SCREENING, ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL/ANTIFUNGAL PEPTIDES PRODUCED BY LACTIC ACID BACTERIA ISOLATED FROM 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.

Joanne Morgan **Date**

SUMMARY

Winemaking is an age-old tradition that dates back to as early as 6000 BC. In our modern era there are several insects and microorganisms that pose a threat to the grapevine, the environment and the final wine product. Farmers and winemakers are becoming aware of the threat and the fight against disease, spoilage and/or pathogenic microorganisms is on the rise. Currently, the natural environment is being altered through rural developments, pollution and disaster, which in turn is responsible for altering the natural micro flora. The result is a harsh battle between man and microorganism. The weapon used often against microorganisms is chemical preservatives, such as sulphur dioxide. These chemical preservatives change the nutritional value, quality and wholesomeness of the wine. Chemical preservatives suppress the quality of the wine with a reduction in wine consumption by the consumers.

Until the 18th century, wine was regarded as a safe drink and prescribed by doctors. In the 20th century alcohol consumption became the focus point of some health campaigners. Medical science restored the good name of wine in the 1990s when it came to light that moderate red wine consumption may aid in preventing heart disease and assist in stress management. The only drawback that lowers consumption levels is the use of chemical preservatives.

It is of utmost importance to place the focus on health issues and the development of natural preservation methods that are environmentally friendly and contributes to the overall wholesomeness of the wine. Due to these demands, the scientific community placed the focus of research projects on the development and enhancement of biopreservation methods, in order to minimise chemical preservation use.

One of the most promising biocontrol agents is bacteriocins. These proteinaceous molecules produced by various lactic acid bacteria exert antimicrobial activity towards closely related organism. Research has shown that bacteriocins may aid in the prevention of wine-spoilage and enhance natural preservation techniques.

Most of the research on biopreservation in food and beverages has been performed on the bacteriocins of LAB. No evidence could be found that indicated bacteriocin production by wine isolated LAB in South Africa. This study is therefore, of utmost importance and is considered to be novel pioneering work for the South African wine industry.

The main objective of this study was to screen wine isolated LAB for the production of antimicrobial and/or antifungal compounds. This was followed by the isolation and characterisation of the produced bacteriocins. This study forms part of a greater project that focuses on wine preservation, under the auspices of the Institute for Wine Biotechnology.

The research results in this study indicated the production of bacteriocins by wine isolated LAB of South African origin. It was found that numerous isolates exerted antimicrobial activity towards other wine associated LAB. The most predominant species that gave the highest activity was *Lactobacillus brevis* and *Lactobacillus paracasei*. Experimental results indicated that the bacteriocins produced by these two species were thermo-stable and active over a wide pH range, including the temperatures and pH values that reign in the South African wine environment. The antimicrobial activity was lost after treatment with proteolytic enzymes, such as proteinase K and lysozyme. The size, production and growth kinetic curves of the bacteriocins under investigation showed similar results that are comparable to other findings in the literature. Antifungal activity was detected against *Botrytis cinerea* that indicated limited inhibitory activity towards spore germination, but had no effect on hyphal growth.

This study provides novel information regarding bacteriocin production by LAB isolated from the South African wine industry. The results indicate the suitability of these bacteriocins as possible biopreservatives in the wine environment. The proposed results obtained in this study will aid in the development of bacteriocin-producing, tailored made wine yeast or LAB that may in future, play vital roles in the winemaking process.

OPSOMMING

Wynmaak is 'n eeu oue tradisie wat terugdateer tot so vroeg soos 6000 jaar v.C. In ons moderne eeu is daar verskeie insekte en mikro-organismes wat 'n bedreiging vir die wingerdstok, asook die omgewing en die finale wynproduk inhou. Boere en wynmakers word al hoe meer bewus van hierdie bedreiging, terwyl die stryd teen siektes, bederf en/of patogene mikro-organismes ook aan die toeneem is. Tans word die natuurlike omgewing deur landelike ontwikkeling, besoedeling en natuurlike rampe verander, wat op sy beurt weer verantwoordelik is vir die verandering van mikroflora. Die gevolg is 'n harde stryd tussen die mens en mikro-organismes. Die wapen wat gereeld ingespan word in die stryd teen mikro-organismes, is chemiese preserveermiddels, soos swaweldioksied. Hierdie chemiese preserveermiddels verander die voedingswaarde, kwaliteit en die voedsaamheid van die wyn. Dit onderdruk ook die gehalte van wyn, wat meebring dat minder wyn deur die verbruiker gedrink word.

Tot en met die agtiende eeu is wyn deur dokters as 'n veilige drankie voorgeskryf. In die twintigste eeu het alkoholverbruik die fokuspunt van gesondheidskamvegters geword. In die 1990's het die mediese wetenskap wyn se goeie naam in ere herstel toe dit aan die lig gekom het dat 'n matige verbruik van rooiwyn moontlik hartsiektes kan voorkom en help om stres te beheer. Die enigste nadelige faktor wat verbruikersvlakke verlaag, is die gebruik van chemiese preserveermiddels.

Dit is uiters noodsaaklik om die fokus op gesondheidskwessies te plaas en die ontwikkeling van natuurlike preserveermetodes wat omgewingsvriendelik is en tot die algehele voedsaamheid van wyn bydra. As gevolg van hierdie eise het wetenskaplikes die fokus geplaas op navorsingsprojekte vir die ontwikkeling en verbetering van biopreserveringsmetodes met die doel om die gebruik van chemiese preserveermiddels te verminder.

Een van die belowendste biokontrolemiddels is bakteriosiene. Hierdie proteïenagtige molekule word deur verskeie melksuurbakterieë vervaardig en oefen anti-mikrobiese aktiwiteit teenoor nabyverwante organismes uit. Navorsing het getoon dat bakteriosiene moontlik kan help in die voorkoming van wynbederf en natuurlike preserveertegnieke kan verbeter.

Die meeste van die navorsing op biopreservering in voedsel en drank is op die bakteriosiene van melksuurbakterieë uitgevoer. Geen bewys kon gevind word in Suid Afrika wat bakteriosienproduksie deur wyn-geïsoleerde melksuurbakterieë aangedui het nie. Hierdie studie is daarom baie belangrik en word as baanbreker werk vir die Suid Afrikaanse wynbedryf beskou.

Die hoofdoel van hierdie studie was om wyn-geïsoleerde melksuurbakterieë vir die produksie van anti-mikrobiese en/of anti-fungiese substanse te toets. Dit is gevolg deur die isolasie en karakterisering van die geproduseerde bakteriosiene. Hierdie

studie maak deel uit van 'n groter projek wat fokus op wynpreservering en wat onder leiding van die Instituut van Wynbiotegnologie uitgevoer word.

Navorsingsresultate van hierdie studie dui op die produksie van bakteriosiene deur wyn-geïsoleerde melksuurbakterieë van Suid Afrikaanse oorsrong.

