recovery from complex fermentation broths that contain high levels of peptides (Parente and Ricciardi, 1999). A general scheme for the purification of low molecular mass bacteriocins of lactic acid bacteria has been developed.

This protocol involves four general steps (Muriana and Klaenhammer, 1991):

(i) ammonium sulphate precipitation
(ii) cation exchange chromatography
(iii) hydrophobic interaction chromatography
(iv) reverse-phase high-performance liquid chromatography

After the first step of ammonium sulphate precipitation, a lipid-like floating material can often be observed. Most of the activity is contained in the hydrophobic lipid-protein material (De Vuyst and Vandamme, 1994a). This method may produce good results in terms of yield and purification, but is unsuitable for large-scale bacteriocin recovery and purification, as it is expensive and labour intensive (Parente and Ricciardi, 1999). This method has been used extensively in the purification of a number of bacteriocins, such as curvacin A, sakacin P, lactocin S and bavaracin A (Tichaczek et al., 1992; Carolissen-Mackay et al., 1997). Ammonium sulphate precipitation, followed by gel permeation chromatography, was used to purifiy helviticin J and lacticin F (Joerger and Klaenhammer, 1986; Muriana and Klaenhammer, 1991). Ion-exchange, ultrafiltration and gel permeation chromatography were used to purify lactacin B (Barefoot and Klaenhammer, 1984).

2.4.4.3 Adsorption/desorption

Several other protocols, suitable for large-scale recovery and purification, have been developed and are based on the adsorption/desorption of the bacteriocin to the producing cell or by phase partitioning. Bacteriocins can be recovered by adsorption to producer cells at pH 6.0-6.5, followed by cell separation and desorption at pH 2.0. This method was suitable for some strains and bacteriocins, such as pediocin AcH, nisin, sakacin A and leucocin Lcm1, but was limited for others. The yield from the isolation procedure was found to be higher than methods relying on ammonium sulphate or solvent precipitation, while also being suitable for large-scale extraction (Yang et al., 1992; Daba et al., 1994).

De Vuyst et al. (1996a) developed an additional procedure that seems to be promising for large-scale bacteriocin isolation - expanded bed adsorption. A large column packed with a strong cation exchanger was used to purify amylovorin L471. This method is unique in that it does not require centrifugation to remove cells or cell debris. Complex media can be used and it is not necessary to harvest floating pellicles, which can be difficult. The cost of the equipment is quite low, the process shortened because of the possibility of a continuous operation and the adsorbents can be recycled. A disadvantage of this process, however, is the large volumes of buffers or solvents used to equilibrate and wash the columns, as well as for eluting the bioactive material from the adsorbents. The bioactive material can be spray-dried immediately for use as a biopreservative. This procedure may be applied for the purification of almost all bacteriocins.

2.4.4.4 Exchange chromatography

These protocols can be inefficient, with only partial extraction, when the bacteriocin concentration is very high and the cells' ability to absorb the bacteriocins is exceeded (Parente and Ricciardi, 1999). Bacteriocins can also be adsorbed on cation exchange resins. Wan et al. (1996) used ingestible porous silica compounds to adsorb the bacteriocins nisin, pediocin PO2, brevicin 28 and piscicolin 126 from fermentation broths. This purification method gave 110 to 130-fold purification. Adsorbed bacteriocins were desorbed with sodium dodecyl sulphate (SDS). However, the removal of the SDS was only partial and higher levels of desorption were achieved by repeated elution or an increase in surfactant concentration (Coventry et al., 1996).

Métivier et al., (2000) used a combination of Triton X-114 phase partitioning and cation exchange chromatography for divercin V41 isolation from a complex medium. This method is fast and versatile and can be carried out on whole broth. A rapid three-step purification method for mesentericin Y105 was developed by Guyonnet et al. (2000). An overnight culture is half-diluted with water and applied to a cation exchange column (carboxy-methyl-cellulose-filled column). The active fractions are applied to a C_{18} cartridge. The active sample was then concentrated on a HPLC column, resulting in a 60% yield.

A simple and rapid extraction method was developed for the extraction of nisin, pediocin RS2, leucocin BC2, lactocin GI3 and enterocin CS1 from crude broth culture supernatants by adsorption onto acid or alkaline rice hull ash or silicic acid. The bacteriocins were adsorbed onto the compounds by a pH-dependent method and desorbed by decreasing the pH to 2.5 or 3.0 and heating to 90°C for 5 minutes. The amount of bacteriocin can be altered by adjusting the concentration of the adsorbent (Janes et al., 1998).

2.4.4.5 Solvent extraction

Another method used to purify the bacteriocin, amylovorin L471, involves precipitating a cell-free supernatant at pH 6.5 with ammonium sulphate, followed by centrifugation. The surface pellicle is then suspended in 50 mM sodium phosphate buffer, and further treated with at least 15-25 volumes of a chloroform/methanol (2:1, v/v) mixture for 1-2 hours at 4°C without stirring. The resulting fine-grained precipitate is collected by centrifugation, air-dried and dissolved in a minimal amount of buffer. The sample is then stored at -20°C. This method is also suitable for large-scale production and purification, especially when combined with expanded bed adsorption, resulting in a high bacteriocin yield with a low loss of activity during the purification procedure (De Vuyst et al., 1996b; Callewaert et al, 1999). Contreras et al. (1997) used a modified version of this method for lactobin A. The fermentation broth was precipitated with ammonium sulphate, as mentioned above, and further treated by adding two volumes of ethanol-diethyl ether mixture (1:2, v/v). The sample was centrifuged, the water phase was evaporated, and the organic phase resuspended in ultrapure water. The sample was further purified with chloroform/methanol (25 volumes; 2:1, v/v, 4°C with stirring for 1 hour).

There is another general method for purifying LAB bacteriocins. The bacteriocin is purified first by an ethanol (cold) precipitation step, followed by secondary preparative pI-electrophoreses. This method has been used to purify lactococcin A, lactococcin B and pediocin PA-1 (De Vuyst and Vandamme, 1994a). Burianek and Yousef (2000) used another solvent extraction method to concentrate lacidin from the fermentation broth. The bacteriocin is concentrated at the interface between chloroform and the aqueous culture of the producing bacterium. A higher bacteriocin yield was reported when compared to the ammonium sulphate precipitation and cell acidification methods, along with relatively clean preparations. The method was also quick and easy to perform.

Hastings and Stiles (1991) developed a purification scheme using only low pH precipitation, gel filtration and hydrophobic interactions. Ion-exchange chromatography, dialysis and high pH conditions were avoided, because they resulted in a high loss of activity.

2.4.5 Nisin

Nisin was first detected in the late 1920s and early 1930s, when it was described as a toxic substance that adversely affected the performance of cheese starter cultures (Delves-Broughton et al., 1996). Rogers and Whittier (1928) and Rogers (1928) were the first investigators to show that some lactococcal strains can inhibit the growth of other lactic acid bacteria. At the time phage infection of the starter cultures was the main cause of failure in cheese production and no further attention was paid to the matter. The inhibitory compound isolated by Meanwell (1943) was concentrated and was found to have inhibitory activity against several pathogenic bacteria and thus called it an antibiotic at that point in time. The name nisin was derived from Group N Inhibitory Substance (De Vuyst and Vandamme, 1994c).

2.4.5.1 Characteristics

2.4.5.1.1 *Physical and chemical properties*

Nisin is a bacteriocin produced by certain strains of *Lactococcus lactis,* subsp. *lactis*. The strains naturally produce different variants, including nisin A, B, C, D and Z (Mulders et al., 1991; De Vuyst and Vandamme, 1994c). The complete structure of the nisin molecule was elucidated by Gross and Morell (1971). The characteristics of nisin are summarised in **Table 2.1**. The peptide behaves like a cationic polypeptide, with a positive charge of 3 (2 for nisin Z) – thus having an isoelectric point in the alkaline range (Jung, 1991a, b).

The nisin molecule is acidic in nature and exhibits greatest stability under acidic conditions. The solubility, stability and biological activity of nisin are highly dependent on the pH of the solution, with the molecule becoming more susceptible to the effect of heat with a concomitant increase in the pH from 3 to 7 (Delves-Broughton, 1990a, b). As the pH is increased, the above activities drop sharply and steadily. At pH 2.0 the solubility of nisin is 57 mg/mL, decreasing to 1.5 mg/mL at pH 6.0, and it is practically insoluble in neutral and alkaline conditions. In diluted HCl solutions with a pH of 2.5 or less, solutions can be boiled without any loss of activity, and even autoclaving does not cause serious loss of activity (Davies et al., 1998). Above pH 7, irreversible inactivation occurs even at room temperature. Large molecules, such as in milk or broth, have a protective effect, so that the degree of inactivation may be less drastic in foods than in a buffer solution alone (Hurst, 1981; De Vuyst and Vandamme, 1994c).

2.4.5.1.2 *Biochemical properties*

Nisin is inactivated by some, but not all, of the proteolytic digestive enzymes. Pancreatin, subtilopeptidase, proteinase K and α-chymotrypsin inactivate nisin, and the occurrence of a nisinase was reported in a number of nisin resistant lactic acid bacteria. The nisinases appear to be quite specific and could be used for identification of the substance in food (Hurst, 1981; De Vuyst and Vandamme, 1994c).

2.4.5.2 Toxicology

There appears to be general consensus that nisin is non-toxic when eaten, as it is proteinaceous and the digestive system degrades the peptide to its constitutive amino acids (Hansen, 1993). Nisin is produced by lactococci that occur naturally in raw milk supplies. This indicates that it is harmless by nature by virtue of its intake by

humans over the past centuries. Nisin therefore has been consumed by people in their normal life spans without apparent ill effects (Hurst, 1981; Delves-Broughton, 1990a, b). Claypool et al. (1966) found that not only did stomach enzymes rapidly degrade nisin, but it could also not be detected in human saliva 10 minutes after consuming liquid containing nisin. This would indicate that the oral bacterial population would not be affected by the consumption of nisin. No sensitisation to nisin has been found in humans. Extensive microbiological studies have not shown any cross resistance to other microorganisms that might affect the therapeutic use of antibiotics (Delves-Broughton, 1990a, b).

2.4.5.3 Inhibition spectrum

Nisin, like other bacteriocins, possesses antimicrobial activity against a limited range of microorganisms. It does not inhibit Gram-negative bacteria, yeasts or fungi (Hurst, 1981). **Table 2.1** summarises the sensitive organisms. It does, however, inhibit a wide range of Gram-positive bacteria, lactococci being the most sensitive, and bacteria that produce spores (Delves-Broughton et al., 1996). Furthermore, it also inhibits corynebacteria, lactobacilli, leuconostocs, micrococci, pediococci, pneumococci, streptococci and actinomycetes, as well as certain strains of *Mycobacterium tuberculosis, Erysipelothrix rhusiopathiae* and *Listeria monocytogenes* (Ogden and Tubb, 1985; Delves-Broughton, 1990a, b; Radler, 1990a; De Vuyst and Vandamme, 1994c). The majority of spore-forming species of *Clostridium* and *Bacillus* are also inhibited, with the spores being more sensitive than the vegetative cells (Campbell and O'Brien, 1955; Delves-Broughton, 1990b). Nisin sensitivity to both vegetative cells and spores can vary between genera and even between strains of the same species (Delves-Broughton et al., 1996). The fact that yeasts are insensitive to nisin indicates that nisin can be used in fermentations alongside yeasts to control the growth of lactic acid bacteria (Delves-Broughton et al., 1996).

2.4.5.4 Applications as a biopreservative

Nisin was first used as a commercial food preservative in the UK approximately 35 years ago. The initial use was as a preservative in processed cheese products, but many other applications have been identified since then (De Vuyst and Vandamme, 1994c; Delves-Broughton et al., 1996). Its use as a safe food preservative has since been authorised in at least 48 countries and it was awarded FDA approval in 1998 (Ross et al., 1999). Nisin has several advantages as a preservative, as it can be used in a spectrum of foods and does not inhibit the growth of Gram-negative intestinal flora (Kaletta and Entain, 1989).

2.4.5.4.1 *Dairy*

The greatest potential for nisin as a preservative seems to be in the area of dairy technology: in dairy products, nitrate is commonly added to cheese milk to prevent clostrial spore outgrowth (Abee et al., 1995). Nisin can easily replace nitrate as preservative and has been used in the preservation of processed cheese and cheese spreads, the levels used in the raw cheese depending on the bacterial and spore loads of *Clostridium butyricum*, *C. tyrobutyricum*, *C. sporogenes* and the toxin-forming *C. botulinum* organisms (Hurst, 1981; Delves-Broughton, 1990a, b; Delves-Broughton et al., 1996). Hard and semi-hard cheeses are protected from "blowing"

caused by the outgrowth of *C. butyricum* and *C. tyrobutryricum,* which produce hydrogen gas and carbon dioxide as part of their lactic acid metabolism, by nisin addition. Protective starter cultures that produce bacteriocins can also be used for *in situ* bacteriocin production, instead of adding bacteriocins to the cheese milk. Nisin has also found an application in reconstituted and recombined milk products, as well as in heat-processed milk and milk drinks, as they are only 'commercially sterile' and often contain heat-resistant spores.

Another successful application among dairy products has been in the extension of the shelf-life of dairy desserts. The products usually undergo taste, appearance or texture modifications when subjected to full heat sterilisation (Delves-Broughton et al., 1996).

2.4.5.4.2 *Canned products*

Nisin can be used in combination with heat treatment to prevent the spoilage of high or low acid canned foods by thermophilic spore-forming bacteria, such as *Clostridium botulinum*, *Clostridium thermosaccharolyticum* and *Bacillus stearothermophilus* or aciduric Gram-positive bacteria, such as *Clostridium pasteurianum*, and *Bacillus macerans*. The use of nisin will reduce the heat processing intensity, thus resulting in canned foods with improved nutritional value, appearance and texture, and a concomitant reduction in energy consumption of the process (Hurst, 1981; De Vuyst and Vandamme, 1994c; Delves-Broughton et al., 1996).

2.4.5.4.3 *Meat products*

The toxicological safety of nitrite in cured meat has raised some questions relating to safety, as it forms carcinogenic N-nitrosamines (Abee et al., 1995). This has led various researchers to consider alternative preservative systems, which include nisin (Delves-Broughton, 1990a, b). Reports regarding the usefulness of nisin in meat preservation seem to be contradictory (Hurst, 1981). *List. monocytogenes* is a food-borne pathogen associated with meat products and it may be present in many meat-processing plants. Nisin is active against this bacterium and would be effective in inhibiting its growth (Abee et al., 1995). The application of nisin in meat products has been limited, because of antagonising effects such as low solubility, uneven distribution, heat sensitivity at neutral pH values, possible binding to meat proteins, lack of stability, etc. (Delves-Broughton, 1990a; De Vuyst and Vandamme, 1994c). The spoilage organisms in meat products are generally LAB, which should be susceptible to nisin (Hurst, 1981). The use of nisin has been quite successful in the preservation of Italian delicatessen sausages and similar products (Hurst, 1981). As nisin is expensive, the levels of nisin required for preservation generally would be uneconomical (Delves-Broughton et al., 1996).

In the case of fish products, few attempts have been made to evaluate the potential of nisin, as the deterioration of fish at chill temperatures is caused primarily by Gram-negative bacteria (De Vuyst and Vandamme, 1994c).

2.4.5.4.4 *Alcoholic beverages*

Research in the UK and Germany has shown that nisin has potential for controlling spoilage LAB in wine and beer. Beer is a hostile environment for bacterial growth, as it has a low pH and contains little fermentable sugar, but contains alcohol and hop substances and lacks oxygen. The spoilage of beer is limited to a few species of bacteria, such as LAB. Beer containing contaminating bacteria show excess turbidity

and acidity and have off-flavours (Ogden et al., 1988). The use of bacteriocins in a pure form is not possible in beers made under the beer "purity law". Some of the LAB used in the acidification of beer have been found to produce bacteriocins. This may be exploited by selecting cultures that produce bacteriocins for the acidification of the wort (Idler and Annemüller, 2000). Further applications have been identified for the brewing industry and include adding bacteriocins to fermentors to prevent or control contamination; washing pitching yeast to eliminate contaminating bacteria, as an alternative to acid washing, which could negatively affect yeast viability; the reduction of pasteurisation regimens; and increasing the shelf-life of unpasteurised cask or bottle-conditioned beers (Ogden, 1987, Ogden et al., 1988). Nisin is quite stable when autoclaved and heated and would be able to withstand high processing temperatures (Davies et al., 1998). Similar applications exist in the wine industry.

The use of nisin in beer would be ideal, as it survives post-fermentation treatments, such as filtration and fining (Delves-Broughton, 1990a), without affecting the taste of the beer (Ogden, 1986).

In wine, the malolactic fermentation is deemed to be favourable in most red wines and unfavourable in fruitier white wines. SO_2 is widely used in the preservation of wines, but it is regarded as good practice to minimise its use. The addition of nisin after the MLF will protect the wine against later spoilage by any remaining LAB (Radler, 1990a, b).

Nisin could also have an application in the fermentation industry dealing with the production of fruit brandies. These are naturally mixed fermentations contaminated by LAB, which compete with yeasts for substrate. The alcohol content in the distillate could rise by more than 10% if nisin is used in the fermentation mash, providing the yeast with less competition (Delves-Broughton, 1990a). LAB isolated from wine were successfully inhibited by nisin without affecting the yeasts performing the alcoholic fermentation at an experimental scale level. When nisin was used as a preservative against LAB in small-scale fermentations, no influence was detected on the sensory quality of the wine (Radler, 1990a, b).

2.4.5.4.5 *Other*

Another application is in high moisture hot plate bakery products: Products such as crumpets can benefit from the addition of nisin as a preservative to the batter, inhibiting food poisoning organisms such as the spore-forming *B. cereus* (Delves-Broughton et al., 1996).

Pasteurised liquid egg products receive a heat treatment to destroy *Salmonella*. However, such heat treatments will not destroy spores and some Gram-negative or Gram-positive bacteria. The addition of nisin will significantly increase the shelf-life of these products (Delves-Broughton et al., 1996).

In salad dressings with a higher pH, the growth of spoilage LAB could be inhibited successfully by the addition of nisin (Delves-Broughton et al., 1996).

Important industrial fermentations carried out by Gram-negative bacteria, yeasts or fungi, such as the production of single cell proteins, organic acids, polysaccharides, amino acids and vitamins, can benefit from nisin use to prevent contamination by Gram-positive bacteria (Delves-Broughton, 1990a, b).

2.4.6 Pediocin

2.4.6.1 Characteristics

Bhunia et al. (1987) first reported the isolation of bacteriocin-producing strains of *Pediococcus acidilactici*, that inhibited the growth of strains of other LAB, from fermented sausages. The bacteriocin from *P. acidilactici* strain H was designated pediocin AcH. **Table 2.1** summarises pediocin AcH characteristics.