Daar is gevind dat verskeie isolate anti-mikrobiese aktiwiteit teenoor ander wynverwante malksuurbakterieë uitgeoefen het. Die oorheersende spesie wat die hoogste aktiwiteit getoon het, was *Lactobacillus brevis* en *Lactobacillus paracasei.* Eksperimentele uitslae dui daarop dat die bakteriosiene wat deur hierdie twee spesies geproduseer word, termostabiel en aktief is oor 'n wye pH reeks, insluitende die temperature en pH-waardes wat in die Suid Afrikaanse wynomgewing voorkom. Die anti-mikrobiese aktiwiteit het verlore gegaan na behandeling met proteolitiese ensieme soos proteïnase K. Die groote, produksie en groeikinetika kurwes van die bakteriosiene wat ondersoek is, toon vergelykbare resultate met ander bevindings in die literatuur. Anti-fungiese aktiwiteit is opgemerk teen *Botrytis cinerea*, wat beperkte inhiberende aktiwiteit ten opsigte van spoorontkieming aangedui het, maar geen effek op hifegroei gehad nie.

Hierdie studie verskaf nuwe inligting aangaande bakteriosienproduksie deur melksuurbakterieë wat van die Suid Afrikaanse wynomgewing geïsoleer is. Die resultate dui op die geskiktheid van hierdie bakteriosiene as moontlike biopreserveermiddels in die wynbedryf. Die voorgestelde resultate deur hierdie studie verkry sal help in die ontwikkeling van bakteriosien produserende, spesifiek vervaardigse wyngis of melksuurbakterieë, wat in die toekoms 'n baie belangrike rol in die wynmaakproses sal speel.

This thesis is dedicated to my parents, *Allen* and *Paula Morgan*.
Hierdie tesis word aan my ouers, *Allen* en *Paula Morgan*, opgedra.

BIOGRAPHICAL SKETCH

Joanne Morgan was born in Cape Town, South Africa on 11 October 1978. She matriculated at Randburg High School in Randburg in 1996. Joanne enrolled at Stellenbosch University in 1997 and obtained a BSc degree, majoring in Microbiology and Genetics, in 1999 and a BScHons degree in Wine Biotechnology in 2000. In 2001, she enrolled for an MSc degree in Wine Biotechnology at the same University.

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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**

Bacteriocins of lactic acid bacteria: Fundamentals and biotechnology

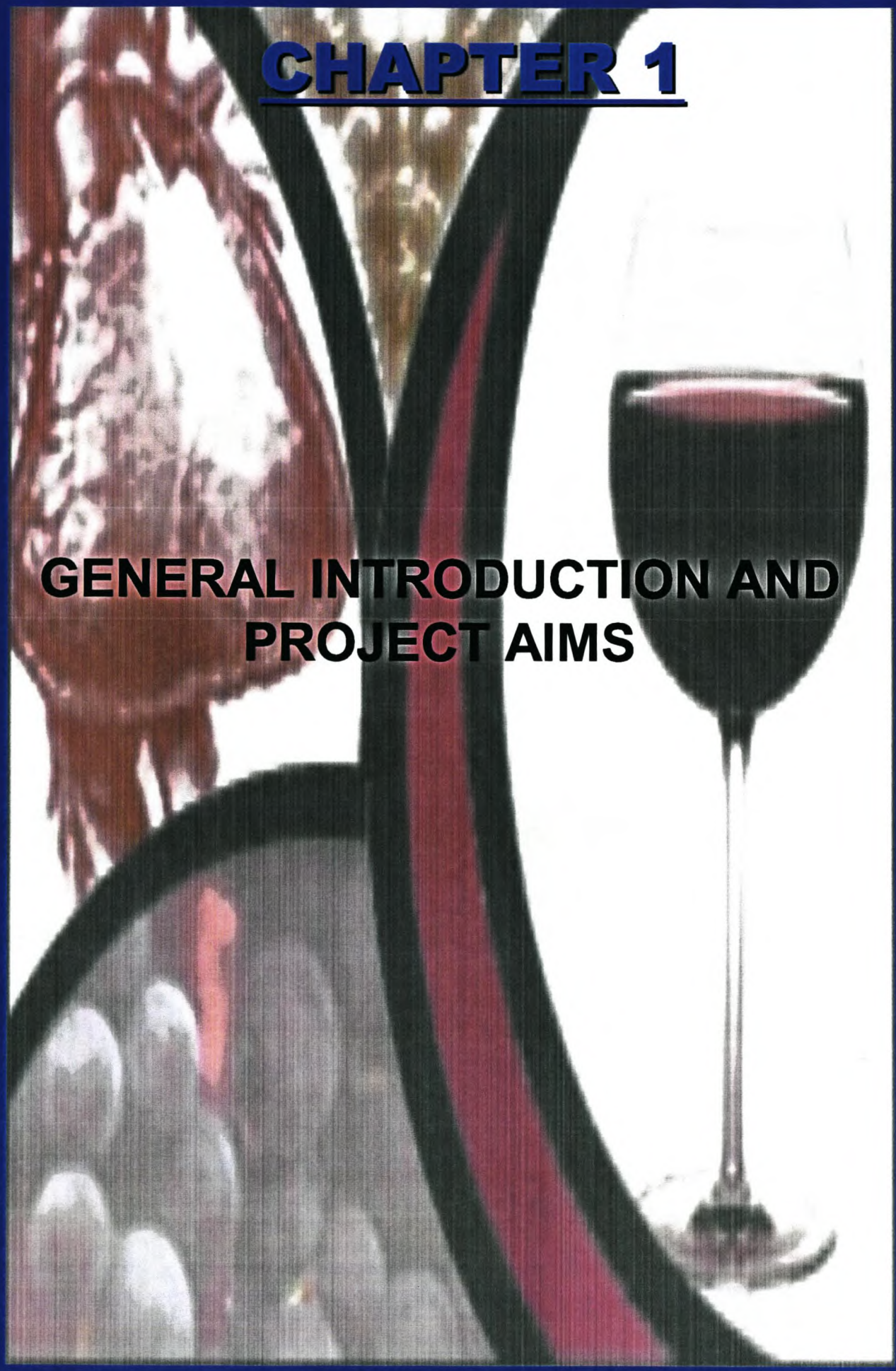
Chapter 3 **Research Results**

Isolation, screening and characterisation of antimicrobial/antifungal peptides produced by lactic acid bacteria isolated from wine

Chapter 4 **General Discussion and Conclusions**

CONTENTS

CHAPTER 1



GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Lactic acid bacteria (LAB) have been around for centuries. They previously were referred to as the 'milk souring' organisms due to their ability to produce acidic molecules, which resulted in milk turning sour (Axelsson, 1998).

Another age-old tradition in which LAB play a pivotal role is winemaking. Winemaking is a complex process during which various microorganisms, including yeast, LAB, acetic acid bacteria and fungi, interact with one another to form part of the ecological and biochemical processes of converting grape juice into quality wines. In the wine industry, LAB are used as starter cultures during the malolactic fermentation (MLF) process. During this second fermentation, LAB may be beneficial to the wine. During MLF, the growth of LAB results in the conversion of dicarboxylic malate to monocarboxylic lactate, which results in an increase in the pH of the wine (Kunkee, 1967; Daeschel et al., 1991). The presence of LAB, however, are in many cases also associated with the spoilage of wine due to their ability to cause ropiness, mousy taints, acidification and the production of high levels of acetic acid that may cause stuck fermentations.

In the 1900s, technological advancements, mechanisation, factory enlargements and process streamlining with regards to LAB-fermented products, such as cheese and wine, increased at a rapid pace (Mäyrä-Mäkinen and Bigret, 1998). As part of this process of industry optimisation, LAB were introduced into the manufacturing process as starter cultures because of their reliable activity, stability and resistance to bacteriophages (Klaenhammer and Fitzgerald, 1994). Although these characteristics ensured them a place in the manufacturing process, it was their ability to produce antimicrobial substances that made them famous in the food and beverage industries, as these peptides could be used as biopreservatives.

During the fermentation process, LAB induce the formation of several organic molecules that exhibit antimicrobial activity (De Vuyst and Vandamme, 1994; Caplice and Fitzgerald, 1999). The most commonly found molecules are lactic, acetic and propionic acid. Other antimicrobial components with similar effects are hydrogen peroxide, carbon dioxide, diacetyl and low molecular weight substances, such as reuterin (Axelsson et al., 1989; Daeschel, 1989). LAB have also been shown to produce antimicrobial peptides that possess antifungal activity. To date, antimicrobial activity against fungi have been reported only in the genera of *Lactobacillus* and *Lactococcus* species (Magnusson and Schnürer, 2001).

However, one of the most promising antimicrobial substances produced by LAB is the bacteriocins. These substances are of a proteinaceous nature and show activity against closely related species (De Vuyst and Vandamme, 1994; Jack et al.,

1995). Many LAB that produce bacteriocins are currently used in various industries with great success (Hill, 1995).

One of the current trends amongst consumers is the promotion of a healthier lifestyle. Consumers expect products to be wholesome and to be produced in an environmentally sustainable manner. For the food and beverage industries to survive this trend, producers need to understand the latest developments in both science and technology (Bisson et al., 2002). Researchers have to assist in this regard and need to explore further possibilities for the use of organic and natural preservatives, which are referred to as biopreservatives (Schillinger et al., 1996; Stiles, 1996).

Currently, the food and beverage industries mainly use chemical preservatives to suppress unwanted spoilage and/or pathogenic microorganisms. These chemicals, however, are capable of causing health risks, changing the nutritional value and influencing the sensorial quality of the product (Kunkee, 1984). In the wine industry, sulphur dioxide is commonly used as a chemical preservative during the production of wine. The levels of sulphur dioxide may vary, depending on the wine and the fermentation conditions. This is unacceptable to the health-conscious consumer and an alternative method must be found to substitute the use of chemical preservatives.

This led to the study of bacteriocins as a possible solution or alternative to the extensive use of chemical preservatives. The most promising results regarding the use of bacteriocins in the food and beverage industries have been seen with the use of nisin, a bacteriocin produced by *Lactococcus lactis*. Nisin is the only bacteriocin that is used commercially and its use is approved in more than 40 countries worldwide. It is sold under the trade name, Nasaplin. Nisin application occurs in various food systems, including the dairy, canning and meat industries (Delves-Broughton, 1990). Nisin also has been evaluated for its use as a biopreservative under winemaking conditions (Radler, 1990a, b; Du Toit, 2002). It was shown that, during the winemaking process, nisin can improve the quality of both white and red wines. In the case of red wine fermentations, nisin and nisin-resistant starter cultures can be added to red wine in order to prevent the growth of other undesirable LAB and to ensure a reliable MLF. In white wines, nisin alone can be added to prevent the growth of all LAB and therefore to ensure that no MLF takes place (Daeschel et al., 1991; Hill, 1995). Other bacteriocins that have been evaluated experimentally as possible biocontrol agents in the wine industry include pediocin PA-1 and leucocin B-Ta11a (Du Toit, 2002).

Another substance that is currently used as a natural antimicrobial agent in the wine industry is lysozyme. This enzyme is extracted from chicken egg white and plays an important role in establishing a healthy environment for alcoholic fermentation (Charter and Lagarde, 1999). The addition of low levels of lysozyme during various stages of the winemaking process will limit the growth of spoilage bacteria that may result in stuck and sluggish fermentations, an increase in volatile acidity and the onset of premature MLF (Gerbaux et al., 1997, 1999).

It is clear that the use of bacteriocins and other natural substances as biopreservatives has become an area of great importance to the food and beverage industries in general. They also show immense promise for the future production of more wholesome and environmentally friendly products.

Research has shown that bacteriocins have the potential to manipulate the properties of fermented foods, to be utilised in complex food systems and to be genetically engineered to alter their target ranges, stability and applications (McKay and Baldwin, 1990; Schoeman et al., 1999). Although these antimicrobial bacteriocins showed promising results in the fight against unwanted microbes, their application is currently limited due to their low cost-effectiveness in bacteriocin preparation (Ross et al., 2002). To overcome this problem, several bacterial starter cultures can be selected that already produce the desired bacteriocins. Another alternative would be the development of appropriate bacterial or yeast strains, engineered to produce bacteriocins at elevated levels

Although the characterisation and genetic analysis of bacteriocins has proceeded at an astonishing pace, most of the proposed application studies still remain in the development stage rather than the commercial implementation phase (Hill, 1995). This is mostly due to technical hitches and complications that suppress the progress towards the use of these compounds. Some of the problems that have to be solved in order to realise the use of bacteriocins as biopreservatives include the identification of corresponding genetic determinants of physiological characteristics. Researchers have to be able to use stable gene transfer methods, as well as be able to effectively dispose of the resulting excess to obtain a 'GRAS' (Generally Regarded as Safe) microorganism. Regulatory problems concerning genetically modified or recombinant microorganisms must be solved to improve cost-effectiveness (Mäyrä-Mäkinen and Bigret, 1998). In order to help overcome these obstacles, the genetic analysis of bacteriocin operons and regulation systems must continue. The search for novel bacteriocins with unique stability, target ranges and modes of action should be an aspect that receives high priority, while the understanding of bacteriocin resistance remains equally important.

1.2 PROJECT AIMS

This study forms an integral part of a crucial research project on the biopreservation of wine in the Institute for Wine Biotechnology. Biopreservation refers to the use of natural substances to promote the preservation of wine. The improvements in the control of wine spoilage microorganism are essential for the prevention of altered sensory characteristics, appearances of the wines, aroma and flavour. In severe cases the wine may even become unpalatable to the consumer. In the fight against these spoilage microbes, the focus is placed on the use of bacteriocins as biopreservatives. These antimicrobial compounds potentially can be introduced into the wine by means of the native producers, by engineered *S. cerevisiae* yeast

strains, or by LAB that have been engineered or manipulated to produce the desired bacteriocin.

Many bacteriocins have been studied intensively for their use in the food industry, but few reports have been found either on bacteriocins produced by wine-isolated lactic acid bacteria or on the possible use of bacteriocins as natural antimicrobial substances in wine. The specific aims of this study were as follows:

(i) to screen LAB isolated from grape berries and throughout the winemaking process for the production of antimicrobial peptides;
(ii) to screen the wine-isolated LAB for the production of antifungal peptides;
(iii) to confirm the proteinaceous nature of the antimicrobial compounds by digestion with proteolytic enzymes;
(iv) to evaluate the influences of pH, temperature and filtration on the activity of the antimicrobial peptides;
(v) to establish the antimicrobial peptide production kinetics of the native LAB producers;
(vi) to determine the mode of action of the antimicrobial peptides;
(vii) to examine the inhibition spectrum of the peptides against wine-associated LAB; and
(viii) to determine the size of the peptides.