2.4.6.1.1 *Physical and chemical properties*

Heating pediocin to 121°C led to the partial destruction of its activity. The activity was found to be most stable from pH 4 to 7, with some loss at pH 2, 3, 9 and 10. Most of the activity was lost at pH 11 (Gonzalez and Kunka, 1987). Similar results were reported for Pediocin AcH (Bhunia et al., 1987). The amino acid sequences of pediocin AcH and pediocin PA-1 were compared, and it was found that the two pediocins were the same (Henderson et al., 1992; Motlagh et al., 1992; Klaenhammer, 1993; Abee et al., 1995; Martínez et al., 1997; Miller et al., 1998).

It was indicated that the pediocins acted rapidly against sensitive cells and had a bactericidal action (Gonzales and Kunka, 1987; Bhunia et al., 1988). In the group IIa bacteriocins, pediocin-like bacteriocins are the largest and also most extensively studied sub-group (Ennahar et al., 1999). Pediocin PA-1 is a class IIa bacteriocin, consisting of 44 amino acids with a molecular mass of 4623 Da and a net charge of +6, and has two disulphide bonds. This might account for its broad range of antimicrobial activity (Montville and Chen, 1998). The antimicrobial range of class II bacteriocins seems to relate to their disulphide bond content (Jack et al., 1995). Pediocin PA-1 is the only known class II bacteriocin with two disulphide bonds and it has the broadest range of activity. The defined structural elements, especially the second disulphide bond formed after pediocin PA-1 binds to the membrane lipids, may play an important role (Montville and Chen, 1998). A *Lactobacillus plantarum* strain isolated from cheese was found to produce pediocin AcH. This was the first reported case of the same bacteriocin being produced naturally by different genera (Ennahar et al., 1996). Several reports associate the production of bacteriocins by pediococci with plasmid DNA (Marugg et al., 1992).

2.4.6.1.2 *Biochemical properties*

Pediocin PA-1 was tested for sensitivity to various enzymes, heat and pH. The bacteriocin was deemed sensitive to protease, papain and α-chymotrypsin. No effect was observed with lipase, phospholipase C, lysozyme, DNase or RNase, or when it was heated to 80 and 100°C (Ray, 1994).

2.4.6.2 Inhibition spectrum

Pediocins from *Pediococcus* spp. seem to have a wide inhibition range, in contrast to other LAB bacteriocins, which have antibacterial action against only a few related strains (Ray, 1992a, b). Pediocin PA-1 has been found to be inhibitory to strains and species from the genera *Pediococcus*, *Lactobacillus* and *Leuconostoc,* such as *P. acidilactici, P. pentosaceus*, *Lb. plantarum*, *Lb. casei*, *Lb. bifermentans* and *Leuc. mesenteroides* subsp. *dextranicum*, and *Listeria* (Pucci et al., 1988; Ennahar et al., 1996). Some of the pediococcal bacteriocins have been shown to inhibit foodborne bacteria, such as *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium botulinum*, *C.*

perfringens, *C. sporogenes* and *Listeria* species (Kalchayanand et al., 1989; Jager and Harlander, 1992).

2.4.6.3 Application as a biopreservative

Pediocin is one of the most characterised bacteriocins and will probably be the second bacteriocin, after nisin, to find practical applications in the food industry (Chikindas et al., 1995; Venema et al., 1995b). The use of pediocin PA-1 is covered by several patents in the US and Europe (Montville and Chen, 1998). To obtain approval for their use from bodies such as the FDA, the complete chemical, physical and genetic characterisation of potential new antimicrobials will be necessary (Jager and Harlander, 1992). Limited studies have indicated that pediocin PA-1 and pediocin AcH can be used as biopreservatives to control both spoilage and pathogenic Gram-positive bacteria. Efficient technologies still need to be developed for the proper application of the pediocins in food systems (Ray, 1994).

A number of tests have been performed to examine the suitability of pediocin as a biopreservative. The production and stability of pediocin N5p from *P. pentosaceus* isolated from wine was examined in grape juice medium. This pediocin is active against other LAB found in wine, such as *Lactobacillus*, *Leuconostoc* and *Pediococcus*. When tested against a range of pH values, the activity was found to be stable at pH values from 2.0-5.0 at 4 and 30°C. It was inactivated completely at pH 10. Ethanol up to 10% and SO_2 levels of 40-80 mg/L did not affect the pediocin activity at acid pH, either in combination or independently (Strasser de Saad et al., 1995).

LAB bacteria are widely used as starter cultures in dairy, meat and vegetable fermentation, with pediococci used commonly in the manufacture of fermented sausages (Cintas et al., 1995). While both nisin and pediocin PA-1 have applications in the meat industry, studies of model food systems demonstrate that pediocin-like bacteriocins are better at killing off pathogens in meat products (Montville and Chen, 1998).

2.4.7 Leucocin

Bacteriocin production by *Leuconostoc* spp. was first observed in the 1950s, but more extensive studies of their bacteriocins have only been conducted since 1984 (Stiles, 1994). The *Leuconostoc* species that were found to produce bacteriocins have so far been isolated from meat, processed meat, raw milk and soft cheese (Hastings and Stiles, 1991; Felix et al., 1994; Stiles, 1994; Papathanasopoulos et al., 1997)

A number of bacteriocins produced by *Leuconostoc* spp. have been reported, such as Leucocin A-UAL 187 from *Lc. gelidum* UAL 187 (A-UAL 187/A-TA11a/A-TA33a are all identical to each other) (Hastings and Stiles, 1991; Felix et al., 1994; Papathanasopoulos et al., 1997), mesentericin Y105/5/187 from *Lc. mesenteroides* subsp. *mesenteroides* Y105, mensenterocin 52A from *Lc. mesenteroides* subsp. *mesenteroides* FR52, leuconocin S from *Weisella paramensenteroides*, carnocin 54 from *Lc. carnosum* LA54a and leucocin B-TA11a from *Lc. carnosum* TA11a (Stiles, 1994; Papathanasopoulos et al., 1997; Ennahar et al., 2000a). *Leuconostoc mesenteroides* TA33a was the first *Leuconostoc* strain found to produce three distinct bacteriocins, namely leucocin C-TA33a, B-TA33a and A-TA33a. Leucocin B-TA33a does not belong to the pediocin family of bacteriocins. Leucocin C-TA33a

belongs to the class II bacteriocins, the third bacteriocin A-TA33a being identical to leucocin A-UAL 187 (Papathanasopoulos et al., 1997, 1998).

2.4.7.1 Characteristics

Leucocin A-TA33a forms part of the class IIa bacteriocins and shares considerable sequence similarity with the group. Leucocin A-TA33a has a molecular mass of 3390 Da, consists of 37 amino acids and has a net charge of +4 (Ennahar et al., 2000b).

2.4.7.2 Inhibition spectrum

Leucocin A-TA33a has only one disulphide bond and has a narrower spectrum of activity than pediocin PA-1/AcH that contains two disulphide bonds (Jack et al., 1995). Leucocin A-TA33a (Papathanasopoulos et al., 1997) and Leucocin B-Ta11a (Felix et al., 1994) were found to inhibit closely-related LAB and *List. monocytogenes*.

2.5 ENZYMES

2.5.1 General

The stability of an enzyme is difficult to predict in the presence of food components such as lipids. A final evaluation of an enzyme as a potential preservative is necessary and should include tests in realistic model systems. The use of enzymes in food preservation will be aided by the availability of cheaper, well-defined preparations (Meyer and Isaksen, 1995).

A number of enzymes that represent new types of potentially applicable natural preservatives have recently been researched. Two groups of enzymes have received some attention, namely the hydrolases and oxidoreductases. The hydrolases target key structural components in the cell walls of microorganisms and the cell is inactivated by the degradation of these components. The oxidoreductases generate reactive molecules that exert their effect *in situ*, destroying vital proteins in the cell. Glucose oxidase (EC 1.1.3.4.), which is produced by moulds such as *Aspergillus niger* and *Penicillium* spp., catalyses the formation of H_2O_2. The antimicrobial acitivity is due to the cytotoxicity of the H_2O_2 formed (Fuglsang et al., 1995).

Bacteriolytic enzymes are generally grouped into three different classes:

(i) *N*-acetylhexosaminidases that catalyse the cleavage of the β(1→4)-glycosidic linkages in the carbohydrate backbone in peptidoglycan

(ii) *N*-acetylmuramyl-L-alanine amidases that catalyse the cleavage between the carbohydrate moiety and the peptide moiety of the peptidoglycan

(iii) Endopeptidases, which hydrolyse the peptide bonds in the peptide crosslinks of the peptidoglycan

Fungal cell wall-degrading enzymes have been found to be effective against plant pathogenic fungi, but have not received much attention in relation to food preservation. This group is active against most of the fungi associated with food and includes the chitinases - both endochitinases and exochitinases - as well as β-glucanases (Fuglsang et al., 1995). Antimicrobial oxidoreductase enzymes do not possess antimicrobial activity, but reaction products from the reactions catalysed by these systems are cytotoxic and exhibit antimicrobial activity. The best studied enzymes in this group are glucose oxidase and lactoperoxidase (Fuglsang et al.,

1995). Lysozyme, however, has found some applications in the food and beverage industry and will be discussed in greater detail in the following sections.

2.5.2 Lysozyme

It has been reported that the early Romans used egg white or human milk in the treatment of eye infections. Both these substances contain appreciable amounts of lysozyme (McKenzie and White, 1991). Alexander Flemming discovered lysozyme in 1922, while he was suffering from a cold and observed that nasal mucus dissolved bacteria on an agar plate. Soon thereafter, he found that an enzyme was responsible for the antibacterial action. This enzyme was effective only against certain bacteria and not those infectious for humans (Proctor and Cunningham, 1988). Lysozyme is commonly found in cow's milk, domestic egg white, nasal secretions and human tears (McKenzie and White, 1991).

2.5.2.1 Characteristics

The make-up of the protein molecule of lysozyme was discovered and its amino acid sequence was mapped. The hen egg white lysozyme molecule consists of a single polypeptide chain of 129 amino acids. Four different disulphide bridges cross-link the molecule. Two of these bonds have to be intact for the lysozyme to function (Jolles et al., 1963; Blake et al., 1965). Amphiphilic helix stretches in the C-terminus of T4 lysozyme mediate its bactericidal and fungistatic activities. The enzymatic activity is completely abolished by heat denaturation of lysozyme but, unexpectedly, the antimicrobial functions remain preserved, resulting in the non-enzymatic microbicidal activity of lysozyme (Düring et al., 1999). The molecular weight is approximately 14 300 to 14 600 and the isoelectric point is at pH 10.7. The activity rate is the highest from pH 3.5 to 7 and the pH range of 5 to 7 is the best for lysing of *Micrococcus lysodeikticus* (Salton, 1957; Wilkinson and Dorrington, 1975). Dimerisation can occur in a pH range of 5 to 9, in which the net charge changes a little while the molecule undergoes no major structural modification (Sophianopoulos and Van Holde, 1964). In contrast, when SO_2 is used in preservation, the lysozyme activity increases with an increase in pH (Gerbaux et al., 1997). The mechanism of irreversable inactivation of lysozyme at pH 4 and 100°C was investigated. The inactivation was caused by the production of molecules in an irreversibly denatured state, with the inactivation not evoked by a single chemical reaction (Tomizawa et al., 1994).

2.5.2.2 Mode of action

Lysozyme (EC 3.2.1.17) is officially described as N-acetylhexosaminodase and is classified as a muramidase (Wilkinson and Dorrington, 1975). This enzyme contributes to bacterial killing by degradation of the peptidoglycan component of the bacterial outer membrane (Ohno and Morrison, 1989). The name was given to lysozyme because of its lysing action on bacterial cells through the digestion and weakening of the rigid cell walls, rendering the cells susceptible to osmotic lysis.

The enzyme functions by splitting or hydrolysing the β(1-4)-linkages between both *N*-acetylmuramic acid and *N*-acetylglucosamine (Davies et al., 1968). **Figure 2.5** represents the structure of the polymer that forms the peptidoglycan component of the bacterial outer membrane. The linkages connect the carbon 1 in *N*-acetylmuramic acid to carbon 4 in *N*-acetylglucosamine. The residues are linked

together in an alternating manner, with the bond available for lysozyme splitting at every fourth residue (Proctor and Cunningham, 1988). These molecules form part of a long and complex polysaccharide that forms the backbone of the outer membrane of bacterial cells. The lysozyme also cleaves the β(1-4)-linked oligosaccharides of N-acetylglucosamine (Davies et al., 1968). Due to the differences in cell wall make-up, the Gram-positive bacteria are more susceptible than the Gram-negative bacteria (Proctor and Cunningham, 1988; Cunningham et al., 1991).

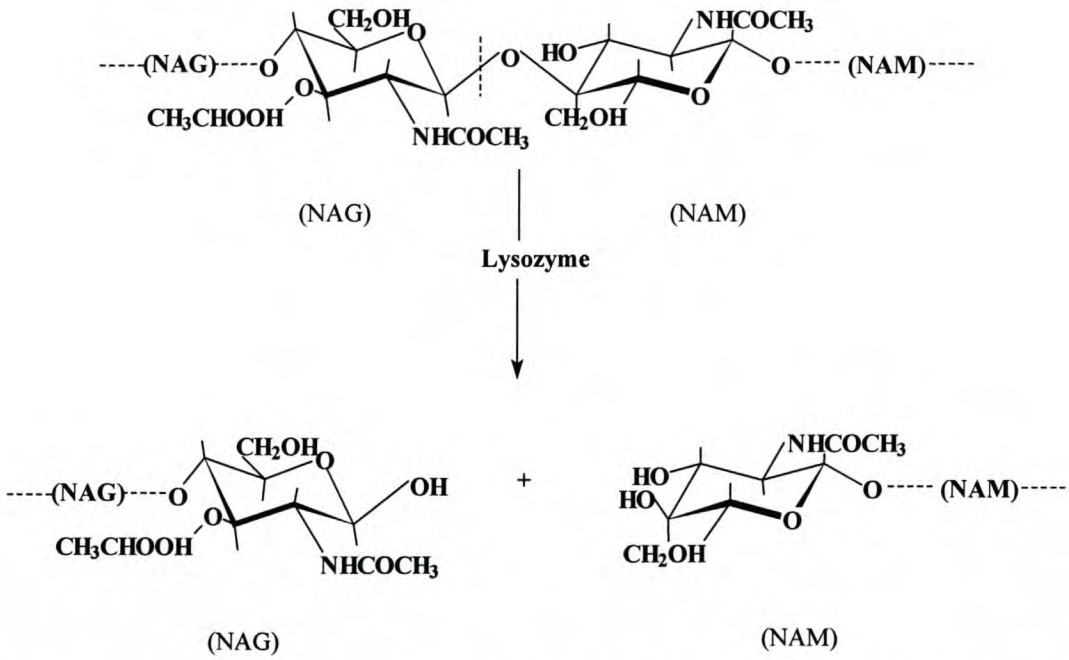

Figure 2.5 Catalysis by lysozyme of the cleavage of the glycosidic link between the C-1 of N-acetylmuramic acid (NAM) and the C-4 of N-acetylglucosamine (NAG) in a polymer of NAM and NAG. The vertical line shows the point of cleavage (McKenzie and White, 1991).

2.5.2.3 Lysozyme sources

Lysozyme is found in many substances, with the highest concentration occurring in human tears. Hen egg white is used as the commercial source for extraction. The classic method of lysozyme extraction involves absorption on bentonite and the elution of contaminating proteins with phosphate buffers and aqueous pyridine. The eluate is dialysed and freeze-dried (Alderton et al., 1945; Proctor and Cunningham, 1988). This method was improved by crystallising lysozyme as the salt of several acids directly from hen egg white with a NaCl solution. A number of methods also use adsorption on chromatography columns (Proctor and Cunningham, 1988).

2.5.2.4 Inhibition spectrum

As previously mentioned, Gram-positive bacteria are more susceptible to the lytic action of lysozyme than Gram-negative bacteria, due to the composition of the respective cell walls. Salton and Pavilik (1960) studied a number of Gram-positive bacteria for varying degrees of susceptibility. The strains studied were *Bacillus, Corynebacterium, Lactobacillus, Micrococcus, Sarcina, Sporosarcina, Staphylococcus* and the Gram-negative *Streptococcus*. Cell walls isolated from these organisms were all sensitive to lysozyme. It has been shown that lysozyme is

more detrimental to spoilage and pathogenic bacteria than lactic acid-producing bacteria in milk. Egg white lysozyme was able to effectively kill a population of resting *Cl. tyrobutyricum* vegetative cells (Wasserfall and Teuber, 1979). A Japanese study isolated lactobacilli that were responsible for spoilage from mirin liquor, namely *Lactobacillus heterohiochii, L. fermenti, L. plantarum* and *L. casei*. The LAB isolated from saké were also sensitive to lysozyme and included *L. heterohiochii, L. homohiochii* and *L. fermentum*, but not *L. acidophilus* (Cunningham et al., 1991). The action of lysozyme against LAB therefore has been documented and the enzyme should have the poptential to inhibit the LAB bacteria in wine.

2.5.2.5 Lysozyme as a biopreservative

A great deal of interest and research have gone into the use of egg white lysozyme for application as a food and beverage preservative in many different foodstuffs. Cheese has perhaps received the most attention, where lysozyme is used to prevent the late blowing of cheese by *Clostridium tyrobutyricum* vegetative cells and spores, for example (Wasserfall and Teuber, 1979; Proctor and Cunningham 1988). The majority of work on other foodstuffs has been done in Japan, where a number of applications have been patented (Cunningham et al., 1991). Lysozyme killed or prevented the growth of *List. monocytogenes* in several foods, but had a more effective inhibitory action in vegetables than in animal-derived foods (Hughey et al., 1989). Lysozyme has been used as a preservative in conjunction with other additives in kimchi, sushi, Chinese noodles and creamed custard (Proctor and Cunningham, 1988). Its use as a preservative in beer has been demonstrated by Makki and Durance (1996). Low levels of lysozyme will delay the growth of spoilage bacteria, but not prevent their growth. Furthermore, attention has been given to its use in heat-sterilised products to reduce thermal treatments. Further applications include its use as a supplement in foods like poultry, shrimp and sausage, and it has been immobilised on food packaging films to prevent contamination by or the growth of microorganisms (Hughey and Johnson, 1987; Appendini and Hotchkiss, 1997).