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CHAPTER 2

LITERATURE REVIEW

Bacteriocins of lactic acid bacteria: Fundamentals and biotechnology

2. LITERATURE REVIEW

Lactic acid bacteria (LAB) historically have been associated with food and beverage fermentations as they occur naturally in the raw material. LAB play an important role in these fermentations, as they provide the characteristic flavour profile and texture. In addition, they produce antimicrobial agents that will increase the shelf life of the finished product by inhibiting the growth of spoilage bacteria, by competing for nutrients and by producing antimicrobial compounds, such as organic acids, ethanol, hydrogen peroxide and bacteriocins (Daeschel, 1993; Cleveland et al., 2001). A wide range of LAB have the ability to produce bacteriocins and the fundumental knowledge gained on the biochemical and genetic characteristics of these substances in the last decade has increased their potential application as biopreservatives in the food and beverage industries. This literature review will focus firstly on LAB bacteriocins and their genetic characteristics, and secondly on the genetic tools available for the heterologous expression of bacteriocins in various LAB hosts. The review will conclude with an overview of future possible applications of bacteriocinogenic LAB and/or their bacteriocins.

2.1 BACTERIOCINS OF LACTIC ACID BACTERIA

2.1.1 LACTIC ACID BACTERIA IN GENERAL

LAB can be described as a group of Gram-positive bacteria with similar morphological, metabolic and physiological characteristics. They are non-spore-forming, non-respiring cocci, coccobacilli or rods, and produce lactic acid as an end product during carbohydrate fermentation. The most prominent strains of LAB belong to the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus.* LAB are associated with habitats rich in nutrients, including milk, meat, vegetables and beverages, but may also be found in the normal mouth flora, intestine and vagina of mammals. Due to the fact that LAB have a very diverse metabolic capacity, they are able to adapt to most growth conditions. This has allowed them to operate in dual functions, such as *Carnobacterium*, which normally is associated with meat, but also may be found in fish as a pathogen (Axelsson et al., 1998).

LAB are also found in the wine environment, where they are known for their ability to cause spoilage. The same LAB are also used as inocula for the onset of malolactic fermentation (MLF), which in most cases is a desirable process during which malic acid is converted to lactic acid, causing an increase in wine pH and enchancing the sensorial quality of the wine. The genera found in the wine environment include *Pediococcus, Leuconostoc, Oenococcus* and *Lactobacillus* (Daeschel et al., 1991; Du Toit and Pretorius, 2000).

In general, these bacteria are involved in the acidification of food and feed products. They play a vital role during food fermentation processes and act as a

preservative for raw foods such as cheese, milk, fruit and meat. The antimicrobial effect may be due to the production of hydrogen peroxide, diacetyl or organic acids or the secretion of antimicrobial peptides, such as bacteriocins (Daeschel, 1989; Carolissen-Mackay et al., 1997).

2.1.2 BACTERIOCINS IN GENERAL

2.1.2.1 Definition

The term bacteriocin initially was coined by Jacob et al. (1953) as a descriptive term for the inhibitory activity displayed by some bacteria towards closely related species.

Today, bacteriocins can be defined as ribosomally synthesised antimicrobial peptides of proteinaceous nature that inhibit the growth of closely related bacteria by destabilising the function of the cytoplasmic membrane.

Bacteriocins comprise a heterologous group with respect to molecular size, ranging from a few thousand daltons to complex protein structures that may contain carbohydrate or lipid moieties. They also show considerable variation in stability, genetic location, post-translational modifications, modes of action and chemophysical properties (Hill, 1995; Van Belkum and Stiles, 2000).

2.1.2.2 Origin and classification of bacteriocins

Bacteriocins are considered to be a relatively new class of antimicrobial peptides. The first description of bacteriocin-like antagonism between Gram-positive bacteria (staphylococci) was described in 1885 by Babes. Clinical observations then suggested that streptococci also were able to inhibit the growth of *Corynebacterium diphtheriae;* this discovery led to the extensive use of staphylococcal nasal and throat sprays for the treatment of diphtheria infections (Jack et al., 1995). In the past 20 years, bacteriocins have drawn the interest of food biotechnologist due to their possible role as natural biocontrol agents.

Based on their proteinaceous character, their molecular mass, structure and mechanism of action, the bacteriocins of LAB can be divided into four distinct classes (Klaenhammer, 1993) (**Table 2.1**). Not all of the published classification models include all four classes, due to the fact that the compounds of the fourth class have not yet been purified (Nes et al., 1996; Van Belkum and Stiles, 2000).

The first class (I) is called the lantibiotics. They are small membrane-active peptides (<5 kDa) that contain the unusual amino acids, lanthionine and β-methyl lanthionine, and dehydrated residues. These peptides undergo extensive post-translational modifications and have a broad host range. The best-known example in this class is nisin; others include carnocin U149, lacticin 481 and lactocin S (Klaenhammer, 1993; Van Belkum and Stiles, 2000).

The second class (II) consists of the small, heat stable, non-lanthionine-containing membrane active peptides (<10 kDa) that undergo minimal post-translational modifications. They have diverse genetic and chemical characteristics

and can therefore be divided into three main subclasses (Klaenhammer, 1993; Van Belkum and Stiles, 2000).

The first subclass, IIa, consists of *Listeria*-active peptides, which are single peptides that often have a characteristic amino acid motif near the N-terminal of Tyr-Gly-Asn-Gly-Val-Xaa-Cys, generally refered to as the YGNGV-C consensus sequence. Examples of these peptides are pediocin PA-1, sakacin A, sakacin P, leucocin A and curvacin A (Hastings et al., 1991; Holck et al., 1992; Lozano et al., 1992; Tichaczek et al., 1992; Van Belkum and Stiles, 2000).

The second subclass, IIb, consists of two-peptide bacteriocins that form poration complexes that are necessary for activity. Examples of these peptides are lactococcin G and lactacin F (Nissen-Meyer et al., 1992; Van Belkum et al., 1992; Allison et al., 1994; Van Belkum and Stiles, 2000).

The third subclass, IIc, contains the bacteriocins that are secreted by the general secretory pathway and are referred to as thiol-activated peptides. These require cysteine residues for activity. An example of these peptides is lactococcin B (Venema et al., 1993; Van Belkum and Stiles, 2000). It since has been shown that subclass IIa bacteriocins can also use this secretory pathway and class IIc therefore could possibly be eliminated (Cleveland et al., 2001).

The third class of bacteriocins (class III) is made up of large heat-labile proteins (<30 kDa) that are non-lantibiotic and are relatively uncommon amongst the antibacterial compounds of LAB. Examples of these peptides are helveticin J, helveticin V-1829, acidophilucin A and lactacins A and B (Joerger and Klaenhammer, 1986; Toba et al., 1991; Vaughan et al., 1992; Van Belkum and Stiles, 2000).

The fourth class of bacteriocins (class IV) consists of complex bacteriocins that are composed of a mixture of undefined proteins added to one or more chemical moieties, which are required for antimicrobial activity. Evidence for their presence is based on the loss of activity following carbohydrate or lipid-hydrolysing enzyme treatments (Van Belkum and Stiles, 2000). There is good reason to believe that this type of bacteriocin is an artefact caused by interaction between constituents from the cell or the growth medium (Jiménez-Díaz et al., 1995; Nes et al., 1996). Examples of these peptides, which are composed of protein and lipid or carbohydrate moieties, are plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1 (Upreti and Hinsdill, 1975; Lewus et al., 1992; Jiménez-Díaz et al., 1993; Klaenhammer, 1993; Schved et al., 1993).

2.1.3 MOLECULAR PROPERTIES

2.1.3.1 Gene location

The genes encoding bacteriocin production can be located on chromosomes, plasmids or transposons, as in the case of nisin (Dodd et al., 1990; Horn et al., 1991; Engelke et al., 1992). Most bacteriocin genes are plasmid-borne, however, although there are some exceptions, such as those encoding lactacin F, which is carried on a

recombinant plasmid or episome, and plantaracin A and sakacin 674, which are chromosomally encoded (Jack et al., 1995). These plasmids vary greatly in size, ranging from 6.0 kb (pediocin SJ-1) to 131 kb plasmid (lactococcin A). It has been shown that two or more bacteriocins may be encoded by the same strain. Both can be located either on a plasmid or on chromosomes, or, alternatively, one can be on a plasmid and the other on a chromosome. Another variation is when a single plasmid may carry the genetic determinants for the production of several bacteriocins, as well as for their corresponding immunity proteins. Bacteriocin producers have developed a type of protection against their own bacteriocins in the form of their immunity systems. Each bacteriocin has its own dedicated protein conferring immunity and these proteins are expressed in conjunction with the bacteriocin. The encoding genes are located next to or downstream of the structural genes of the bacteriocin (Holo et al., 1991; Venema et al., 1995).

Table 2.1
Classification of bacteriocins (adapted from Cleveland et al., 2001)

Class	Class description	Bacteriocin examples
I	Lantibiotics, small (<5 kDa) peptides containing lanthionine and β-methyl lanthionine.	Nisin.
II	Small heat-stable non-lantibiotics that show activity against *Listeria*.	
IIa	Pediocin-like bacteriocins with strong antilisterial activity.	Pediocin PA-1, sakacins A and P, leucocin A, carnobacteriocins.
IIb	Two-peptide bacteriocins.	Lactococcin G and F, lactacin F, Plantaricin EF and JK.
III	Large heat-labile proteins.	Helveticins J and acidophilucin A, lactacins A and B.
IV	Large complex bacteriocins containing lipid/carbohydrate moieties in addition to protein moieties.	Plantaricin S, lactocin 27, pediocin SJ-1 and leuconocin S.

2.1.3.2 Gene organisation

LAB possess the genes encoding the structural peptide, proteins that aid in the processing of the active form, the genes that encode the transporters that assist the bacteriocin across the membrane, regulatory proteins and proteins that provide the host producer with immunity.

The most important genes are the structural, immunity and modification genes. These genes are usually in close proximity to one another and form the basis of a

bacteriocin gene operon (Siegers and Entian, 1995). It is common for the structural bacteriocin and immunity genes to be located on the same operon and often next to one another. The immunity proteins are highly specific for a particular bacteriocin (Lakey et al., 1994).

Though bacteriocins are ribosomally synthesised, the resulting pre-peptides must be modified before becoming active. The genes responsible for encoding the enzymes that facilitate the modification are usually in close proximity to the structural gene (Kupke and Götz, 1997). These modifications are necessary for the secretion and transport across the cell membrane, a crucial part in the processing of the bacteriocin (Cleveland et al., 2001).

Since bacteriocins are encoded by one structural gene, the active sites and structure-function relationships can be examined through genetic manipulation. Nisin is one such bacteriocin that has been studied intensively. The genetic organisation and location of nisin can be described as shown in **Fig. 2.1.**

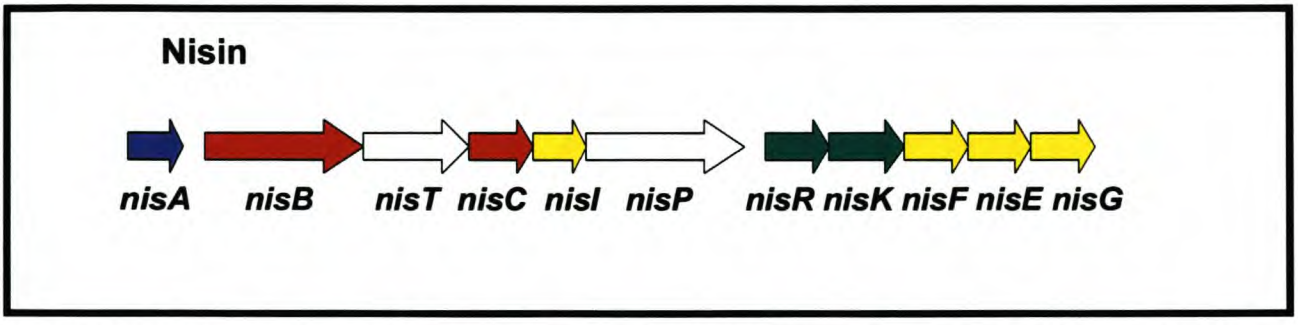

Fig. 2.1. The organisation of the well-characterised nisin gene cluster (McAuliffe et al., 2001).

The genetic location of the nisin biosynthesis genes is on the chromosomally located 70 kb conjugative transposon, named Tn 5276 or Tn 5301, which also encodes sucrose utilisation (Dodd and Gasson, 1994).

The entire 10 kb nisin-coding region includes seven genes, the first of which is *nisA*, the structural gene encoding pre-nisin. This is followed by *nisB*, which is involved in the modification of pre-nisin, possibly in the dehydration steps. *NisT* encodes a protein that plays a role in the translocation of nisin across the membrane of the producer cell. The fourth gene, *nisC*, is involved in the post-translational modification of pre-nisin. *NisI* plays a role in immunity, whereas *nisR* directs the synthesis of a regulatory protein. *NisR, K, F, E,* and *G* are grouped together and all play a role in the immunity function (McAuliffe et al., 2001).

2.1.3.3 Structure

Lantibiotics

Of all the bacteriocins produced by LAB, the lantibiotics (I) have been studied and characterised the most intensively. Nisin is the best-known lantibiotic and therefore will serve as a model bacteriocin for the purpose of this discussion.

Lantibiotics consist of not less than 19, and not more than 38, amino acids. The percentage of residues involved in the modification ranges from 24% to 47% in the cinnamycin group of lantibiotics (Sahl and Bierbaum, 1998). They are characterised by the presence of a high proportion of unusual amino acids, as well as a number of amino acid-derived residues. These include the thioether amino acid lanthionine and 3-methyllanthionine, as well as a number of dehydrated amino acids, such as the α,β-unsaturated amino acids didehydroalanine (Dha) and didehydrobutyrine (Dhb). This sequence-specific dehydration of serine (to Dha) and threonine (to Dhb) results in modified amino acids with electrophilic centres. These centres allow the amino acids to react with neighbouring nucleophilic groups to form intramolecular bridges that contribute to the globular structure of some of the lantibiotics (Skaugen et al., 1994; Kupke et al., 1995; McAuliffe et al., 2001).

Lantibiotics are grouped into type-A and type-B peptides based on their structural and functional features. Their characterisation is dependent on the amount and orientation of their polycyclic bridge structures, which contain a number of lanthionine rings (Jung, 1991). Type-A lantibiotics are elongated, cationic peptides of up to 34 residues in length that show similarities in the arrangement of their lanthionine-bridges. These peptides act primarily by disrupting the membrane integrity of target organisms, and include nisin, subtilin and epidermin (**Fig. 2.2**). Type-B peptides are globular, up to 19 residues in length and act through the disruption of enzyme function, i.e. inhibition of cell wall biosynthesis. Examples of these are duramycin and mersacidin (Hansen, 1993) (**Fig. 2.2**).