Much attention has been given specifically to the replacement of SO_2 in the microbiological and physico-chemical stabilisation of foods and wines (Amati et al., 1992). Many attempts to replace it with other preservatives have been insufficient from a technological point of view, in terms of ease of use (Amati et al., 1992). A number of researchers evaluated the use of lysozyme in winemaking for its ability to reduce the lactic acid bacteria in musts and wine. The control of lactic acid bacteria during the winemaking process is essential to obtain wines of consistent and high quality. Wines with a low acidity (high pH) are generally more prone to microbial spoilage, especially due to the growth of lactic acid bacteria. The antimicrobial activity of SO_2 decreases with an increase in pH, which is in contrast to lysozyme, in which case the antimicrobial activity increases (Gerbaux et al., 1997). This explains why there is such a good synergy between the two products. However, as is the case with many antimicrobials, some strains of bacteria have been isolated that have resistance to lysozyme (Cunningham et al., 1991). The action of lysozyme is immediate and, after a few hours, the lysozyme becomes inactive in white wine, or is eliminated by binding with tannins (Gerland, note).

Amati et al. (1992) tested the application and performance of lysozyme to control the malolactic fermentation by lactic acid bacteria in oenology. The experiment first established whether certain components in wine influenced the

lysozyme activity, then tested its stability at normal wine pH. The conclusion of the study is that the enzyme can inhibit bacterial growth without adversely affecting the wine composition or its taste qualities. Levels of 500 mg/L were used to stabilise wines, and these wines were stable throughout the nine months of storage. The antimicrobial action was most pronounced when lysozyme was added before the commencement of the malolactic fermentation, thus preventing the malolactic fermentation from occurring and providing microbiological stability to the wine. When staggered levels of SO_2 were added, it was found that lysozyme did not interact with this preservative. Lysozyme-supplemented wines exhibited greater levels of tartaric acid, ash and potassium. These changes can be attributed to the reduced precipitation of tartrates, despite the slightly higher alcohol levels. Due to the muramidasic action of lysozyme, it can be expected that the wine will contain increased colloidal substances because of the detachment of micro-molecules.

Lysozyme has several applications in oenology, a number of which require technical knowledge and expertise to be efficient. Gerbaux et al. (1999) found that lysozyme could be used to control the onset of MLF. The addition of low levels of lysozyme at the onset of alcoholic fermentation did not inhibit the eventual development of an inoculated MLF. This would be particularly applicable in red wine vinification, where problems with lactic acid bacteria might occur. Lysozyme was also used at higher dosages (250-500 ppm) for the total inhibition of the MLF where it was deemed unwanted. The MLF was also managed to such an extent that it slowed down, or stopped completely, by adding an intermediate dosage of lysozyme (350-500 ppm). Bacteria remaining in the wine can cause organoleptic defects during aging or post-bottling. Lysozyme in dosages of 250-500 ppm can protect the wine by inhibiting the bacteria. In the case of sluggish alcoholic fermentations, LAB can increase, with an accompanying increase in volatile acidity, leading to wine of poor quality. In such cases, the addition of SO_2 would inhibit not only the LAB, but also the yeasts, thereby sometimes necessitating a second yeast inoculation. Due to the specificity of lysozyme, the enzyme can be added without making a new yeast culture. Bacteria were most effectively inhibited at low levels, thereby maintaining good hygienic conditions (Gerland, note).

Chung and Hancock (2000) researched the action of a combination of lysozyme and nisin against LAB and found that the combination produced a synergistic effect, whereby a combination of the two molecules was more effective than the parent molecules alone. The physical-chemical stability of wine cannot be achieved without the addition of SO_2, as it also acts as an antioxidant (Amati et al., 1992).

Glucose oxidase is an oxygen scavanger, using oxygen (O_2) as part of its action, and it is considered a means by which to maintain wine quality. The enzyme acts as an antioxidant by excluding O_2 from the wine, as well as inhibiting some microorganisms. The combination of lysozyme and glucose oxidase in a wine system offers the possibility of having both an oxygen scavenger and an antimicrobial in place, allowing for a large reduction in the amount of SO_2 to be used. This combination still has to be given some attention (Fuglsang et al., 1995; Pickering, 1998).

Lysozyme has been authorised by the European Community for the production of all types of cheeses without limits on the doses. This testifies to the harmlessness of the enzyme. It was also authorised as a food ingredient by the FAO-WHO's JECFA in 1992, and is to receive GRAS status from the FDA (Amati et al. 1992). In

Italy and France, lysozyme has been tested for use in wine, in many conditions under the authorisation of the *Office International de la Vigne et du Vin* (OIV) and the European government since 1990. The OIV has prescribed some guidelines for the use of lysozyme in wine and must (Resolution Oeno15/2001). The accumulated dose of lysozyme should not exceed 500 mg/L, as this level is sufficient to control the bacteria responsible for the MLF during the alcoholic fermentation. It was also recognised that lysozyme cannot totally substitute SO_2, but that the association between the two substances provides more stable wines.

In summary, lysozyme will be an effective tool to assist SO_2 as an antimicrobial agent and therefore lower the levels of this chemical preservative used in the wine industry, as it does not impair the alcoholic fermentation and does not negatively impact wine characteristics, such as colour, flavour, taste or aroma.

2.6 ANTIMICROBIAL SUBSTANCES USED IN COMBINATION

The antimicrobial efficiency of a bacteriocin may be enhanced or broadened by combining it with other bacteriocins, antimicrobial substances or sterilisation techniques. Leistner (1992) called this "hurdle technology" for the preservation of foods. The combined effects of water activity, pH, temperature and preservatives are studied in conjunction with added bacteriocinogenic LAB or their bacteriocins. For optimum effectivity, bacteriocins have to be used as part of a general multihurdle food preservation system, which would involve a set of other antimicrobial factors (Ennahar et al., 2000b). A synergistic effect, by which combinations of the antimicrobials are more effective for growth inhibition than either one alone, has been observed with a number of bacteriocin combinations. These are listed, in combination with other preservative substances, in **Table 2.2**.

Bacteria can be further sensitised to the action of bacteriocins by heat treatment, for example (Modi et al., 2000). Such combinations of preservatives can be highly advantageous over the use of the individual agents, as many preservatives have quite different modes of action (Chung and Hancock, 2000).

The emergence of organisms resistant to certain classes of bacteriocins has become quite common and is a potential obstacle to their application as antimicrobial agents. Bacteriocins with different modes of action may have practical consequences, effectively excluding the occurrence of resistance to a certain bacteriocin (Horn et al., 1999).

Table 2.2 Combinations of antimicrobial substances, such as bacteriocins, enzymes, environmental factors and chelating agents, displaying synergistic inhibition.

Antibacterial substances	***Active against***	***Application***	***Synergy***	***Ref***
Nisin, NaCl, pH	*List. monocytogenes*	Experimental system	Yes	*a*
Nisin, leucocin F10, pH, NaCl, EDTA	*List. monocytogenes*	Experimental system	Nisin Leucocin pH	*b*
Sakacin A, nisin A	*List. monocytogenes*	Experimental system	Yes	*c*
Nisin A, pediocin PA-1	Several Gram-positive bacteria, incl. *List. monocytogenes*, clostridia	Experimental system	Yes	*d*
Pediocin PA-1, Nisin A, lacticin 481, lacticin B, lacticin F	Lactic acid bacteria	Experimental system	Yes	*e*
Nisin, EDTA, lysozyme	Lactic acid bacteria, *List. monocytogenes, E.coli, B. thermosphacta*	Ham and bologna	Yes	*f*
Nisin, heat treatment	*L. plantarum, List. monocytogenes* (nisin resistant)	Experimental system	Yes	*g*
Nisin, heat treatment, pH	*Pectinatus frisingensis*	Beer	Yes	*h*
Nisin, sodium lactate	*List. monocytogenes*	Trout	Yes	*i*
Nisin, lysozyme	Lactic acid bacteria	Experimental system	Yes	*j*
Acidocin CH5, NaCl, $NaNO_3$, lysozyme	Lactic acid bacteria	MRS broth, milk	Yes	*k*

(a) Bouttefroy and Milliere, 2000; *(b)* Parente et al., 1998; *(c)* Schillinger et al., 1996; *(d)* Hanlin et al., 1993; Horn et al., 1999; *(e)* Mulet-Powell et al., 1998; *(f)* Gill and Holley, 2000; *(g)* Modi et al, 2000; *(h)* Chihib et al., 1999; *(i)* Nykänen et al., 2000; *(j)* Chung and Hancock, 2000; *(k)* Chumchalova et al., 1998.

2.7 FUTURE PROSPECTS

There are a number of research challenges that can be addressed within the next years. Bacteriocins are next in line to be used more widely as preservatives in the food industry. Another consideration is the use of bacteriocins as replacements for antibiotics for therapeutic use, with the emergence of antibiotic-resistant Gram-

positive pathogenic bacteria (Van Belkum and Stiles, 2000). Research can be done on the application of bacteriocins, and to increase the level of commercial development and the economic production thereof. Multihurdle preservation systems (Leistner, 1992) and technology transfer should also be given attention. The improvement of existing microbial strains and the development of novel strains by genetic engineering are active research areas worldwide.

2.7.1 Engineering of bacteriocins and production of heterologous proteins by *Saccharomyces cerevisiae* and lactic acid bacteria

The limited efficiency of bacteriocin-producing cultures in fermented foods may be attributed to various factors, such as low production, regulatory systems, genetic instability, inactivation and occurrence of resistance in target bacteria. An excellent tool in overcoming these obstacles is the heterologous expression of bacteriocins in various LAB strains. Bacteriocin regulation systems have been overcome through the cloning and expression of class IIa bacteriocins genes in new hosts (Chikindas et al., 1995; Schoeman et al., 1999; Ennahar et al., 2000b). The yeast *Saccharomyces cerevisiae* has been used as a model eukaryotic organism to produce useful heterologous proteins. Various food-grade bacteria can also be selected for use as hosts for defined bacteriocins of interest, based on the characteristics relevant to a given food system. LAB strains that are adapted to a specific kind of food can be constructed to produce bacteriocins, helping to overcome colonisation and bacteriocin production problems. The use of heterologous expression can be used additionally to develop LAB-producing multiple bacteriocins, each one having a specific range of targets and possibly enhancing the antimicrobial efficiency of LAB in food. Developing such bacteriocin-producing strains will yield an organism active against a broad range of undesirable organisms and possibly decrease the risk of developing bacteriocin resistance among target bacteria (Ennahar et al., 2000b). The enhanced production of pediocin PA-1 and the co-production of nisin and pediocin PA-1 by *Lactococcus lactis* through a heterologous expression system have been achieved by Horn et al. (1998, 1999). Chikindas et al. (1995) achieved the expression of lactococcin A and pediocin PA-1 in heterologous hosts. Peptide synthesis was used to create four new, biologically active hybrid bacteriocins by interchanging corresponding modules from various pediocin-like bacteriocins. The relative sensitivity of bacterial strains was quite similar, and a loss of activity, when present, was detected only after months of storage (Fimland et al., 1996).

As more knowledge about the LAB accumulates, it becomes possible to construct genetically useful LAB strains with characteristics shaped for specific purposes. The quality and preservation of fermented foods and beverages can be enhanced by LAB that have been improved through genetic methods (McKay and Baldwin, 1990). New biologically important peptides with improved activity and stability can be engineered through chemical or genetic modifications. Sequence modification, including single-residue modifications, generally will result in peptides with diminished activity compared to the native bacteriocin (Fimland et al., 1996; Chen et al., 1997). Miller et al. (1998), however, recently found that the substitution of a Glu residue for Lys-11 showed a significant increase in pediocin PA-1 activity, which suggests possible interesting future developments. Pediocin PA-1 will lose its antimicrobial activity when stored at 4°C. Johnsen et al. (2000) engineered pediocin PA-1 so that it has increased stability by replacing the methionine (Met31) residue.

This protects the bacteriocin from oxidation, while affecting the antimicrobial activity minimally.

2.7.2 Engineering and production of lysozyme by *Saccharomyces cerevisiae*

The glycosylation of proteins by yeast is a promising approach to increase the stability of lysozyme against heating and proteases (Kato et al., 1994). Lysozyme deaminated by protein engineering has been shown to have almost the same activity as that of wild lysozyme, but with a slight shift of the optimum pH to an acidic range (Kato et al. 1992). A number of genetically modified lysozyme forms have been engineered. Generally, these novel forms have improved activity or wider inhibition spectra. Hen egg white lysozyme has been modified genetically by glycosylation to have extreme heat stability, and to have strong antimicrobial activity against Gram-negative bacteria through the attachment of a hydrophobic pentapeptide at the C-terminus (Nakamura et al., 1993a, Ibrahim et al., 1993). Kato et al. (1998) expressed a glycosylated lysozyme, having enhanced heat stability and antimicrobial activity, in a yeast. Polymannose chain attachment to lysozyme also showed extreme heat stability when expressed in yeast containing the modified cDNA of lysozyme (Nakamura et al., 1993a, b). Ibrahim et al. (1991) chemically modified a lysozyme with the N-hydroxysuccinimide ester of palmitic acid. The palmotoyl-attached derivatives exhibited substantial activity against Gram-negative bacteria. Hen egg white lysozyme was also covalently modified with perillaldelhyde, which is a phenolic aldehyde. The addition of perillaldehyde did not have a significant effect on the solubility of the lysozyme derivatives. This altered enzyme had substantially more activity against both Gram-negative and Gram-positive bacteria (Ibrahim et al., 1994).

In another experiment, lysozyme was modified by the covalent attachment of selected phenolic compounds to characterise non-enzymatic reactions of food proteins with secondary plant metabolites. This modification led to a decrease in the solubility of the derivatives over a broad pH range and an increase in hydrophobicity. The digestion of these modified lysozyme molecules was affected adversely, and a reduction in lytic activity was also noted (Rawel et al., 2001). Lysozyme has further been modified to alter the surface hydrophobic/hydrophilic character. An increase in hydrophobicity marked an improvement in thermostability, with an opposite effect in hydrophilic character. These results indicate further possibilities in the use of lysozyme in food systems (Longo and Combes, 1999).

2.8 CONCLUSION

The late Prof. Dr. Kei Arima expressed his belief in microorganisms as an inexhaustible source of interesting metabolites as follows: "Microorganisms will never betray our needs if we create rational, sensitive screening and assay methods, since microorganisms are extremely excellent chemists" (Vandamme, 1994).

Bacteriocins have received considerable research interest in different applications, such as the biopreservation of food, in the past 20 years. This was stimulated specifically by the fact that they could be used as a preservative agent in foods and that LAB that grow in foods could be selected as preservative cultures (Van Belkum and Stiles, 2000). The amount of information on bacteriocins at the basic and applied levels is accumulating rapidly. The food industry and governments can now evaluate the acceptability of the biopreservation of foods with

bacteriocinogenic LAB, especially if genetically modified LAB are used, even though there seem to be many constraints (Stiles, 1996).

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CHAPTER 3

RESEARCH RESULTS

The application of nisin, pediocin, leucocin and lysozyme as biopreservatives in winemaking

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3. RESEARCH RESULTS

THE APPLICATION OF NISIN, PEDIOCIN, LEUCOCIN AND LYSOZYME AS BIOPRESERVATIVES IN WINEMAKING

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Abstract

The bacteriocins nisin, pediocin and leucocin and the bacteriolytic enzyme lysozyme were evaluated for use as biopreservatives in wine for a possible decrease in the levels of the commonly-used chemical preservative, sulphur dioxide (SO_2). Several type and reference strains of wine-associated lactic acid bacteria (LAB), as well as wine-isolated LAB, were screened against the three bacteriocins and were found to be sensitive in varying degrees. The bacteriocins had no effect on the wine-associated acetic acid bacteria and yeasts. It was found that pediocin was stable for a sufficient period of time in a simulated wine environment. Varying concentrations of nisin, pediocin and leucocin were used in combination against increasing cell concentrations of *Leuconostoc mesenteroides* DIIIM:I, a wine isolate. The combinations were most effective against cell numbers of 10^2 and 10^4 cfu/mL, whereas cell numbers of 10^6 and 10^8 cfu/mL were affected very little by the bacteriocins. Of the pairs of bacteriocins used, nisin-leucocin was the most effective, followed by nisin-pediocin and leucocin-pediocin. All three bacteriocins in combination proved to be the most effective overall. Scanning electron microscopy was employed to examine the effect of the bacteriocins on the cell morphology of *Leuconostoc mesenteroides* DIIIM:I. Major cell disturbances were detected, especially at the septa where the cells are joined. The leucocin-pediocin combination elicited the least inhibition, as exhibited by the most undamaged living cells between damaged cells. Varying sensitivity was determined by means of the microtitre broth dilution method. Synergism exists between certain pairs of bacteriocins, but the degree of synergism depended on the concentration of one bacteriocin used in relation to another. The addition of leucocin at 250 AU/mL to decreasing concentrations of pediocin and nisin was the most effective, with the inhibitory activity of the pairs being markedly higher than that of bacteriocins used alone. The addition of nisin to pediocin and leucocin had intermediary results, while pediocin addition to leucocin and nisin was the least effective. Pediocin added to decreasing concentrations of nisin proved to be less effective than nisin alone. The ratio of bacteriocins in combination proved to be the most important factor governing the synergistic or antagonistic effect. When considering the use of bacteriocins in combination, such interactions should be examined carefully. No definite conclusions could be drawn about the action of lysozyme and pediocin combinations.

Keywords: Wine, Lactic Acid Bacteria, Bacteriocins, Lysozyme, Biopreservation

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3.1 INTRODUCTION

Winemaking is an ancient biotechnological process, even older than bread-making. Yeasts, such as *Saccharomyces cerevisiae,* are responsible for the alcoholic fermentation of grape must to wine, converting sugar to alcohol with a concomitant production of flavour compounds. In addition to yeasts, bacteria play a pivotal role in determining the quality of wine. Lactic acid bacteria (LAB), including *Lactobacillus*, *Leuconostoc* and *Pediococcus* spp., as well as *Oenococcus oeni* (formerly *Leuconostoc oenos*), are Gram-positive, facultatively anaerobic bacteria that are present during the vinification process (Vaughn, 1955, Radler, 1990a, b; Dicks et al., 1995; Ribéreau-Gayon et al., 2000; Du Toit and Pretorius, 2000). These organisms are deemed either favourable to the winemaking process, such as *O. oeni*, which performs the **m**alol**a**ctic **f**ermentation (MLF), or as spoilage organisms, producing off-flavours and other defects in the wine (Lafon-Lafourcade et al., 1983; Wibowo et al., 1985; Van Vuuren and Dicks, 1993; Du Toit and Pretorius, 2000). Acetic acid bacteria (AAB) are Gram-negative, aerobic bacteria, and species of the genera *Acetobacter* and *Gluconobacter* have been associated with the vinification process. These bacteria are known to oxidise ethanol to acetic acid, which affects the quality of the wine negatively if produced in amounts exceeding the threshold value of 0.7 – 1.2 g/L, depending on the style of the wine (Drysdale and Fleet, 1988; Boulton et al., 1996, Ribéreau-Gayon et al., 2000).