Non-lantibiotics

Class II, the non-lantibiotic bacteriocins, consists of a wide range of bacteriocins that are divided into three subclasses. Within each subgroup there are characteristic features in the structures of the peptides that makes it easier to distinguish between the wide variety of bacteriocins belonging to class II and its subclasses.

In general, the non-lantibiotics have similar primary structures to the YGNGVXC amino acid motif near the N-terminus of the mature peptide, the cysteine residues at or close to position 9 and 14 of the peptide, and an amphipathic α-helix near the C-terminus. The N-terminal region is hydrophilic and conserved, whereas the C-terminal region is hydrophobic and diverse (Van Belkum and Stiles, 2000). The class IIa bacteriocins are the largest subclass and have been studied intensively due to their potential as biopreservatives in food. The class IIa bacteriocins are known for their role in inhibiting the food-borne pathogen, *Listeria monocytogenes*. These bacteriocins contain between 37 and 48 residues and share considerable sequence similarity. Another unifying feature of the class IIa bacteriocins is their net positive charge and isoelectric points (pI), varying from 8.3 to 10.0, thereby displaying similar net charges at various pH values (Ennahar et al., 2000).

Class IIa bacteriocins exist primarily in unstructured conformations, generally random coils in an aqueous solution and a partly helical structure in a non-aqueous

environment with varying amounts of hydrophobicity. The bacteriocins are cystibiotics and have at least two cysteines with disulphide bridges. In all class IIa bacteriocins, the two cysteine residues in the N–terminal domain are present in conserved positions, and consequently is the six-member ring disulphide bridge that is formed over these two residues. The N-terminus also contains β-sheets maintained in a β-hairpin conformation that is stabilised by the N-terminal disulfide bridges. This conformation results in an amphiphilic characteristic inside this specific region. The C-terminal adopts an amphiphilic α-helix, spanning similar regions in different molecules, and therefore leaves a non-helical portion of only one or two C-terminal residues (Ennahar et al., 2000).

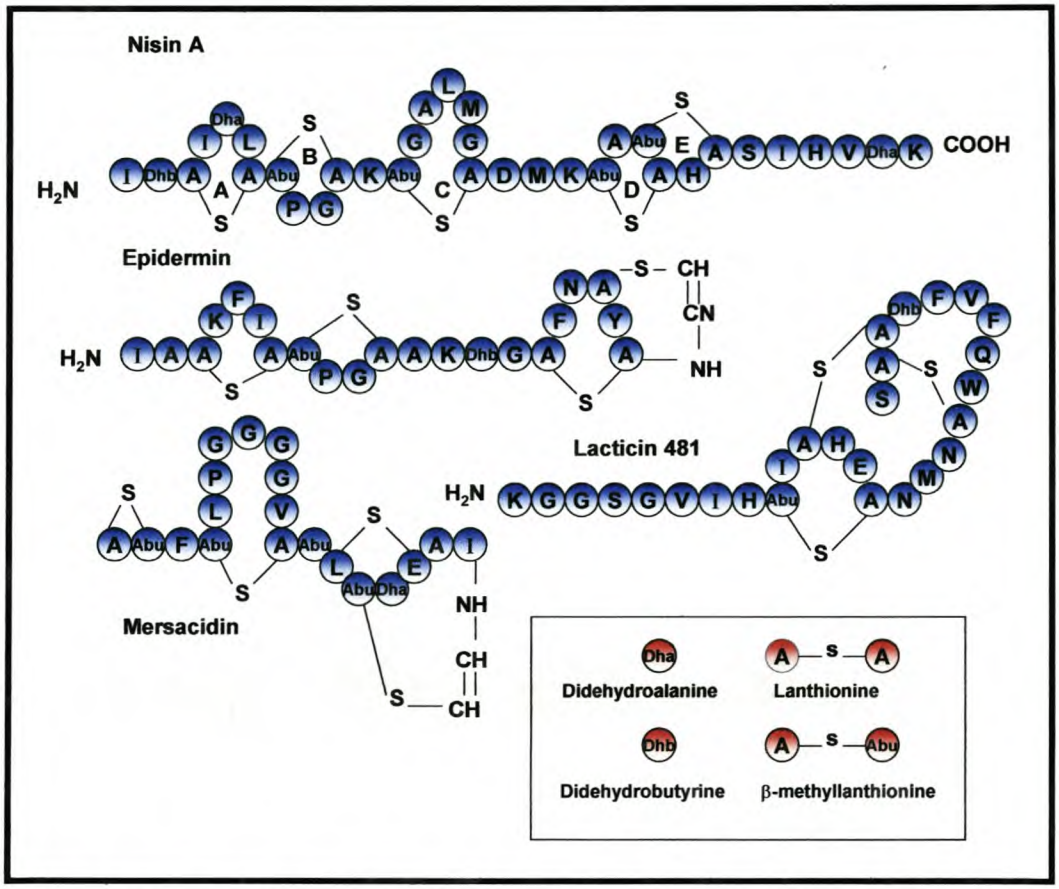

Fig. 2.2. Examples of globular and elongated structures of representative lantibiotics. Nisin A and epidermin represent type A: elongated, flexible peptide. Lacticin 481 represents a group with a cross-bridged C-terminus and an unbridged N-terminal domain. Mersacidin represents type B: globular peptides (Jung, 1991).

2.1.4 ANTIMICROBIAL ACTIVITY

2.1.4.1 Mechanism of action

Bacteriocins produced by Gram-positive bacteria are directed primarily against other Gram-positive species, with the range of organisms inhibited by each bacteriocin varying greatly (McAuliffe et al., 2001).

Bacteriocins inhibit target cells by dissipating the proton motive force (PMF) through the formation of pores in the membrane, thereby depleting the transmembrane potential and/or the pH gradient and resulting in the leakage of cellular material. This action promotes the rapid efflux of ions, solutes and small metabolites, such as amino acids and nucleotides, which rapidly depolarises the cytoplasmic membrane and leads to an instant cessation of all biosynthetic processes (Sahl and Bierbaum, 1998; Cleveland et al., 2001).

The "barrel-stave" model is used to describe the formation of the poration complexes during bacteriocin-membrane interactions. The mode of action of the class IIa bacteriocins is usually based on the "barrel-stave" model. The model is divided into four simple stages. The first stage is the recognition of the receptor molecule that is present on the target cell membrane. The second stage is the electrostatic interaction of the bacteriocin with the membrane surface. The third step involves the interaction of the hydrophobic domain with the C-terminal half of the bacteriocin and the lipid acyl chains; this step is crucial for the pore formation process. The last stage follows the hydrophobic interactions; the bacteriocin is now reoriented into a more energetically favourable orientation, which can result in its insertion into the membrane, followed by aggregation (Ennahar et al., 2000). The aggregation stage involves the formation of water-filled pores inside the target cell wall membrane (**Fig. 2.3**).