Sulphur dioxide (SO_2) has a two-fold purpose in the winemaking process and is added to the wine and must as an antioxidant and antimicrobial substance. Firstly, it protects the wine from oxidation by acting as an antioxidant and, secondly, it acts as an antimicrobial agent, inhibiting wine spoilage LAB, AAB and yeasts (Casey, 1992; Ribéreau-Gayon et al., 2000). Consumer awareness and resistance towards chemical preservatives, such as SO_2, have increased due to the suspected role of sulphites in lowering the nutritional value of foods and the initiation of allergic and asthmatic reactions in certain individuals. The oenological industry has responded by lowering sulphur levels to produce more "natural" wines. Furthermore, this has encouraged researchers to search for alternative preservatives that would be applicable in wine systems. One such example is biopreservation, which is a novel, scientifically-based approach to improve the microbiological safety of foods that has attracted much attention in recent years. This preservation method entails the use of the natural microflora of the product and/or their antimicrobial products to inhibit or destroy undesired microorganisms, thereby extending the storage life and enhancing the safety of foods (Schillinger et al., 1996; Stiles, 1996). Examples include bacteriocins that have applications in food preservation, such as nisin in dairy products (Hurst, 1981). Pediocin (Strasser de Saad et al., 1995), acidocin (Chumchalová et al., 1998) and leucocin (Parente et al., 1998) have also been examined for possible use in foods and beverages. The bacteriolytic enzyme, lysozyme, has also found some applications in dairy products (Cunningham et al., 1991).

Bacteriocins are defined as proteinaceous complexes with antagonistic activity against species that are usually closely related to the producing bacterium. The effect can be bactericidal or bacteriostatic (Venema et al., 1995; Schillinger et al., 1996; Ennahar et al., 2000; Jack and Jung, 2000; Van Belkum and Stiles, 2000). The bacteriocins of Gram-positive bacteria are active against sensitive Gram-positive bacteria, but not against Gram-negative bacteria, yeasts or fungi (Radler, 1990b;

Kalchayanand et al., 1992; Abee et al., 1995; Dielbandhoesing et al., 1998). Most bacteriocins are reported to act primarily on the cytoplasmic membrane of Gram-positive bacteria, rendering the cells permeable to small ionic components; cell death is caused by a loss of these components and a concomitant dissipation of the proton motive force (Bruno and Montville, 1993; Ennahar et al., 2000). McKenzie and White (1991) proposed that lysozyme acts as a muramidase, enzymatically breaking the sugar linkages of peptidoglycan and causing the cells to burst due to the weakened cell wall. Lysozyme can also kill cells by a non-enzymatic mode of action (Düring et al., 1999).

Three bacteriocins were researched in this study, namely nisin, pediocin and leucocin, as well as the enzyme lysozyme. The best characterised and researched bacteriocin is nisin, belonging to the class I lantibiotics. These are cationic polypeptides with an amphiphilic character, containing 34 amino acids with activity in the pH range 2-7. Their activity decreases as the pH increases and they can withstand heating up to 100°C. Nisin has a broad inhibition spectrum, which is active against a wide range of Gram-positive bacteria, such as LAB, *Listeria*, *Bacillus* and *Clostridium* and their spores (Hurst, 1981; Delves-Broughton, 1990a, b; Radler, 1990a; De Vuyst and Vandamme, 1994).

Pediocin belongs to the class IIa bacteriocins, the *Listeria* active peptides, and is identical to pediocin AcH (Motlagh et al., 1992; Miller et al., 1998). It consists of 44 amino acids (Chen et al., 1998), with activity in a pH-range of 2-10, and can withstand heating up to 100°C (Gonzalez and Kunka, 1987). The inhibition spectrum is also wide, as it inhibits a number of species of *Pediococcus, Lactobacillus, Leuconostoc* and *Listeria* (Pucci et al., 1988; Ray, 1994), as well as certain species of *Bacillus, Staphylococcus* and *Clostridium* (Jager and Harlander, 1992).

Leucocin A-TA33a, which is identical to leucocin A-UAL 187 and B-TA11a (Felix et al., 1994; Papathanasopulos et al., 1997), also belongs to the class IIa *Listeria*-active bacteriocins and is one of the bacteriocins produced by *Leuc. mesenteroides* TA33a. The peptide consists of 37 amino acids and is most active in the pH range 2-3, also being able to withstand heating to 100°C. It has a narrower inhibition spectrum than nisin and pediocin, and is active against LAB and *Listeria* (Ennahar et al., 2000).

Lysozyme (EC 3.2.1.17) is a bacteriolytic enzyme, officially described as an *N*-acetylhexosaminodase, and consists of 129 amino acids (Wilkinson and Dorrington, 1975). Lysozyme is effective at lysing bacterial cell walls, with hydrolytic activity against β(1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine peptidoglycan (McKenzie and White, 1991). Davies et al. (1969) have demonstrated that it is active throughout a wide pH range of 4-10. Lysozyme has antibacterial activity against some Gram-positive bacteria, but is ineffective against Gram-negative bacteria, yeasts or fungi, due to the differences in cell wall composition (Proctor and Cunningham, 1988, Cunningham et al., 1991).

To date, nisin is the only bacteriocin to have received GRAS status (**G**enerally **R**egarded **A**s **S**afe) and it has been approved for use in certain foods as it is not only non-toxic to humans, but does not affect the intestinal flora (Claypool et al., 1966; Hurst, 1981; Kaletta and Entain, 1989; Hansen, 1993). Lysozyme was approved for use in wine by the OIV (*Office International de la Vigne et du Vin*) in 2001 (Resolution Oeno15/2001). Nisin (Radler, 1990a, b) and lysozyme (Gerbaux et al., 1997) have previously been evaluated for use in the winemaking process. It was indicated that

both nisin and lysozyme, when added to the must prior to the alcoholic fermentation, effectively inhibited most of the LAB, without affecting the yeasts. Neither substance affected the taste or composition of the wines. Furthermore, both substances were found to be ideal for wines with a slightly higher pH that are prone to spoilage due to diminished SO_2 activity (Gerbaux et al., 1997), as nisin and lysozyme are active in the higher pH range.

In this study, nisin, pediocin, leucocin and lysozyme were evaluated against microorganisms found naturally in wine to determine both their inhibition spectrum and their suitability for use in wine preservation. The effectiveness of the bacteriocins and lysozyme was investigated in wine by simulating wine conditions. To our knowledge this is the first time that bacteriocins in combinations with each other and in conjunction with lysozyme have been used for wine preservation. Possible interactions between the bacteriocins and lysozyme were also assessed by combining them for preservation to determine whether there was any additive or synergistic effect.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria, yeasts and growth conditions

The bacteria and commercial wine yeasts used in this study are listed in **Table 3.1a, b, c**, including the AAB, LAB reference strains and wine isolates (isolated at the Institute for Wine Biotechnology).

The bacteriocin-producing strains used were *Lactococcus lactis* subsp. *lactis* DSM 20729 (hereafter referred to as *L. lactis*), which produces nisin, *Pediococcus acidilactici* PAC1.0 (received from T.J. Montville), which produces pediocin PA-1 (hereafter referred to as pediocin) and *Leuconostoc mesenteroides* TA33a (received from J.W. Hastings), which produces multiple bacteriocins, namely leucocin A-TA33a, B-TA33a and C-TA33a (hereafter referred to as leucocin).

The LAB were cultivated throughout in De **M**an, **R**ogosa and **S**harpe medium (MRS) (De Man et al., 1960) broth and agar (Biolab, Merck, South Africa; all other nutrient broths and agar used were also from Biolab, unless stated otherwise), with the exception of the *Oenococcus oeni* strains, which were grown in malic medium (MMM, Zuniga et al., 1994). *Lb. kunkeei* was cultivated in MRS of which the pH was adjusted to 5.2 with 10N HCl. AAB were maintained in **G**lucose **Y**east Extract medium (GY) (5% glucose, 1% yeast extract) and on GYC agar plates (3% $CaCO_3$, 5% glucose, 1% yeast extract, 2% Agar) (Drysdale and Fleet, 1988). Yeasts were grown in **Y**east **P**eptone **D**extrose (YPD) broth and *Listeria monocytogenes* B73 (Dykes and Hastings, 1998) in **B**rain **H**eart **I**nfusion (BHI) broth. The bacteria and yeast were maintained and stored at –80°C in their respective growth media with 20% and 30% glycerol each.

The LAB were grown microaerophilically at 30°C. The AAB and yeasts were grown at 30°C and the *List. monocytogenes* at 37°C with aeration. The cultures to be used in the experimental work were subcultured twice for 18 h, and inoculated into the growth medium at 1%.

Table 3.1a. Wine-related lactic acid bacteria type and reference strains used in the sensitivity test assay against purified nisin, pediocin and leucocin.

Microorganism	Strain number		Bacteriocin		
			Nisin	Leucocin	Pediocin
Lb. brevis	ATCC[1]	8291	+++*	+*	++*
Lb. casei	LMG[2]	13552	+++	-	+
Lb. curvatus	LMG	13553	+++	+	+
Lb. fermentum	ATCC	9328[T]	++	+	+
Lb. fermentum	DSM[3]	23271	+++	-	-
Lb. fermentum	LMG	13554	+++	+	++
Lb. fructivorans	ATCC	8288[T]	+++	+	++
Lb. hilgardii	ATCC	8290[T]	+++	+	++
Lb. kunkeei	DSM	12361	+++	+	+++
Lb. plantarum	LMG	13556	+++	++	+
Lb. sake	LMG	13558	+	+	+
Lb. sake	DSM	20017	++	+++	+
Ped. pentosaceus	LMG	13561	+++	+	++
Ped. pentosaceus	LMG	13560	+++	+++	++
Ped. pentosaceus	NCDO[4]	514	+++	+	+++
Ped. pentosaceus	NCDO	813	+++	+++	+++
Leuc. mesenteroides			+++	+	++
Viniflora oenos[5]			+++	++++	+++
VinoD nuovo[5]			+++	+++	+++
VinoD bitec[5]			+++	+++	+++

[1]ATCC: American Type Culture Collection, Rockville, Maryland, U.S.A.
[2]LMG: Culture Collection Laboratorium Mikrobiologie, Gent, Belgium
[3]DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, F.R.G.
[4]NCDO: National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, England
[5]*O. oeni* MLF starter cultures
*+++ Highest level of inhibition: Inhibition Titre: 6-10
++ Intermediary inhibition: Inhibition Titre: 3-5
\+ Minimal inhibition: Inhibition Titre: 1-2
\- No inhibition

3.2.2 Preliminary screening of bacteriocin producers against wine microorganisms

Cell cultures producing nisin, pediocin and leucocin were spotted onto a buffered bacteriocin screening medium (BSM) (Tichaczek et al., 1992) and grown at 30°C for 24 h. The plates were subsequently lawned with 10 ml of MRS, GY and YPD soft agar (0.7% bacteriological agar) for LAB, AAB and yeasts, respectively, and inoculated with a test panel organism. The plates were incubated aerobically at 30°C and examined after 18-24 h for growth inhibition zones. The degrees of inhibition were documented by measuring the diameter of the zones of inhibition (in mm).

Tabel 3.1b. Wine-isolated LAB used in the sensitivity test assay against purified nisin, pediocin and leucocin.

Microorganism	Bacteriocin		
	Nisin	Leucocin	Pediocin
AIIIM1:3	-	+++	++
AIVM2:7	+	+	++
EIIIM3(D):4	+++	++	+
FIVM2(M)4	+++	+	++
HIIIM1:4	+++	+	+
FIIIM2D:4	+++	-	++
MRS 2 al	++	+	+
GYC 42d	++	+	+
BM2(1)	+	+	+
AM3 (7)	++	-	+
AM2(7)	++	-	+
DIIIM:I	+++	++	++
HIIIM2:7	+++	-	++
AIIIM1:3	++	-	++
AIIIM3:2	+++	-	+
DM(3):I	++	++	+
DM1:4	+++	-	+++

Table 3.1c. Wine-associated acetic acid bacteria and yeasts used in the sensitivity test assay against purified nisin, pediocin and leucocin.

Microorganism	Strain number	Bacteriocin		
		Nisin	Leucocin	Pediocin
Acetic acid bacteria				
A. aceti	DSM[1] 3508			
A. hansenii	DSM 5602			
A. liquefaciens	DSM 5603		**No Inhibition**	
A. pasteurianus	DSM 3509			
G. oxydans	DSM 7145			
Wine yeasts				
N96[2]				
NT116[2]				
VIN7[2]				
Bourdeaux Red[3]				
228[2]			**No Inhibition**	
WE14[2]				
VIN13[2]				
EC1118[2]				
NT112[2]				
WE372[2]				

[1] DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, F.R.G.
[2]Anchor Yeast, Epping, South Africa.
[3]Lallemand, France.

3.2.3 Leucocin and pediocin isolation

Bacteriocin activity was assayed by the spot-on-lawn method (De Vuyst et al., 1996) by spotting 10 µl of the bacteriocin-containing solution on a seeded lawn. The unit of bacteriocin activity, often called the Arbitrary Activity Unit (AU), was calculated as follows: AU/mL = 2^xx100, where *x* is the number of the last dilution showing inhibition (the bacteriocin titre), and the AU was converted to mL by multiplying it by 100 (Chumchalová et al., 1998). The AU is usually defined arbitrarily as the reciprocal of the highest dilution showing growth inhibition under standardised conditions (De Vuyst and Vandamme, 1994).

For the isolation procedure, MRS medium was made up from individual components according to De Vuyst et al. (1996). The glucose was autoclaved separately and added after the media had cooled down. Large-scale isolation was performed, using 4-5 L for each bacteriocin. The producing bacterial strains were upscaled systematically in MRS broth. The resulting cell culture was centrifuged at 10 400 *g* for 12 min at 4°C (Sorvall, MC 5C plus, Du Pont) and the supernatant was adjusted to pH 6 with 10 M NaOH. The active component in the supernatant was precipitated slowly with ammonium sulphate (Merck, South Africa) up to 85% saturation (56.8 g/100ml supernatant) (Bollag and Edelstein, 1991) by gentle stirring at 8°C. The precipitated cell-free supernatant was centrifuged at 10 400 *g* for 10 min at 4°C. The precipitate formed after centrifugation was dissolved in a minimal amount of sterile distilled water and was termed the crude bacteriocin fraction. This resulting fraction was subjected to chloroform/methanol precipitation. The sample was added to 18 volumes of a chloroform/methanol (2:1, vol/vol) mixture and stored at 4°C for 2 h without stirring. The mixture was centrifuged at 3 300 *g* for 60 min (De Vuyst et al., 1996; Callewaert et al, 1999; Zamfir et al., 1999), and the resulting fine-grained white precipitate was dissolved in a minimal amount of sterile 50 mM sodium-phosphate buffer, aliquoted and stored at –20°C. This fraction was regarded as partially purified. The activity titres were followed throughout the isolation process against the indicator strain *List. monocytogenes* B73 by the spot on lawn procedure.

3.2.4 Purity and molecular mass confirmation

The molecular mass of the partially purified bacteriocins was confirmed by Tricine-Sodium Dodecyl Sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). The samples for Tricine-SDS-PAGE were boiled for 3 min with one volume of sample buffer (2x for SDS ultra low molecular weight marker kit, Sigma Aldrich Chemicals, Germany), and the molecular weight marker ultra-low range (Sigma Aldrich Chemicals, Germany) was diluted 1:20 with 1x sample buffer and heated for 2 min at 65°C. The marker was loaded in the first lane, and the samples loaded in duplicate on two halves of the gel. The samples used were leucocin and pediocin. The protocol was then followed as described by Schoeman et al. (1999), dividing the gel for staining and overlay with *List. monocytogenes* B73.

3.2.5 Screening with partially purified and commercial bacteriocins

The bacteriocins pediocin and leucocin, as partially purified bacteriocins, and commercial nisin were used and standardised to contain 102 400 AU/mL (or a titre of 10) against the indicator organisms *List. monocytogenes* B73. The solution was serially two-fold diluted and 10 µL was spotted onto the lawn (prepared as in 3.2.2 above) containing a test organism as listed in **Table 3.1a, b, c**. The plates were

incubated for 18-24 h. The bacteriocin titre was determined for each bacteriocin against the test panel organisms listed in **Table 3.1a, b, c**.

3.2.6 Effect of pH, ethanol and SO_2 on leucocin and pediocin stability

Pediocin stability was evaluated in white grape must (Colombard), sterilised by autoclaving, containing no SO_2. The grape must was adjusted to simulate wine by adjusting the pH to 3.0, 3.5, 4.0 and 4.5 with 2 M HCl or 10 M NaOH. The SO_2 levels were adjusted to 0, 40, 60 and 80 mg/L in the form of potassium metabisulphite, and the alcohol levels were adjusted to 0, 10 and 14% with 99.9% pure ethanol (Strasser de Saad et al., 1995). Pediocin was added to a final concentration of 12 800 AU/mL and the samples were incubated at 23ºC. Cell counts were performed on MRS agar plates at intervals from serial ten-fold dilutions and incubated at 30ºC for 48 h. The experiment was repeated in duplicate.

3.2.7 Effect of bacteriocin combinations against lactic acid bacteria and acetic acid bacteria

Nisin, pediocin and leucocin were used in combination against LAB and AAB to determine if there was any enhanced or diminished antimicrobial activity, as described in the following two sections.

3.2.7.1 Effect of varying bacteriocin concentrations against increasing bacterial concentrations

A modified grape must was created by adding 3% yeast extract to sterile white grape must, without SO_2. A sensitive organism was selected from the test panel of wine-isolated LAB, and the must was inoculated with *Leuc. mesenteroides* DIIIM:I at cell counts of 0, 10^2, 10^4, 10^6 and 10^8 cfu/mL. Combinations of commercial nisin (Sigma Aldrich Chemicals, Germany) and partially purified pediocin and leucocin were used as follows: nisin-leucocin; leucocin-pediocin; pediocin-nisin and nisin-pediocin-leucocin, at concentrations of 0, 100, 500 and 1000 AU/mL, were added to the samples inoculated with increasing cell concentrations. Cells counts were performed on MRS agar plates at intervals from days 1-40, as before. The experiment was repeated in triplicate.

3.2.7.2 Combinations of bacteriocins against acetic acid bacteria

Four *Acetobacter* type strains, namely *A. aceti, A. pasteurianus, A. liquefaciens* and *A. hansenii*, were used in this study and inoculated into grape must, as prepared above, at 10^4 cfu/mL. 1 000 AU/mL of each bacteriocin was added as follows: Nisin-pediocin, pediocin-leucocin and nisin-leucocin, and the samples were maintained at 30ºC. Cell counts were performed in duplicate from serial ten-fold dilutions on GYC agar plates and incubated at 30ºC for 48-72 h. The experiment was repeated in duplicate.

3.2.8 Combinations of pediocin and lysozyme

Combinations of pediocin and lysozyme were used to determine if any enhanced or diminished action could be detected.