Due to the narrow spectrum of antibacterial activity of bacteriocins, it is suggested that there is a receptor molecule at the surface of the target cell. In the case of lantibiotics, these are positively charged molecules with hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on cell membranes are thought to contribute to the initial binding with the receptors located on the target membrane. The bactericidal action is then based on the formation of short-lived transmembrane pores that are non-selective, oligodynamic and require energy for formation and opening. Generally, the electrical transmembrane potential, as generated by metabolising bacterial cells, represents the major driving force for activity (Sahl and Bierbaum, 1998; Cleveland et al., 2001).

Studies performed by Tagg et al. (1976) suggested that the lethal action of a bacteriocin takes place in two stages. In the initial step, the bacteriocin adsorbs to specific receptors of the cell envelope of the sensitive organism and, in the second stage, lethal biochemical lesions occur in the membrane. The killing of the cells sets in immediately after the addition of peptides, while inhibitors of cell wall biosynthesis usually include slow lysis and killing does not occur before the completion of at least one cell cycle. The first step seems to be reversible and the cells can remain viable by the inactivation of the adsorbed bacteriocin by means of proteases. The second step, however, is irreversible and results in cell death (Klaenhammer, 1993; Cleveland et al., 2001).

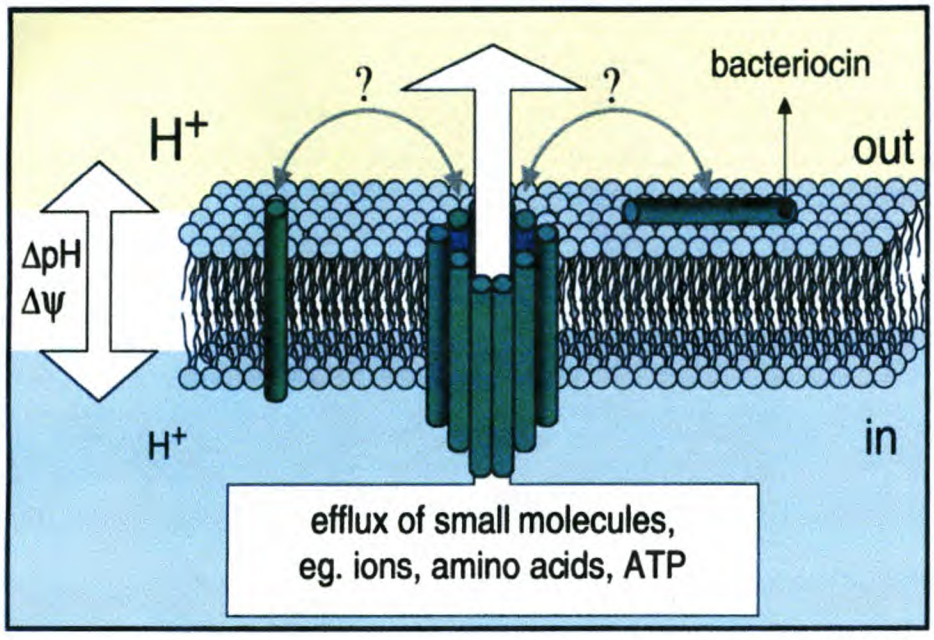

Fig. 2.3. Pore formation by bacteriocins: model of 'barrel stave' mechanism (McAuliffe et al., 2001).

2.1.4.2 Inhibitory spectrum

Bacteriocins are active against a wide range of Gram-positive bacteria, depending on the characteristics of the bacteriocin. The degree and occurrence of the activity may vary, depending on the presence of chemical agents that weaken cell-wall integrity or on a change in pH values. In some cases, bacteriocins of Gram-positive bacteria can inhibit Gram-negative bacteria once the cell wall has been damaged (Jack et al., 1995).

Bacteriocins can be divided into two groups according to their inhibition spectra. Those inhibiting a relatively wide range of Gram-positive organisms, such as the lantibiotics, and those exhibiting antibacterial activity confined to species closely related to the producer strains, such as some of the class IIa bacteriocins. The activity range may vary amongst these bacteriocins (Schillinger and Lücke, 1989).

General observations show the following to be characteristic of the low molecular weight bacteriocins (e.g. nisin) of Gram-positive bacteria:

- Within a given species, some strains may be sensitive and others may be resistant to particular bacteriocins
- A strain that appears to be sensitive to a bacteriocin may also have members of the population that are resistant to it
- A strain can be sensitive to one bacteriocin while being resistant to other similar types of bacteriocin
- Under normal conditions, Gram-negative bacteria are not sensitive to bacteriocins produced by Gram-positive bacteria. It has been shown that certain bacteriocins, such as nisin and pediocin AcH, can act on several species of Gram-negative bacteria, provided that the integrity of the cell wall is disrupted (Jack et al., 1995).

2.2 GENETIC MODIFICATION TECHNIQUES AND HETEROLOGOUS EXPRESSION SYSTEMS FOR LACTIC ACID BACTERIA

Rapid progress has been made in the last 10 years in the development of tools for the genetic modification of LAB. Advances have been made in the development of transformation systems for both integration and amplification vectors, selection criteria and the generation of food-grade heterologous expression systems. The genome sequences of several LAB that are available today will enhance the field of genomics as the genes and operons become characterised.

2.2.1 TRANSFORMATION SYSTEMS

There are two main pathways in which genetic recombination can take place. The first pathway is referred to as the general recombination pathway and includes natural gene transfer methods such as transduction, phage-mediated genetic exchange and conjugation. During the operation of these systems, genetic material is transferred from the donor to the recipient, requiring considerable sequence homology between the transferred DNA and the recombination functions of the recipient. The second pathway of recombination is mediated by transposable elements. This pathway is independent of host function and only needs short target sequences of about 10 bp or less (Von Wright and Sibakov, 1998).

Transposable elements can contain additional genetic information, such as drug resistance genes, or they can contain only the functions necessary for transposition (Grindley and Reed, 1985). Shimizu-Kadota et al. (1985) identified the first transposon insertion sequence element in *Lb. casei*. The sequence caused the virulence of an otherwise lysogenic phage, possibly by insertional inactivation of phage repressor function. Other well-characterised transposable elements include the streptococcal transposon Tn 916 and the related Tn 919 transposon (Franke and Clewell, 1981; Fitzgerald and Clewell, 1985). They have been introduced into several lactococcal strains, as well as into *Lactobacillus* and *Leuconostoc* species, by conjugation.

Before genetic recombination can take place in LAB, the genes must be transferred from one bacterium to another. There are various ways in which the transfer of genes can take place.

2.2.1.1 Transduction

Transduction is a method of genetic exchange that occurrs when a bacterium incorporates DNA carried into it by a virus. When the virus forms new particles, a small amount of the bacterial DNA can be incorporated into the virus. When the virus attacks a new bacterium, the DNA from the first bacterium is released into the second. Transduction does not have a high efficiency rate, since the virus may kill the second bacterium or the foreign DNA may be depolarised rather than be incorporated into the DNA of the bacterium (Mauseth, 1995).

Transduction experiments were first done with lactococcal strains in 1963, using virulent bacteriophages (Von Wright and Sibakov, 1998). Since 1990, temperate phages have been used as key tools for transduction experiments. This led to the transduction of genes involved in lactococcal lactose metabolism, as well as various proteinase genes. Both plasmid-linked as well as chromosomal genes have been found to be transducible in lactococci. However, few reports of successful transduction with other LAB have been noted. The only exceptions are *Strept. thermophilus* and *Lb. acidophilus*, in which both of these bacterial plasmids were effectively transduced (Fremaux et al., 1993; Mercenier et al., 1988; Raya et al., 1989).