3.2.8.1 Against lactic acid bacteria

The same must as in section 3.2.7.1 was prepared and aliquoted into 250 mL bottles. The first combination tested was that of pediocin and lysozyme. Pediocin and

lysozyme were sterilised by boiling at 100°C for 5 min and filter sterilisation with a 0.22 μm filter (MSI Micro Separation Incorporated, USA), respectively. The modified must was divided into four batches. The first batch was inoculated with 12 800 AU/mL partially purified pediocin, the second with 500 mg/L lysozyme, both levels being suitable for usage in wine, and the third group with a combination of the two substances at the aforementioned levels. A control batch without pediocin or lysozyme was included in the experiment. The must was subsequently inoculated with 10^3 cfu/mL of wine isolate DIIIM:I, a LAB level expected to be found naturally in wine, and stored at 15°C. Cell counts were performed as before. The experiment was repeated in duplicate and the cell counts were presented as an average of the two experiments.

3.2.8.2 Against acetic acid bacteria

The same must as above was aliquoted into 250 mL bottles. The AAB used in 3.3.7.2 were inoculated in the bottles at 10^4 cfu/mL each. The three bacteriocins, at a concentration of 1000 AU/mL, were added to the inoculated grape must, each with lysozyme at 500 mg/L. A control with no added antimicrobial substances was included for each bacterium. The samples were maintained at 30°C and monitored at intervals from days 1-35. Cell enumeration was performed in duplicate as before. The experiment was repeated in duplicate

3.2.9 Scanning electron microscopy: morphological effects

The DIIIM:I wine isolate was incubated in MRS medium for 18 h at 30°C with 5 000 AU/mL of each of nisin, pediocin or leucocin added to the bacterial suspension. A total of 5 000 AU/mL was added to four other samples, as combinations of nisin-leucocin (1:1), nisin-pediocin (1:1), leucocin-pediocin (1:1) and nisin-pediocin-leucocin (1:1:1). A control, containing no bacteriocins, was included. Nucleopore Track-Etch Membranes (0.2 μm) were installed into Millipore non-sterile Swinnex filters. The treated bacterial cells were filtered through the membranes using a 5 mL syringe and the filters were transferred to small glass bottles in which the remainder of the experiment was performed. The protocol described by Chung and Hancock (2000) was followed from this point. The filters were fixed in 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer for 30 min at 4°C and afterwards washed three times for 5 min in 0.1 M sodium phosphate buffer, pH 7.2. The cells were then fixed with 1% osmium tetroxide in a 0.1 M sodium phosphate buffer for 30 min, rinsed in distilled water for 5 min, fixed with 1% tannic acid for 20 min and rinsed again in distilled water for 5 min. The samples were finally fixed with 1% osmium tetroxide and rinsed with distilled water for 5 min. The samples were treated progressively with 50, 70, 80, twice with 95, and three times with 100% alcohol each step was applied for 5 min to dehydrate, after which they were dried in a Balzers critical point dryer, sputtered with gold and viewed under a Leica Stereoscan Scanning Electron Microscope.

3.2.10 Microtitre broth dilution method for sensitivity

A number of versions of the microtitre broth dilution method have been described. A modified version of the assay, as described by Du Toit and Rautenbach (2000), was used. All the microtitre plates (Flat bottomed, 96 well, Sero-wel, Bibby Sterilin, UK) were blocked with 200 μl of 0.5% casein in phosphate buffered saline (PBS) per well (Dulbecco and Vogt, 1954) for 1 h and sterilised and dried under ultraviolet light for a

further 4 h. The AU/mL of test samples of nisin, pediocin and leucocin were determined, and the samples were diluted in sterile analytical quality water to a five times stock solution of 4 000 AU/mL, as required for the remainder of the experiment. Serial two-fold dilutions from 4 000 AU/mL were performed with sterile water in the titre plates. The three bacteriocins were examined alone and in combination with each other. Nisin, pediocin and leucocin were each tested from the two-fold dilutions, at an initial concentration of 4 000 AU/mL. As combinations, two-fold dilutions of nisin were made in the solvent and pediocin and leucocin were added at 250 AU/mL, respectively. This process was repeated for pediocin and leucocin. **Table 3.2** indicates the layout for the experiment.

Table 3.2. Combinations of bacteriocins used in the microtitre broth dilution method.

*[Varied]**	*[Constant]* [250 AU/ml]	*[Varied]**	*[Constant]* [250 AU/ml]	*[Varied]**	*[Constant]* [250 AU/ml]
Nisin	-	pediocin	-	leucocin	-
Nisin	pediocin	pediocin	nisin	leucocin	pediocin
Nisin	leucocin	pediocin	leucocin	leucocin	nisin

*Eight Doubling dilutions from a concentration of 4 000 AU/ml (4 000, 2 000, 1 000, 500, 250, 125, 62.5, 31.3 AU/ml)

The first two rows of each microtitre plate received 80 µl of sterile MRS media and 20 µl of sterile solvent to act as a blank and sterility control, respectively. The DIIIM:I LAB wine isolate was cultivated at 30°C, subcultured twice for 18 h and grown to an OD of 0.6 at 620 nm to ensure that the cells were in log-phase. The cells were further diluted with MRS to an OD_{620} of 0.285 that, when diluted five times, yielded 5 x 10^5 cfu/mL, based on the relationship OD_{620} 0.20 = 5 x 10^5 cfu/mL (Lehrer et al., 1991). The remaining wells received 80 µl of the cell suspension, and 20 µL of the five times sample stock was added to the rows with the cell suspension as quadruplicate values. The first two rows containing cells received only sterile solvent, and served as growth controls. The plates were covered and incubated at 30°C for 12 h. The light dispersion in each well was determined using a microtitre plate reader (Multiscan Titertek) at 620 nm, and again every 2 h thereafter for the next 12 h. The Graphpad Prism version 3.0 for Windows (Graphpad Sotfware, USA) was used to analyse the data obtained. Nonlinear regression was performed on the data and a sigmoidal curve with variable slope was fitted to each of the data sets.

3.3 RESULTS

3.3.1 Preliminary screening of bacteriocin producers against sensitive strains with cell-free culture supernatant

The nisin producer consistently formed the largest inhibition zones, in comparison to all the genera of the LAB tested. Pediocin generally formed zones smaller than those of nisin, but displayed activity against the majority of LAB examined. Leucocin had the weakest inhibitory action against the LAB tested. In some cases the inhibitory zones were so slight that they could have been ascribed to acid formation. The spot on lawn method performed in 3.2.5 eliminated this problem. The results of the overlay method were confirmed by the results obtained in section 3.3.4. The yeasts

and AAB were not sensitive and no zones were observed when screened against the bacteriocin producers.

3.3.2 Leucocin and pediocin isolation

After ammonium sulphate precipitation of the cell-free culture supernatant, the bacteriocin activity increased on average six to eight-fold. The activity of the precipitate formed during the chloroform/methanol extraction increased further by 64 to 128-fold. Initially, dialysis followed ammonium sulphate precipitation, but dialysis was later eliminated from the isolation protocol due to a severe loss of yield. **Table 3.3** illustrates the increase and decrease in titres and AU/mL when several isolation protocols were followed.

Table 3.3. Influence of purification methods on the increase or decrease in titres and Arbitrary Units/ml (AU/ml) according to the purification method used for pediocin.

Purification Method	Titre	AU/ml	Increase or Decrease
Unpurified supernatant	5	3 200	control
Adsorption/desorption	0	0	decrease
Ammonium sulphate precipitation	8	25 600	six to eight-fold increase
Chloroform/methanol precipitation	14	1 638 400	64 to 128-fold increase
Dialysis	5	3 200	decrease
Chromatography	6	6 400	two-fold increase

3.3.3 Purity and molecular mass confirmation

The Tricine-SDS-PAGE gel stained with Coomassie Brilliant Blue (result not shown) showed peptide bands for the bacteriocins, which corresponded to the expected peptide sizes, namely 4 623 Da for pediocin and 3 932 Da for leucocin. After 24 h of incubation at 37°C, clear inhibition zones of *List. monocytogenes* B73, corresponding to the molecular mass of each of the bacteriocins, could be observed on the overlaid gel.

3.3.4 Screening with partially purified bacteriocin solutions

Table 3.1a, b, c lists the bacteria and yeasts used in this study, as well as their sensitivity towards the bacteriocins. The LAB panel showed varying levels of sensitivity. Nisin constantly resulted in the highest level of inhibition, followed by pediocin. Leucocin had the weakest inhibitory action. The *O. oeni* strains were particularly sensitive to the action of all the bacteriocins. As expected, no inhibition was observed in the case of AAB or yeasts.

3.3.5 Bacteriocin stability

The pediocin activity levels decreased systematically in the white must over the period monitored, from a titre of seven (12 800 AU/mL) to a titre of one to two (200-400 AU/mL), a level still sufficient to inhibit some of the LAB occurring in wine to some extent. When considering the decrease in AU/mL, it seems that the decrease was more rapid for the samples at pH 3 than for the other samples at a higher pH

(**Figures 3.1 and 3.2**). Ethanol or SO_2 had no discernable influence on the decrease in activity. The initial sharp decrease in activity is probably due to the proteins binding to the must components.

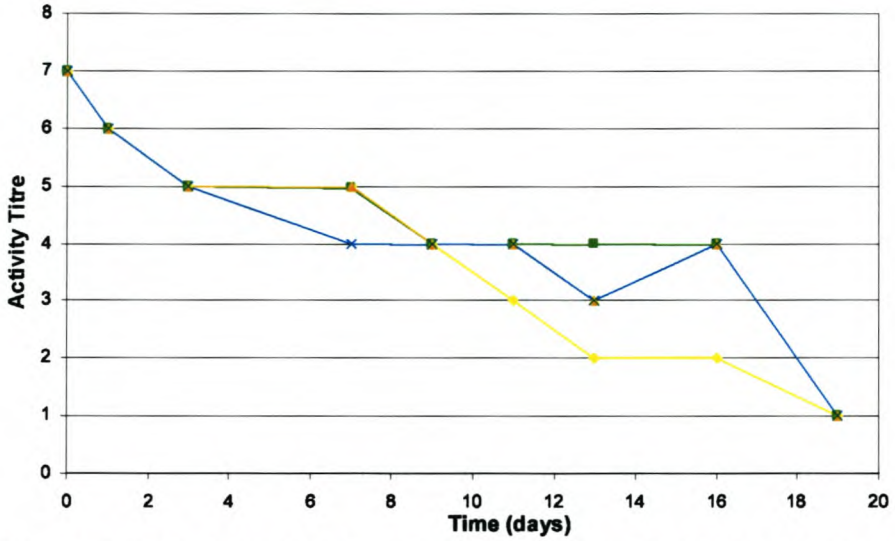


Figure 3.1. The activity titre decrease of pediocin in grape must at 14% alcohol over 20 days at a pH of 3.5 and varying SO_2 levels. ♦ pH 3.5; SO_2 0 mg/l; ■ pH 3.5; SO_2 40 mg/l; ▲ pH 3.5; SO_2 60 mg/l; × pH 3.5; SO_2 80 mg/l.

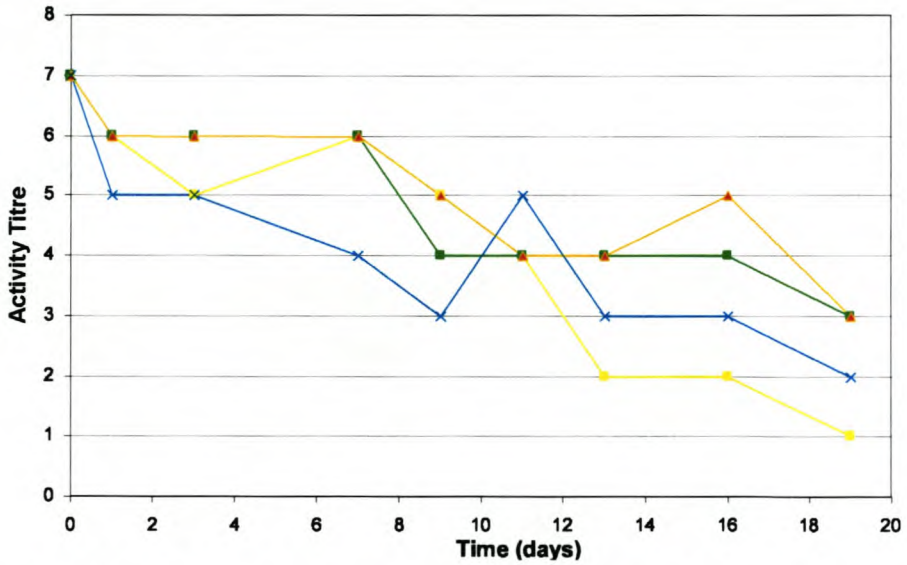


Figure 3.2. The activity titre decrease of pediocin in grape must at 14% alcohol over 20 days at a pH of 4 and varying SO_2 titres. ■ pH 4.0; SO_2 0 mg/l; ■ pH 4.0; SO_2 40 mg/l; ▲ pH 4.0; SO_2 60 mg/l; × pH 4.0; SO_2 80 mg/l.

3.3.6 Effect of varying bacteriocin concentrations against increasing bacterial numbers

3.3.6.1 Increasing bacteriocin levels against lactic acid bacteria

Samples containing levels of 10^2 cfu/mL DIIIM:I were inhibited most effectively, with cell growth only becoming apparent after approximately 14 days (results not shown).

The lower bacteriocin levels proved to be much less effective, specifically the leucocin-pediocin combination.

At 10^4 cfu/mL, the inhibitory activity of the bacteriocins was still quite effective against DIIIM:I in most cases. The samples that showed the highest growth contained the lowest concentration of bacteriocins, specifically the leucocin-pediocin combination. The growth of samples with nisin-pediocin and nisin-leucocin at 100 AU/mL and leucocin-pediocin at 500 and 1 000 AU/mL was comparable to that of the control, especially after day 14 (**Figures 3.3, 3.5 and 3.7**).

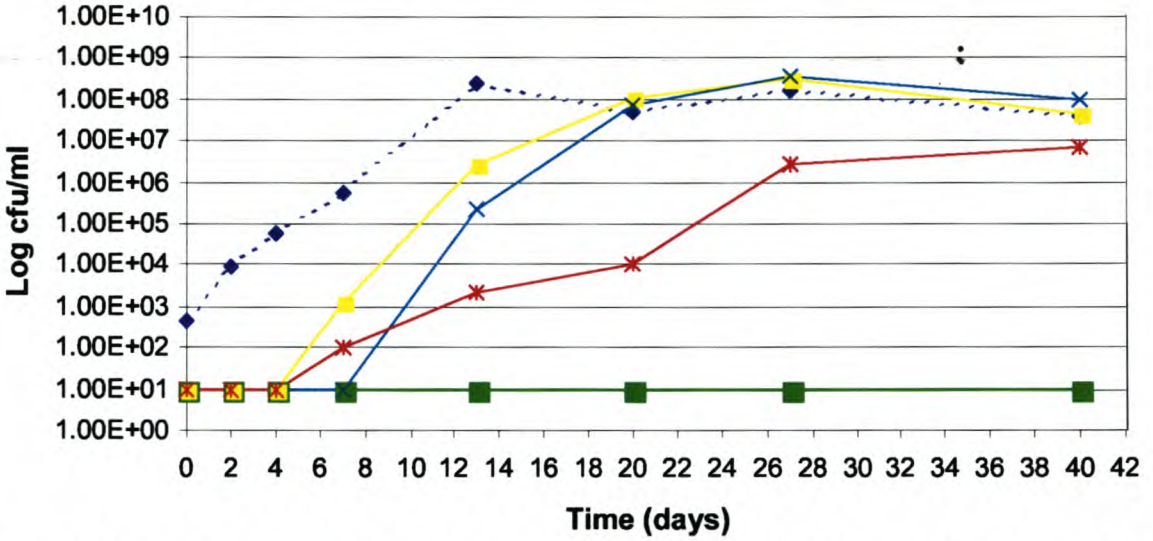


Figure 3.3. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^4 cfu/ml, with 100 AU/ml each of nisin, pediocin and leucocin in various combinations. ■ Nisin-Pediocin-Leucocin; ■ Nisin-Leucocin; × Nisin-Pediocin; ✳ Leucocin-Pediocin; ♦ Control.

The inhibitory effect was not as pronounced in the case of 10^6 cfu/mL, with several samples (all 100 and 500 AU/mL samples, 1 000 AU/mL leucocin-pediocin) growing as well as or slightly less effectively than the control. There was almost no difference in the cell counts of the control and the samples after days five to eight, and an initial lag-phase in the case of higher concentrations of bacteriocins. The nisin-leucocin and nisin-leucocin-pediocin combinations were the most effective at 1 000 and 500 AU/mL, respectively (**Figures 3.4, 3.6 and 3.8**).

In the case of 10^8 cfu/mL, there was little significant difference between the control and the leucocin-pediocin combination of 500 AU/mL from the first day. In most cases, the wine isolate grew to the level of the control by day 20 and by day 28 most samples were at roughly the same cell concentration of 10^8-10^9 cfu/mL. The combination of nisin-leucocin and all three bacteriocins in combination at 1 000 AU/mL exhibited the strongest inhibition, followed by nisin-pediocin at 1 000 units and nisin-pediocin, nisin-leucocin and nisin-leucocin-pediocin at 500 AU/mL.

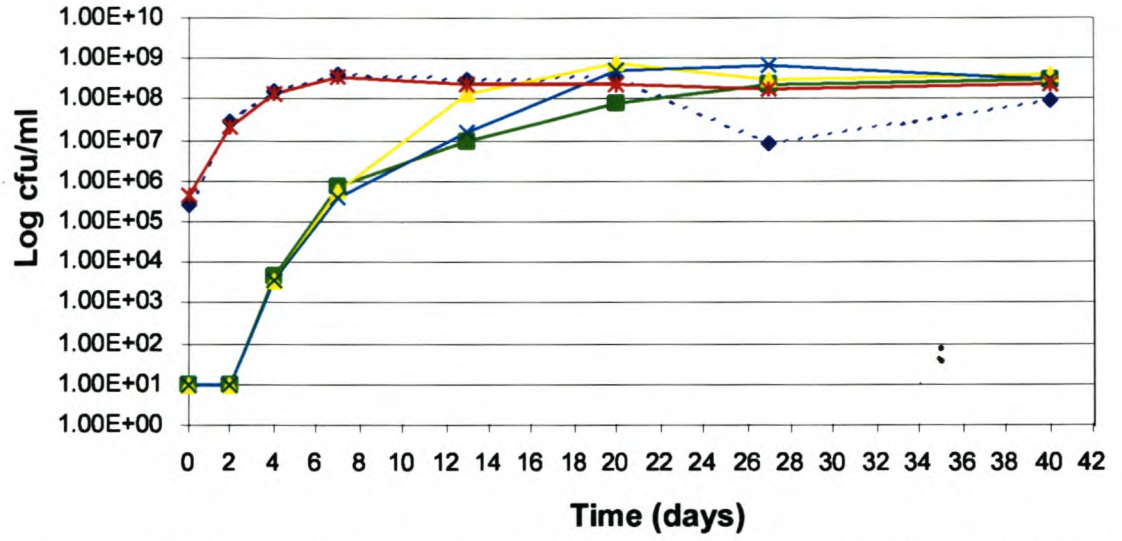


Figure 3.4. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^6 cfu/ml, with 100 AU/ml each of nisin, pediocin and leucocin in various combinations. ■ Nisin-Pediocin-Leucocin; ▲ Nisin-Leucocin; × Nisin-Pediocin; ✳ Leucocin-Pediocin; ♦ Control.

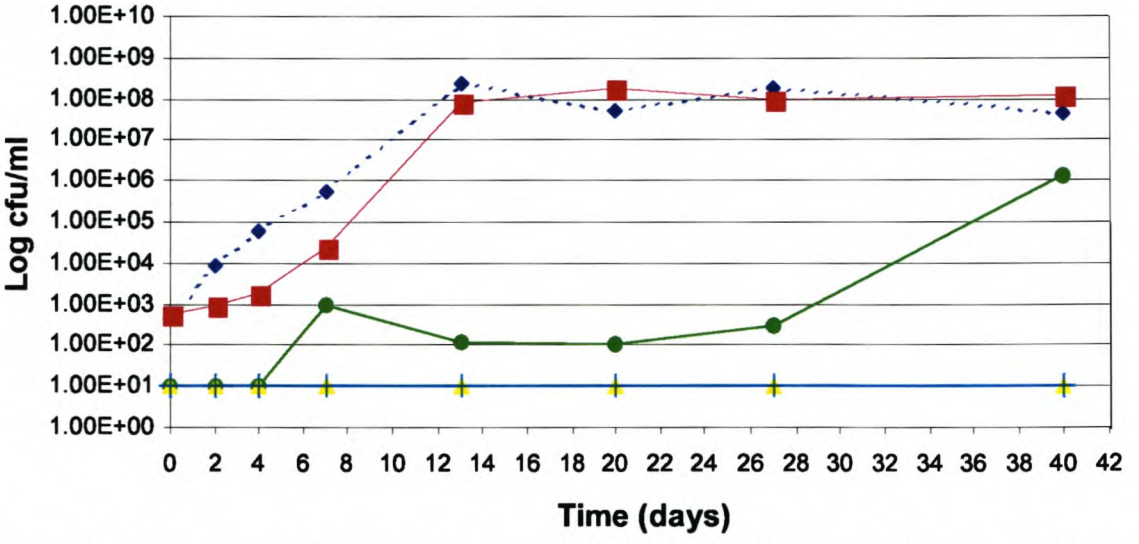


Figure 3.5. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^4 cfu/ml, with 500 AU/ml each of nisin, pediocin and leucocin in various combinations. ● Nisin-Pediocin-Leucocin; ▲ Nisin-Leucocin; | Nisin-Pediocin; ■ Leucocin-Pediocin; ♦ Control.

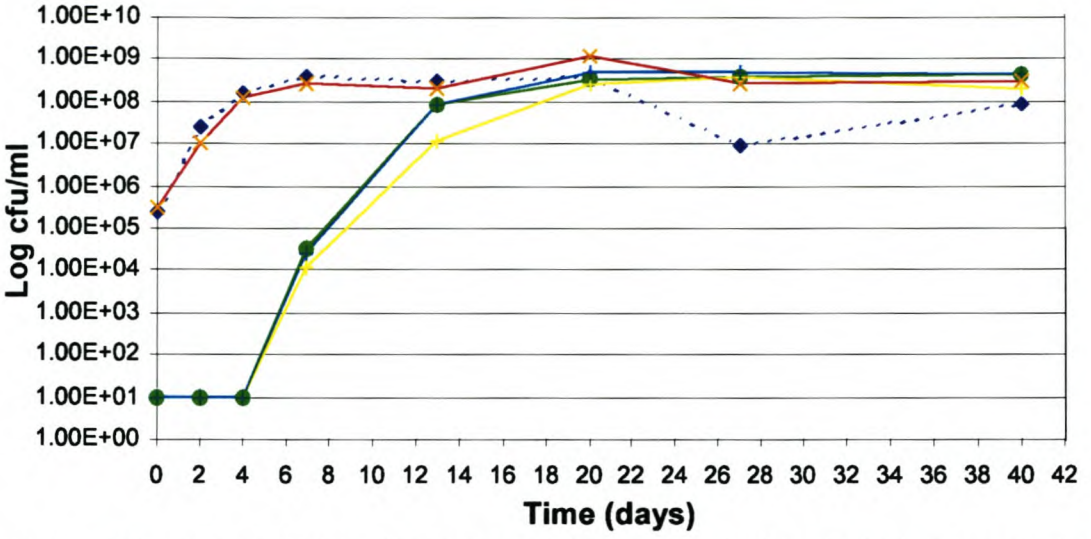


Figure 3.6. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^6 cfu/ml, with 500 AU/ml each of nisin, pediocin and leucocin in various combinations. ● Nisin-Pediocin-Leucocin; | Nisin-Leucocin; | Nisin-Pediocin; × Leucocin-Pediocin; ♦ Control.

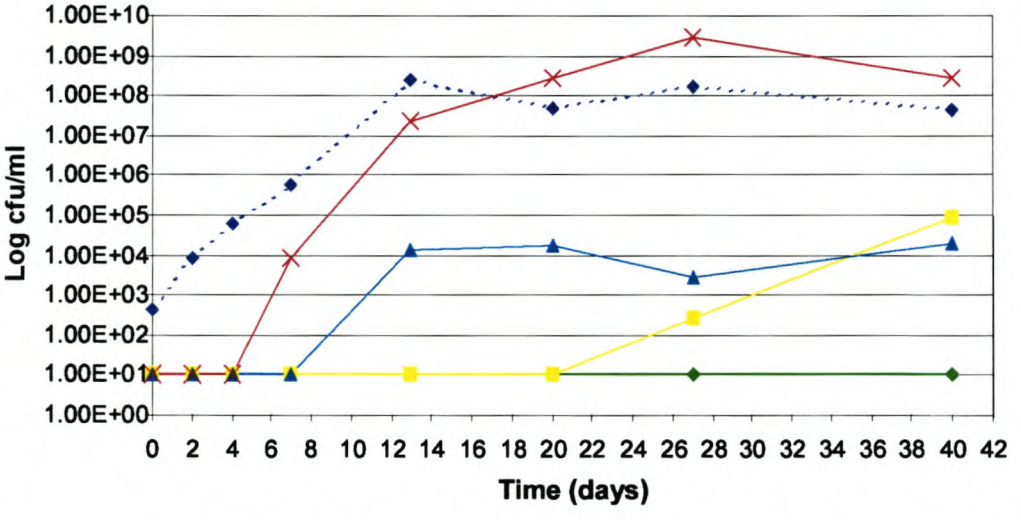


Figure 3.7. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^4 cfu/ml, with 1 000 AU/ml each of nisin, pediocin and leucocin in various combinations. ♦ Nisin-Pediocin-Leucocin; ■ Nisin-Leucocin; ▲ Nisin-Pediocin; × Leucocin-Pediocin; ♦ Control.

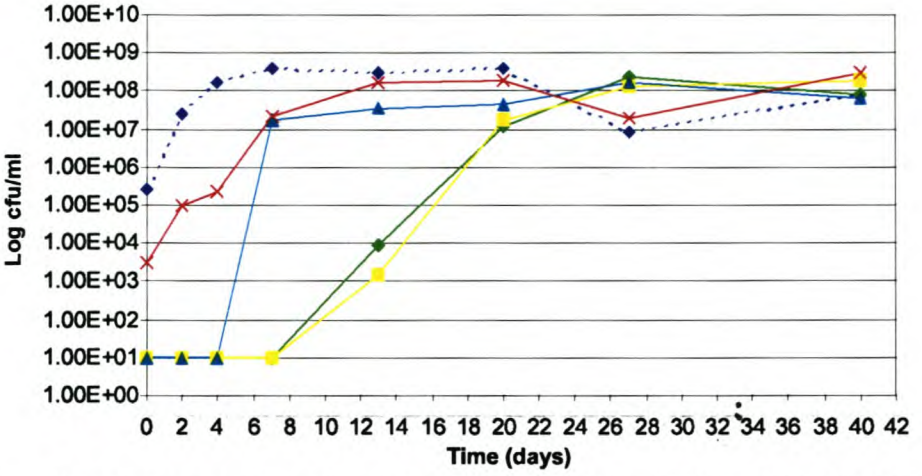


Figure 3.8. The increase in cell counts of LAB wine isolate DIIIM:I from an initial concentration of 10^6 cfu/ml, with 1 000 AU/ml each of nisin, pediocin and leucocin in various combinations. ♦ Nisin-Pediocin-Leucocin; ■ Nisin-Leucocin; ▲ Nisin-Pediocin; × Leucocin-Pediocin; ♦ Control.

3.3.6.2 Combinations of bacteriocins against acetic acid bacteria

The AAB displayed no sensitivity towards the combinations of bacteriocins used (data not shown).

3.3.7 Combination of bacteriocins and lysozyme

3.3.7.1 Against lactic acid bacteria

LAB cell enumeration did not indicate any significant difference between the samples containing pediocin and the combination of lysozyme and pediocin, and the LAB were inhibited almost completely after only 12 h. They remained at levels too low to detect for the next 17 days, indicating complete inhibition. There was no marked difference between the sample containing only lysozyme and the control (**Figure 3.9**). The sample containing lysozyme, in fact, closely followed the cell counts obtained for the control.

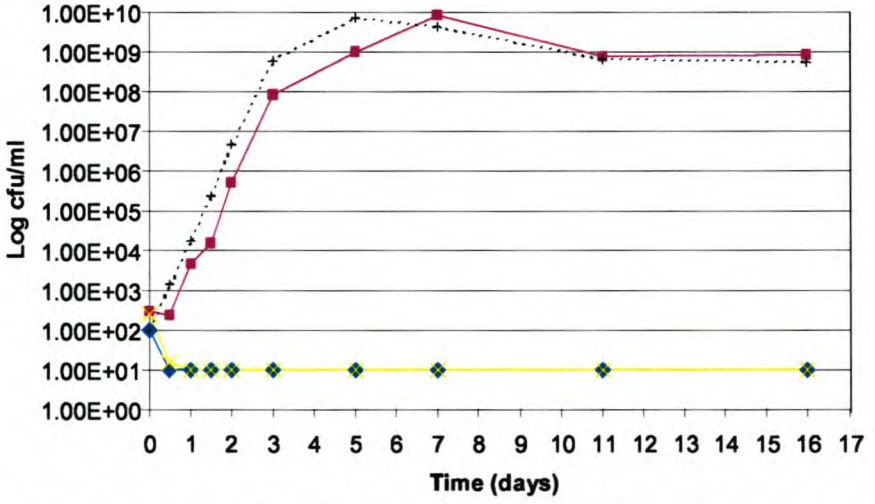


Figure 3.9. The cell growth curves of LAB wine isolate DIIIM:I at an initial concentration of 10^3 cfu/ml with added pediocin, lysozyme and a combination of pediocin and lysozyme. ♦ Pediocin; ■ Lysozyme, × Pediocin-Lysozyme; + Control.

3.3.7.2 Against acetic acid bacteria

The four species of Gram-negative AAB were not inhibited by the combination of pediocin and lysozyme. Cell enumeration indicated no difference between the samples containing lysozyme and bacteriocins in comparison to the controls containing no antibacterial substances (results not shown).

3.3.8 Scanning electron microscopy: morphological effects

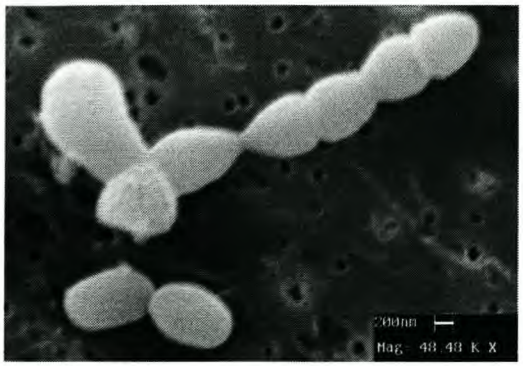
The bacteriocins had an obvious effect on the cell surface, such as pores in the cell surface, causing major disturbances in the cell morphology, in comparison to the control (**Figure 3.10a**). The pore formation led to the release of cytoplasmic material, causing the cells to lose their shape and deform (**Figure 3.10b, c, e**). Another noticeable effect was drastic abnormalities at the position of the septa (**Figure 3.10d**) and the release of large pieces of material. At a total concentration 5 000 AU/mL of each bacteriocin (1:1), the leucocin-pediocin combination (**Figure 3.10g**) had the least drastic effect when viewed, with more intact cells remaining. Nisin-pediocin (**Figure 3.10f**) had a dramatic effect, more so than leucocin-pediocin. Nisin-leucocin (**Figure 3.10e**) had a dramatic effect on the cells, resulting in a major release of cell material and pronounced abnormalities on the cell tips. Nisin-pediocin-leucocin (1:1:1) (**Figure 3.10h**) had a similar effect, causing ruffling of and perturbations on the cell surface.

3.3.9 Microtitre Broth Dilution assay

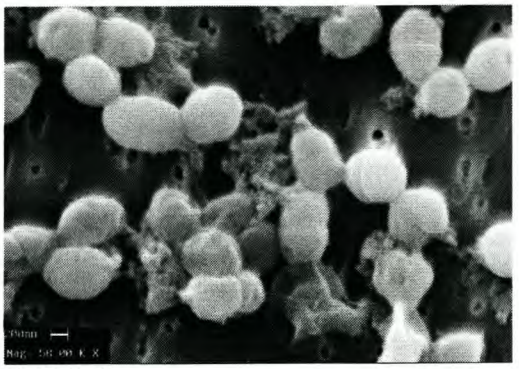
Serial two-fold dilutions were performed with nisin, leucocin and pediocin from an initial concentration of 4 000 AU/mL. The results discussed were taken after 14 and 16 h. Leucocin was the most effective inhibitor, with the lowest concentration needed to inhibit 50% of the population (IC_{50}) of the wine isolate. Nisin and pediocin had matching IC_{50} values (**Figure 3.11a**), which were slightly higher than that of leucocin after 14 h. When 250 AU/mL of a bacteriocin was added to the doubling dilutions of another bacteriocin, a shift in IC_{50} was noted in most cases. Most notably, the combinations of the bacteriocins inhibited the bacterial population more effectively at low AU/mL values. In some cases, the inhibition curve indicated that more than 50% of the population was inhibited, in comparison to other samples, where there was less than 50% inhibition.

The two test series containing two of the three peptides were compared, and of the two, the addition of a constant concentration of leucocin to decreasing concentrations of nisin proved to be the strongest inhibitory combination. The combination of 250 AU/mL leucocin added to the nisin inhibited the bacterial population to a level of approximately 63%, while the samples with a constant concentration of nisin added to a decreasing leucocin concentration had a IC_{50} value of about 230-250 AU/mL. Both series, however, exhibited final inhibition levels much higher than that of leucocin or nisin alone.

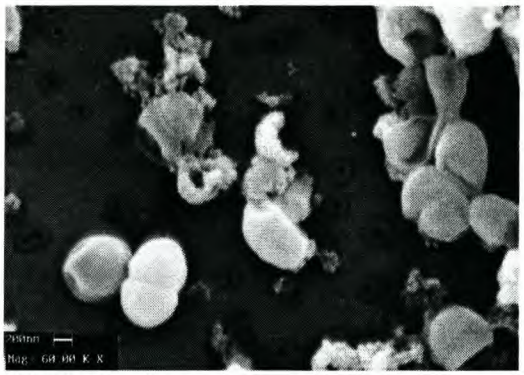
In the case of the leucocin-pediocin combination, the addition of 250 AU/mL leucocin to decreasing pediocin levels was the most effective combination, inhibiting approximately 75-100% of the population, even at levels below 250 AU/mL. Pediocin addition to decreasing levels of leucocin, however, proved to be less effective at lower concentrations, being comparable to the action of pediocin at lower concentrations. However, there was a lowering in the IC_{50} value.



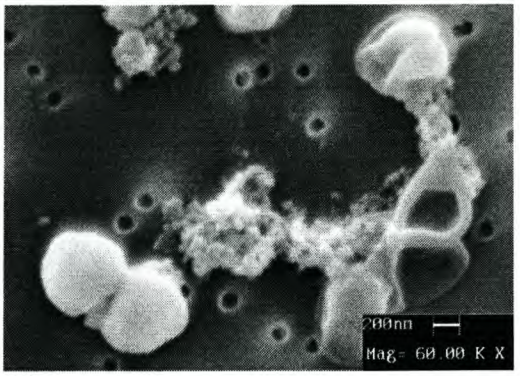
(a) Control



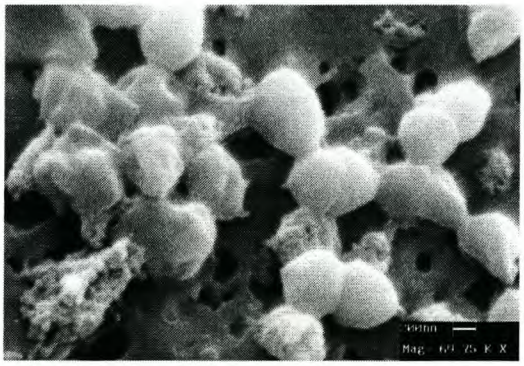
(b) Nisin



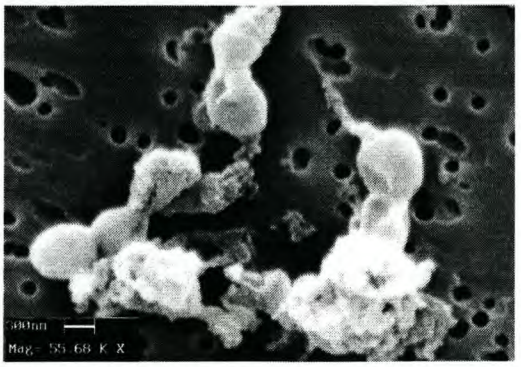
(c) Pediocin



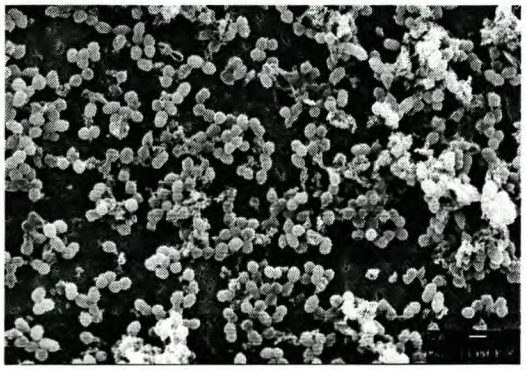
(d) Leucocin



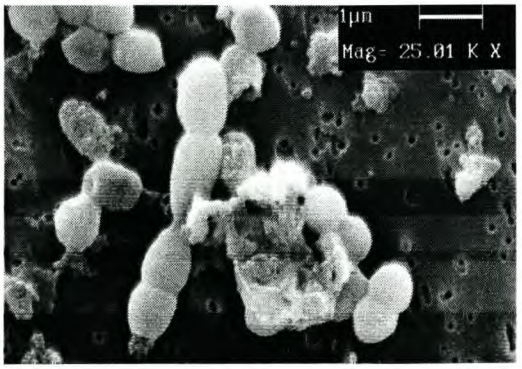
(e) Nisin-leucocin



(f) Nisin-pediocin



(g) Leucocin-pediocin



(h) Nisin-leucocin-pediocin

Figure 3.10. Influence of nisin, pediocin and leucocin and combinations of these at a total concentration of 5000 AU/ml on the morphology of wine isolate DIIIM:I, as assessed by scanning electron microscopy: (a) untreated control; (b) nisin treated; (c) pediocin treated; (d) leucocin treated; (e) nisin:leucocin treated (1:1); (f) nisin:pediocin treated (1:1); (g) leucocin:pediocin treated (1:1); (h) nisin:leucocin:pediocin treated (1:1:1).

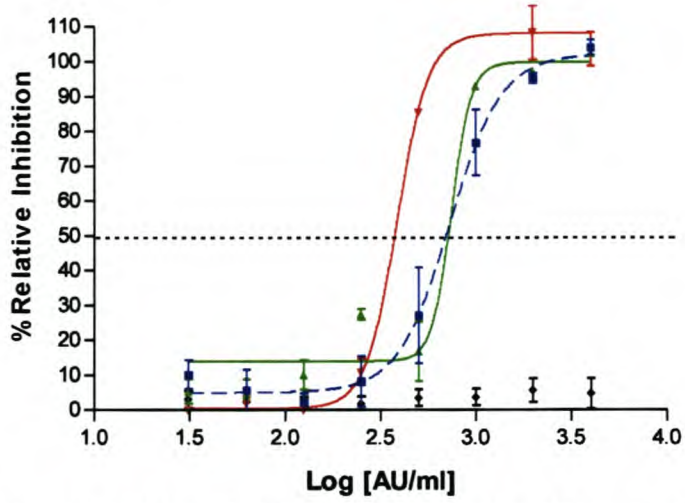


Figure 3.11a. The % relative inhibition curves for nisin, pediocin and leucocin, recorded after 14 h against DIIIM:I. The IC_{50} value is obtained where the dashed line intersects the inhibition curve. ■ Nisin at decreasing concentrations; ▲ Pediocin at decreasing concentrations, ▼ Leucocin at decreasing concentrations; ♦ Control.

When nisin was added to decreasing concentrations of pediocin, the IC_{50} did not shift noticeably, although lower concentrations (500 AU/mL and lower) inhibited approximately 50% of the population, whereas pediocin alone at those concentrations had almost no inhibitory action. The addition of pediocin to nisin had almost no effect on the IC_{50} and the inhibition curve, the two curves being too similar to predict any effect on activity.

When the inhibition values of the one bacteriocin at a certain concentration and the bacteriocin added at a constant concentration were compared and added, the inhibitory effect of the two substances was more than the additive inhibition. For example, at 250 AU/mL each of nisin and leucocin, the percentage relative inhibition is less than 10%, but used in combination, it is approximately 50% for both ratios used. If the effect had been additive, inhibition would have been less than 20%, indicating some synergism at work. The percentage relative inhibition values of nisin, leucocin and pediocin at a concentration of 250 AU/mL each were then subtracted from the samples containing combinations of bacteriocins. The effect of synergism was more noticeable. Leucocin addition at constant levels to both nisin and pediocin proved to be the most effective (**Figure 3.11b, c**). Although the percentage relative inhibition at higher concentrations (500 AU/mL and higher) was less effective than nisin and pediocin alone, lower levels (125 AU/mL and lower) inhibited more than 50% of the bacterial population. In isolation, these bacteriocins have almost no inhibitory effect at concentrations below 250 AU/mL. Nisin addition to pediocin and leucocin had the same effect, lowering the inhibitory effect at higher concentrations, but increasing the effectiveness at lower concentrations. In the case of nisin (**Figure 3.11d, e**) addition to leucocin, the IC_{50} was lowered slightly, while nisin addition increased the IC_{50} slightly in the case of pediocin. Pediocin (**Figure 3.11f, g**) lowered the percentage relative inhibition values of both leucocin and nisin by approximately 30%. As observed before, the combination of pediocin and leucocin lowered the IC_{50}, and this was the case again. However, the overall inhibitory effect was decreased. As observed before, the pediocin-nisin combination increased the IC_{50},

as well as exhibiting less inhibition at low concentrations. When samples measured after 14 h were compared to samples measured after 16 h, it was noticeable that some of the inhibitory effect had diminished (**Figure 3.11e**).

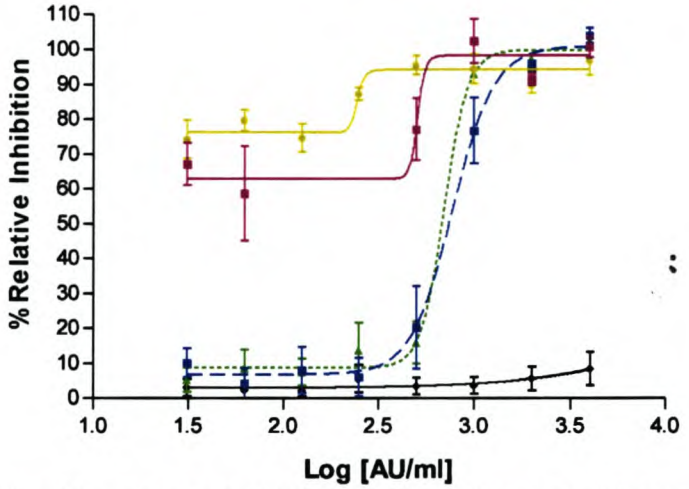


Figure 3.11b. The % relative inhibition curves for 250 AU/ml leucocin added to decreasing concentrations of nisin and pediocin, recorded after 14 h against DIIIM:I. ■ Nisin at decreasing concentrations, with 250 AU/ml leucocin added to each concentration; ● Pediocin at decreasing concentrations, with 250 AU/ml leucocin added to each concentration; ■ Nisin at decreasing concentrations; ▲ Pediocin at decreasing concentrations, ♦ Control.

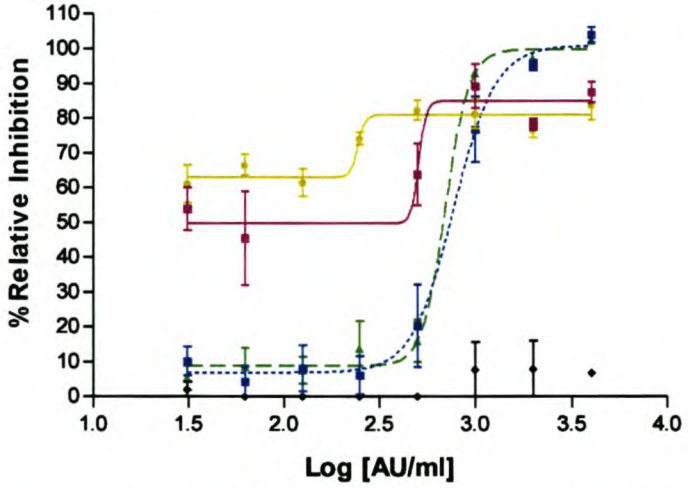


Figure 3.11c. The % relative inhibition curves for samples with 250 AU/ml leucocin added to decreasing concentrations of nisin and pediocin, with the % relative inhibition value subtracted from the values obtained for the combination, recorded after 14 h against DIIIM:I. ■ Nisin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml leucocin subtracted from the combined value; ● Pediocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml leucocin subtracted from the combined value; ■ Nisin at decreasing concentrations; ▲ Pediocin at decreasing concentrations, ♦ Control.

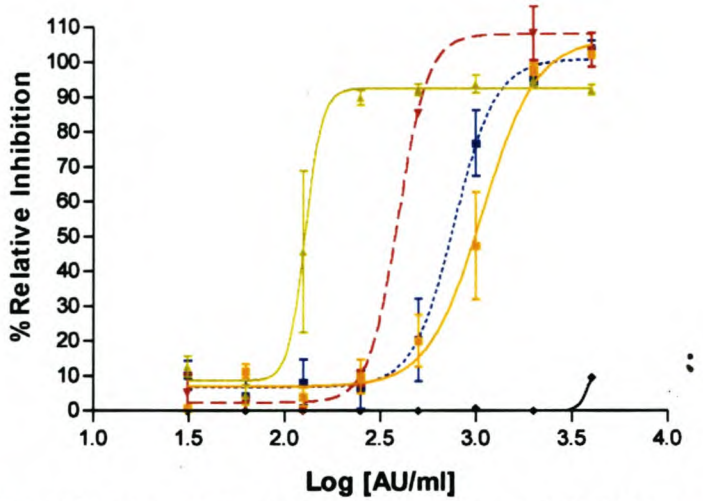


Figure 3.11d. The % relative inhibition curves for 250 AU/ml pediocin added to decreasing concentrations of leucocin and nisin, recorded after 14 h against DIIIM:I. ■ Nisin at decreasing concentrations, with 250 AU/ml pediocin added to each concentration; ▲ Leucocin at decreasing concentrations, with 250 AU/ml pediocin added to each concentration; ■ Nisin at decreasing concentrations; ▼ Leucocin at decreasing concentrations, ♦ Control.

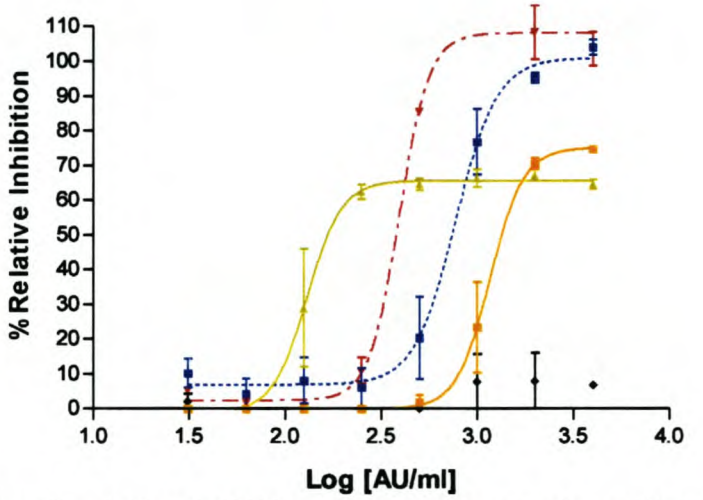


Figure 3.11e. The % relative inhibition curves for samples with 250 AU/ml pediocin added to decreasing concentrations of leucocin and nisin, with the % relative inhibition value subtracted from the values obtained for the combination, recorded after 14 h against DIIIM:I. ■ Nisin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml pediocin subtracted from the combined value; ▲ Leucocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml pediocin subtracted from the combined value; ■ Nisin at decreasing concentrations; ▼ Leucocin at decreasing concentrations, ♦ Control.

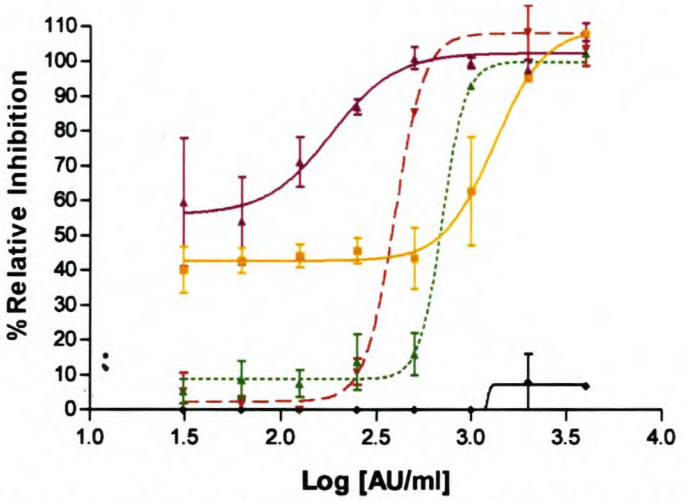


Figure 3.11f. The % relative inhibition curves for 250 AU/ml nisin added to decreasing concentrations of leucocin and pediocin, recorded after 14 h against DIIIM:I. ■ Pediocin at decreasing concentrations, with 250 AU/ml nisin added to each concentration; ▲ Leucocin at decreasing concentrations, with 250 AU/ml nisin added to each concentration; ▲ Pediocin at decreasing concentrations; ▼ Leucocin at decreasing concentrations, ♦ Control.

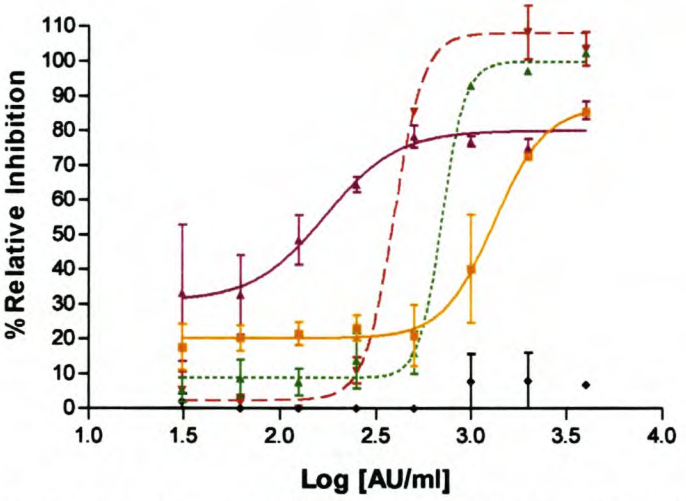


Figure 3.11g. The % relative inhibition curves for samples with 250 AU/ml nisin added to decreasing concentrations of leucocin and pediocin, with the % relative inhibition value subtracted from the values obtained for the combination, recorded after 14 h against DIIIM:I. ■ Pediocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml nisin subtracted from the combined value; ▲ Leucocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml nisin subtracted from the combined value; ▼ Pediocin at decreasing concentrations; ▼ Leucocin at decreasing concentrations, ♦ Control.

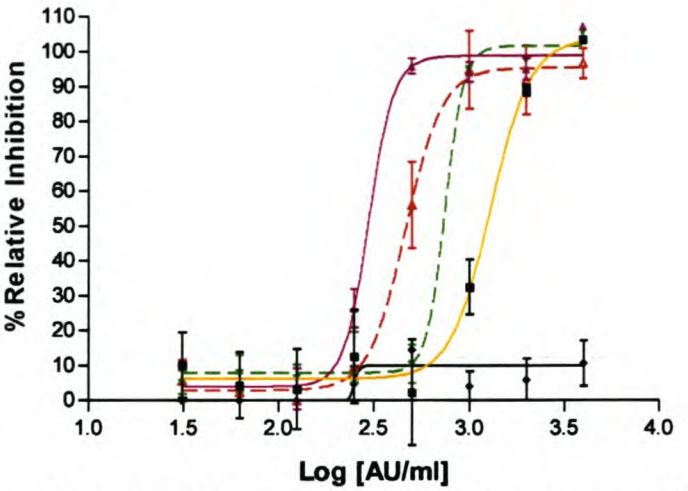


Figure 3.11h. The % relative inhibition curves for samples with 250 AU/ml nisin added to decreasing concentrations of leucocin and pediocin, with the % relative inhibition value subtracted from the values obtained for the combination, recorded after 16 h against DIIIM:I. ■ Pediocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml nisin subtracted from the combined value; ▲ Leucocin at decreasing concentrations, with the relative % inhibition values for 250 AU/ml nisin subtracted from the combined value; ▼ Pediocin at decreasing concentrations; ▼ Leucocin at decreasing concentrations, ♦ Control.

3.4 DISCUSSION AND CONCLUSION

All three bacteriocins, as well as lysozyme, are active in the wine pH range, leading to synergy between SO_2 and the other antimicrobial peptides. One of the main objectives of this study was to determine whether the selected bacteriocins and lysozyme combinations would be applicable as biopreservatives in a wine system. The antimicrobial activity of lysozyme against the LAB occurring in wine is well known and documented (Cunningham et al., 1991; Amati et al., 1992; Gerbaux et al., 1997) and should be active against wine microorganisms. The bacteriocins were used to screen against LAB reference strains and wine isolates, to determine their spectrum of activity. Nisin, known to be a very effective antimicrobial, had the strongest action against the LAB, followed by pediocin. Leucocin has a narrower inhibition spectrum with regard to the wine LAB, and this was apparent in this experiment. All three bacteriocins had varying degrees of activity against most of the LAB tested. This indicates that if the bacteriocins were to be used in combination, they might inhibit a wider range of bacteria due to their different inhibition spectra. As expected, there was no effect on the AAB or yeasts.

Tricine-SDS-PAGE gels were performed to confirm that the proteins isolated throughout the course of the experiments were of the right size and did indeed have antibacterial activity. Three bands of the expected size were observed, and *List. monocytogenes* was inhibited when the gel was overlaid.

Pediocin proved to be stable in a wine system, which would make it suitable for use in wine. The parameters used in the stability test were chosen to simulate wine conditions as closely as possible, taking into account alcohol levels, SO_2 levels and pH. The pH seemed to have the most pronounced effect on the decrease in AUs,

with the activity dropping the quickest at pH 3.0. However, most South African wines are in the region of pH 3.3-3.8, a pH at which the bacteriocins exhibit greater activity. Radler (1990a) found this to be the same for nisin. When leucocin was tested under the same conditions as pediocin, but in MRS medium and not in modified must, it exhibited the same tendencies as pediocin (personal communication, S. J. Krieling, Institute for Wine Biotechnology).

According to results obtained previously (Hanlin et al., 1993; Schillinger et al., 1996; Mulet-Powell et al., 1998; Parente et al., 1998; Horn et al., 1999), combinations of certain bacteriocins display synergism. To test this hypothesis, the bacteriocins (pediocin, leucocin and nisin) were used in combination against a wine-isolated LAB. Bacteriocins are known to be most effective against lower cell loads (approximately 10^3 cfu/mL), and this was confirmed by our results, in which lower cell loads were inhibited more effectively than higher cell numbers by almost all the bacteriocin combinations. In our system, it was found that the nisin-leucocin combination was the most effective of the pairs of bacteriocins tested. Nisin-pediocin was the second most effective. Leucocin-pediocin was the most ineffective combination of all the concentrations tested and had almost no inhibitory action against cell numbers of 10^4 to 10^8 cfu/mL. The nisin-leucocin combination at concentrations of 500 and 1 000 AU/mL was the most effective, inhibiting cell concentrations of 10^4 and 10^6 cfu/mL to the greatest extent. Bacteriocin combinations containing nisin resulted in the most effective inhibition in this experiment. The results obtained suggest that a possible synergistic action occurs in the nisin combinations, or that nisin is a more powerful antimicrobial substance. This will have to be confirmed using the purified peptide with the same specific activity. Samples with all three bacteriocins added, thus containing the most bacteriocin AU/mL, resulted in the most effective inhibition at all concentrations.

Classically, bacteriocins are proposed to cause cell death by permeabilisation of the cell wall of microorganisms and dissipating the proton motive force, with a concomitant release of cell contents (Bruno and Montville, 1993; Ennahar et al., 2000). Scanning electron microscopy indicated that, when used alone, all three bacteriocins exhibited distinct cell damage at a concentration of 5 000 AU/mL, causing large-scale release of the intracellular material. Combinations of bacteriocins, such as nisin-pediocin (1:1), nisin-leucocin (1:1), and leucocin-pediocin (1:1), elicited a similar response, but to varying degrees. The nisin-leucocin combination exhibited the strongest cell damage, followed by nisin-pediocin, while less cell damage was visible in the case of the leucocin-pediocin combination. This confirms the results obtained in the experiment, in which pairs of bacteriocins were added to increasing cell numbers in wine must. The combination containing all three bacteriocins (1:1:1) also caused dramatic cell damage.

Although the above-mentioned two tests indicated an interaction between nisin, pediocin and leucocin, it was difficult to describe precisely. The microtitre broth dilution method (Du Toit and Rautenbach, 2000) presented a sensitive and accurate means of assessing the sensitivity of the DIIIM:I wine isolate.

The use of microtitre plates allowed the simultaneous testing of large numbers of samples without being labour intensive. When used separately against the specific wine isolate, nisin, pediocin and leucocin were almost equally effective, making it possible to compare the results. By adding one of the bacteriocins to decreasing concentrations of the remaining two, any increase or decrease in activity

could easily be detected when compared to samples of bacteriocins alone. It was obvious that the inhibition curve was affected when additional bacteriocins were added to a sample. Generally, the maximum inhibitory concentration (IC_{max}) and minimum inhibitory concentration (IC_{min}) remained the same. In most cases, the IC_{50} was lowered, implying that less of the combination of pairs of bacteriocins was needed to inhibit the population. Most notably, in the case where 250 AU/mL of leucocin was added to nisin and pediocin, more than 50% of the population was inhibited. Leucocin therefore increased the inhibitory action of nisin and pediocin. Pediocin addition at 250 AU/mL to nisin increased the IC_{50} somewhat, while a decrease was observed when added to leucocin. When nisin was added to pediocin and leucocin, it was once again observed that the inhibitory action of the nisin-pediocin combination was diminished, while the leucocin-pediocin combination was more effective.

By subtracting the percentage relative inhibition values of nisin, pediocin and leucocin at 250 AU/mL, the absolute effect of the combinations could be observed, excluding any additive effects created by adding two bacteriocins together. If the effect of increased inhibition was additive, the subtraction would have given a curve similar to that of a bacteriocin tested alone. This was, however, not the case. In all the samples tested, a clear shift was seen in the inhibition curve. At high concentrations of the peptide used in the test, there was a decrease in inhibition, lowering the IC_{max}. This could be ascribed to different mechanisms of action, or to bacteriocins competing for binding receptors or space on the cell surface. At low concentrations of bacteriocin, to which 250 AU/mL of another bacteriocin was added, the inhibitory effect was much greater than that of the bacteriocin alone, increasing the percentage relative inhibition at the IC_{min}. This is best illustrated by the addition of leucocin to nisin and pediocin, in which case the inhibitory action is sufficient to inhibit approximately 50% of the population. Pediocin had a similar effect when added to leucocin and nisin, although the inhibitory potential was much less than when leucocin was added to pediocin. Nisin addition to pediocin and leucocin seemed to be the least effective combination. At low concentrations, the inhibition was similar to that of leucocin or pediocin used in isolation. Once again, the action of nisin and pediocin exhibited an antagonistic tendency.

From these results it is possible to suggest that there is a synergistic effect when certain bacteriocins are used in combination. Leucocin and pediocin exhibited synergistic action, as well as nisin and leucocin together. Nisin and pediocin used together seemed to have an antagonistic interaction. However, the ratio used seems to play an important role in the reaction. For example, low amounts of leucocin added to pediocin exhibited very high inhibition levels, in contrast to samples in which pediocin was added to leucocin. Combinations of nisin and leucocin and of nisin and pediocin did not seem to be affected by the ratios used. This is further illustrated by the bacteriocin combinations used against increasing cell numbers and the scanning electron microscopy images. Nisin-leucocin was the most powerful combination against the LAB, confirming the findings of the microtitre broth dilution method to a degree, with nisin-pediocin being less effective. Leucocin-pediocin was the least effective combination, although the microtitre test indicates that it should have been the most effective. The most important parameter in this study is therefore the ratio of the bacteriocins used with each other. Bacteriocin combinations should thus be

researched carefully in model food and beverage systems to determine their interactions with each other, before their usage is considered.

An additional fact to consider is the action of the bacteriocin over time. The action and stability of bacteriocins are time dependent, as indicated in both the experiments in which increasing cell numbers and the microtitre broth dilution method were used. The bacteriocins were effective only up to a certain time, depending on the experiment, and the remaining cells multiplied after the bacteriocins were exhausted. The time of bacteriocin and lysozyme addition should be decided on carefully, as both bind to wine components. Bacteriocins can be added to both wine and must. White wine contains fewer components, notably fewer colour components, that can bind these proteinaceous substances. The earliest time of bacteriocin addition in the case of white wine should be after must clarification, and if heat treatment is applied, it can be done before addition of the bacteriocin. Bacteriocins should be added to red wine only if no MLF is desired, at the earliest stage after pressing. Addition after racking off the lees is also a favourable stage. The LAB develop some time after fermentation and wines are most susceptible to microbial spoilage at this time (Radler, 1990b). Bacteriocins can be removed by normal fining procedures that remove proteins from wines to prevent haze formation. With the development of bactericidal yeast strains, as described by Schoeman et al. (1999), bacteriocins are produced continuously during the fermentation process and are an ideal alternative to adding purified bacteriocins. The *in situ* production of bacteriocins, whether by yeasts or native LAB producers, is also more economical than adding purified bacteriocins, which can be very expensive.

Chung and Hancock (2000) indicated that a synergy exists between nisin and lysozyme, and while the precise mechanism is not known at this stage, increased cell lysis is a definite possibility. Nisin might inhibit the energy-dependent processes that repair lysozyme damage. Nisin and lysozyme in combination prevent the growth of food spoilage organisms. In this study, pediocin and lysozyme were evaluated in combination, but the results were contradictory to those obtained by Chung and Hancock (2000). Unfortunately, any effect of lysozyme was overshadowed by the pediocin action. This should be explored more extensively in the future. The AAB are immune to the action of both bacteriocins and lysozyme, due to an additional lipopolysaccharide cell wall layer, making them impenetrable to the action of the antimicrobials (Cutter and Siragusa, 1995; Boziaris and Adams, 1999). *G. oxydans* was excluded from the tests in liquid media, as it is ethanol sensitive and would subsequently not occur in wine.

If the MLF is desired, bacteriocins can still be used to prevent spoilage by LAB present in the must and wine, but a bacteriocin-resistant *O. oeni* will have to be used to promote a pure culture MLF (Daeschel, 1989). Bacteriocin or lysozyme would also help preserve the acidity by preventing the MLF from occurring.

A number of bacteriocins, most notably nisin (Hurst, 1981; Ogden et al., 1988; Radler, 1990a, b; De Vuyst and Vandamme, 1994; Abee et al., 1995; Delves-Broughton et al., 1996; Choi and Park, 2000), pediocin (Ray, 1994; Cintas et al., 1995; Strasser de Saad et al., 1995; Montville and Chen, 1998) and, to a lesser extent leucocin, have been tested for applications in food preservation. Pediocin is one of the most characterised bacteriocins and will probably be the second bacteriocin, after nisin, to find practical applications in the food industry (Chikindas et al., 1995; Venema et al., 1995). The use of pediocin PA-1 is covered by several

patents in the US and Europe (Montville and Chen, 1998). To obtain the approval of governing bodies, however, complete chemical, physical and genetic characterisation of potential new antimicrobials will be necessary (Jager and Harlander, 1992).

In conclusion, the results indicate that bacteriocins and lysozyme have vast potential for use as biopreservatives in winemaking. This study indicated that bacteriocins other than nisin, such as pediocin and leucocin, are also stable in a wine environment. The crucial factor affecting stability in white wine is the pH. To broaden the inhibition spectrum, bacteriocins can be used in combinations. As combinations of these antibacterial substances have proven to be synergistic or antagonistic, the combinations should be evaluated carefully. Should genes be selected for heterologous expression in a host such as yeast or bacteria, this factor should be considered carefully.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

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Since less heavily preserved, minimally processed, safer and more natural food and beverages have become in demand internationally, antimicrobial peptides from lactic acid bacteria and bacteriolytic enzymes have received considerable attention to be applied as biological control agents (Cunningham et al., 1991; Schillinger et al., 1996; Stiles, 1996; Cleveland et al., 2001). As long as there is a consumer demand for innovative products that are healthier and contain fewer preservatives, researchers will always be on the lookout for new substances that will allow them to meet this challenge. With this demand in mind, the wine industry aims to reduce the levels of chemical preservatives used, especially sulphur dioxide, which is used mainly as antimicrobial agent. In recent years wine has received much interest, due to its possible health benefits when used in moderation.

It can be seen that the wine industry took this challenge seriously, as the OIV (*Office International de la Vigne et du Vin*) - wine governing body - accepted the proposal for lysozyme use in wine, with due precautions (Resolution Oeno15/2001). This approval is an indication that the use of alternative preservatives is a feasible option. Nisin has been approved for use in specified food and beverages in a number of countries (Abee et al., 1995; Choi and Park, 2000), and wine could be among the next foodstuffs in which its use gains acceptance. Both these substances carry the added benefit of being harmless to humans when ingested (Claypool et al., 1966; Hansen, 1993).

This study outlines the investigation of the suitability of nisin, pediocin, leucocin and lysozyme, specifically in combination, for the preservation of wine. Nisin and pediocin proved to be the most effective against LAB, in both type and reference strains and in wine isolates. As anticipated, no activity was detected against the Gram-negative AAB and yeast. This indicates that the yeast alcoholic fermentation will not be affected when these substances are used, implying a number of advantages. For instance, troublesome spoilage LAB could be inhibited without interfering with the alcoholic fermentation. As SO_2 also inhibits yeasts, its addition could, in the case of concomitant stuck or slow fermentations, necessitate secondary yeast inoculations.

Both leucocin and pediocin stability tests indicated that the bacteriocins were stable for a sufficient period of time under wine conditions, with white wine components not having a significant effect on the inhibitory action against the test organism.

The bacteriocins were tested in increasing concentration against wine isolate DIIIM:I at increasing cell numbers. The bacteriocins were added in combinations of nisin-pediocin, nisin-leucocin, leucocin-pediocin and nisin-leucocin-pediocin at 100, 500 and 1 000 AU/ml each against the LAB in the must. As a rule, the combinations were more effective against lower cell concentrations (10^2 and 10^4 cfu/ml), while exhibiting less marked inhibition against higher cell concentrations (10^6 and 10^8 cfu/ml) over a period of 40 days. LAB usually occur in wine in numbers of 10^3-10^4 cfu/ml. The highest concentration of bacteriocin at 1 000 AU/ml was the most effective, while concentrations of 500 and 100 AU/ml were much less effective in

inhibiting the sensitive LAB used in the study. Of the different combinations, nisin-leucocin was the most effective, followed by nisin-pediocin. Leucocin-pediocin demonstrated the least inhibition, with bacterial growth similar to that of the control without bacteriocins from the first days of observation, or reaching elevated levels within the first 14 days in the case of lower cell concentrations.

The possibility of a synergistic action in the combination of bacteriocins was anticipated (Hanlin et al., 1993; Schillinger et al., 1996; Mulet-Powell, et al., 1998; Parente et al., 1998; Horn et al., 1999). This interaction was investigated by scanning electron microscopy to determine the effect on cell morphology. The microtitre broth dilution method was further utilised to determine the concentrations of bacteriocins needed in combination to inhibit a sensitive organism, in contrast to the concentrations of individual bacteriocins.

For the scanning electron microscopy, combinations were used as in the above experiment and bacteriocins were added at a total concentration of 5 000 AU/ml against the wine isolate used before. The scanning electron micrograph images demonstrated clear damage to the cells, with the bacteriocins having an obvious effect on the cell surface, causing major disturbances to the cell morphology, such as perturbations, holes or craters. The wine isolate displayed drastic abnormalities at the position of the cell wall septa, where the cells were joined, and the release of large pieces of material – presumably the cell contents – was noticeable. Bacteriocin combinations elicited the same response as the individual bacteriocins, but in varying degrees. Nisin-leucocin combinations had the most visible effect on the cell surface and septa. Leucocin-pediocin combinations resulted in the most intact cells between injured cells.

The microtitre broth dilution method (Du Toit and Rautenbach, 2000) presents a simple, accurate and sensitive method for the assay of antimicrobial activity. In this assay, the three bacteriocins were tested alone against the previously-used wine isolate DIIIM:I to determine the inhibitory effect in isolation. All three bacteriocins were serially two-fold diluted from a concentration of 4 000 AU/ml to 31 AU/ml in the titre plates and the LAB was added to the peptides in the titre plates. Further samples were included in the control: Nisin was diluted as described, and 250 AU/ml of either pediocin or leucocin was added to the diluted samples to determine if the addition exhibited any diminished or increased inhibition when used in combination. The same experiment was applied in the case of pediocin and leucocin, with one bacteriocin concentration being diluted and the other two bacteriocins being added at the same concentration.

It was obvious that the inhibition curves were affected when additional bacteriocins were added to a sample. Generally, the maximum inhibitory concentration (IC_{max}) and the minimum inhibitory concentration (IC_{min}) remained the same. In most cases, the IC_{50} was lowered, implying that less of the combination of pairs of bacteriocins was needed to inhibit the population. Most notably, of 250 AU/ml leucocin additions to nisin and pediocin increased the inhibitory action of nisin and pediocin. Pediocin addition at 250 AU/ml to nisin increased the IC_{50} somewhat, while a decrease was observed when it was added to leucocin. When nisin was added to pediocin and leucocin, it was once again observed that the inhibitory action of the nisin-pediocin combination was diminished, while the leucocin-pediocin combination was more effective.

By subtracting the percentage relative inhibition values of nisin, pediocin and leucocin at 250 AU/ml, the absolute effect of the combinations could be observed and a clear shift of the inhibition curve was seen in all the samples tested. At high concentrations of the peptide used in the test, there was a decrease in inhibition due to this subtraction. However, at low concentrations of bacteriocin to which 250 AU/ml of another bacteriocin had been added, the inhibitory effect was much greater than that of the bacteriocin alone, increasing the percentage relative inhibition at the MIC. This is best illustrated by the addition of leucocin to nisin and pediocin. Pediocin addition had a similar effect when added to leucocin and nisin, although the inhibitory potential was much less than when leucocin was added to pediocin. Nisin addition to pediocin and leucocin seemed to be the least effective combination, as at low concentrations the inhibition was similar to that of leucocin or pediocin used in isolation. Once again, the action of nisin and pediocin exhibited an antagonistic tendency.

From the results it is possible to suggest that there is a synergistic effect when certain bacteriocins are used in combination. Leucocin and pediocin exhibited synergistic action in some cases, as did nisin and leucocin together. Nisin and pediocin used together seemed to have an antagonistic interaction. However, the ratio used seems to play an important role in the reaction. For example, low amounts of leucocin added to pediocin exhibited very high inhibition levels, in contrast to samples when pediocin was added to leucocin. Combinations of nisin and leucocin, and of nisin and pediocin, did not seem to be affected by the ratios used. This is illustrated further by the bacteriocin combinations used against increasing cell numbers and the scanning electron microscopy images. Nisin-leucocin was the most powerful combination against the LAB, confirming the findings of the microtitre broth dilution method to a degree, with nisin-pediocin being less effective. Leucocin-pediocin was the least effective combination, although the microtitre test indicates that it should have been the most effective. The most important parameter in this study therefore is the ratio of bacteriocins used with each other. Bacteriocin combinations should thus be researched carefully to determine their interactions with each other, in model food and beverage systems, before their general usage is considered.

An additional fact to consider is the action of the bacteriocin over time. The action and stability of bacteriocins are time dependent, as indicated by both the test against increasing cell numbers and the microtitre broth dilution method. The bacteriocins were only effective up to a certain time, and the remaining cells multiplied after the bacteriocins were exhausted. As bacteriocins and lysozyme both bind to wine components, the time of addition should be decided on carefully, white wine also contains fewer components that can bind these substances than red wine. In the case of white wine, the earliest time of bacteriocin addition should be after must clarification, bacteriocins should only be added to red wine if no MLF is desired, at the earliest stage after pressing. The LAB develop mostly after fermentation and wines are most susceptible to microbial spoilage after racking off the lees (Radler, 1990a, b). Bacteriocins can be removed together with proteins that lead to haze formation in wine by normal fining methods. With the development of bactericidal yeast strains, as described by Schoeman et al. (1999), bacteriocins are produced continuously during the fermentation process and this is an ideal alternative to adding purified bacteriocins. The *in situ* production of bacteriocins, whether by yeasts or

native LAB producers, is also more economical than the addition of purified bacteriocins, which can be very expensive.

The accumulated dose of lysozyme should not exceed 500 mg/L, as prescribed by the OIV, since this level is sufficient to control the bacteria responsible for MLF during the alcoholic fermentation. It was also recognised that lysozyme cannot totally substitute SO_2, but that the association between the two substances provides more stable wines. Further research on this subject should include a more in-depth investigation of the bacteriocins, as well as their interactions with lysozyme.

In conclusion, natural antimicrobial peptides and enzymes have proven to be useful tools for lowering the use of chemical preservatives used in the food and beverage industries. From the results it is clear that the future use of antimicrobial agents as biopreservatives will lie in choosing the appropriate combinations of bacteriocins or bacteriocin/enzyme combinations to broaden the spectrum of inhibition. Bacteriocins and enzymes are suitable tools to be implemented in multihurdle food and beverage preservation systems, resulting in synergistic actions that will prevent or inhibit the growth of spoilage microorganisms more efficiently. Although the bacteriocins of LAB and lysozyme can help to combat LAB spoilage in wine, alternatives need to be found to combat the Gram-negative acetic acid bacteria. In terms of bacterial spoilage, this is possibly one of the next important research topics in the wine industry for the improvement and promotion of clean and green products.

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