2.2.1.2 Conjugation

Transduction is an accidental process that occurs rarely, whereas conjugation involves specific structures and metabolisms that are the result of natural selection favouring genetic exchange.

During conjugation, two compatible bacteria, in close range of one another, donate genetic material. The donor bacterium duplicates one of its DNA circles and donates it to the recipient through a proteinaceous tube, referred to as a conjugation pilus. This tube is fragile and usually breaks before the entire DNA helix is transferred, therefore the recipient becomes only partially diploid. The foreign DNA is then depolarised or inserted into the recipient's own DNA, followed by normal replication (Mauseth, 1995).

The best-characterised conjugation transfer system is the transfer of lactose fermentation plasmids in several lactococcal strains, such as *Lact. lactis* 712 and ML3. The conjugal transfer of the broad host range streptococcal plasmid pAMß1, a 26.5 kb erythromycin-resistant plasmid, was first demonstrated by Gasson and Davies (1980). Since then, pAMß1 and other streptococcal plasmids, such as pIP, have been transferred successfully into various LAB through conjugation (Evans and Macrina, 1983; Leblanc and Lee, 1984).

Despite the usefulness of the *in vivo* methods available for genetic studies, such as transduction and conjugation, the development of different techniques was necessary for actual gene cloning in LAB. Only the *in vitro* protoplast transformation and electroporation techniques have made recombinant DNA techniques feasible in LAB (Von Wright and Sibakov, 1998).

2.2.1.3 Protoplast fusion

A protoplast is a bacterial cell with the cell wall removed and is also sometimes known as a spheroplast. These altered cells can be maintained in culture and stimulated to fuse with other protoplasts (Klug and Cummings, 1997).

The preparation and regeneration of lactococcal protoplasts were reported in 1980. Researchers demonstrated PEG-induced protoplast fusion and the

recombination of both chromosomal and plasmid-linked markers in the regenerated fusion products.

Progress in this field has since advanced at a rapid pace and several LAB have been transformed by means of the protoplast technique. The greatest success has been with the lactococci species and several *Lactobacillus* species that show a lower efficiency of protoplast transformation (Tynkkynen and Von Wright, 1988).

2.2.1.4 Electroporation

The adaptation of electroporation as a transformation method for LAB was due to the limitations and low efficiency of the protoplast technique.

Electroporation is based on the permeabilisation of the cell wall and the membrane by means of an electrical discharge. Electroporation has proven to be a very useful technique to transforming previously non-transformable lactobacilli, leuconostocs, pediococci and certain lactococci (Von Wright and Sibakov, 1998).

Lact. lactis subsp. *lactis* was the first strain to be electroporated. The transformation frequency was very comparable to that achieved by protoplast fusion. High efficiency electroporation results were also achieved with *Lb. casei*, followed by the successful electroporation of several other Gram-positive bacteria (Somkuti and Steinberg, 1988). In 1988 and 1989, further optimisation of this technique ensured the electroporation of lactococci strains, and today electroporation is used as a standard gene transfer method for *Lb. plantarum* (Van der Lelie et al., 1988; Bates et al., 1989).

2.2.2 CLONING VECTORS

After the first successful genetic transformation took place in 1982, it took only a year for the development of the first cloning vector. Since then, many specialised vector systems have been created (Kondo and McKay, 1984).

2.2.2.1 First generation vectors

The most commonly applied vectors, known as first generation cloning vectors, can be divided into two classes. The first class comprises vectors based on large conjugative plasmids that are found in Gram-positive species (Horodniceanu et al., 1976). Class 1 replicons do not appear to produce single-stranded DNA intermediates, a characteristic that has proven to increase genetic stability in cloning experiments. An example of this type of vector can be seen with the deletion derivatives of pIP501 and pAMβ1 that were obtained by spontaneous or deliberate deletions. These derivatives lost their conjugative properties, but retained the ability to replicate and express antibiotic resistance markers while showing an increase in copy number (Von Wright and Sibakov, 1998).

The class 2 vectors are based on small cryptic plasmids found in several lactococcal species. These cryptic plasmids have to be tagged with a selectable marker, usually an antibiotic resistance gene. This method has been used in the

construction of pGK12 and its derivatives on the basis of a cryptic replicon pWV01 from *Lact. lactis* subsp. *cremoris*, as well as in the construction of pCK1 based on replicon pSH71 of *Lact. lactis* subsp. *lactis* (Gasson, 1983). The drawback of class 2-type cloning vectors are that they often show instability when foreign DNA is expressed in them, possibly due to their mode of replication that creates single stranded intermediates. Therefore, the class 1-type vectors are preferred above class 2 due to their superior stability.

With the application of replicon screening techniques, another approach has been developed to construct cloning vectors. The technique is based on the joining of random fragments from different lactococcal plasmids and DNA fragments bearing selectable markers. An example of this type of vector is plasmid pNZ12, bearing the replicon region from pSH71 (Kok et al., 1984).

The two most widely used replicons are pWV01 and pSH71, which both are small plasmids that share nearly identical DNA sequences. What makes these plamids so popular is their unique feature of being able to replicate not only in several Gram-positive bacteria, but also in some *E. coli* strains (Kok et al., 1984). This unique characteristic makes the development of more sophisticated methods possible because of the fact that the *E. coli* system is considered the primary prokaryotic host due to its extensive genetic characterisation. Many of its biological processes are well understood and there is a wide range of genetic tools readily available for its manipulation (Billman-Jacobe, 1996).

2.2.2.2 Integration vectors

Once a gene transfer system has been established, it is possible to construct integration vectors that integrate foreign genes into the chromosome of the host cells. This is used especially in dairy starters, since many of the crucial properties are plasmid encoded and hence prone to instability. It has been shown that integration occurs via a single or, in rare cases, a double crossing-over mechanism. This makes it possible to construct strains with only the needed piece of foreign DNA in the host's genome (Leenhouts et al., 1989).

2.2.2.3 Food grade vectors

Vectors intended for industrial applications may not carry any DNA that is not fit for human consumption. This implies that antibiotic resistance markers in the plasmid vectors have to be replaced because of the possible transmission of the drug resistance plasmid to other organisms. This gives rise to the construction of alternative food grade selection systems. An example of a suitable marker was found in the lactose operon, where the *lacF* gene provided a direct selection method in the laboratory, as well as on an industrial fermentation scale (De Vos et al., 1990). Another vector, independent of antibiotic resistance markers, involved the use of a 7.6 kb fragment coding for a nisin resistance determinant and an origin of replication from a plasmid present in *Lact. lactis* subsp. *lactis* var. *diacetylactis* DRC3. This

vector existed as an independent replicon after self-ligation and, after further developments was used to clone phage-resistant genes (Froseth et al., 1988).

2.2.2.4 Selectable genetic markers

The most commonly used selectable genetic markers in the selection of transformed LAB are erythromycin, chloramphenicol and tetracycline. Depending on the type of strain and the constructed plasmids, any of these may be incorporated to generate selection criteria (Allison et al., 1995; Biet et al., 1998; O'Sullivan and Klaenhammer, 2001).

2.2.3 HETEROLOGOUS EXPRESSION SYSTEMS

The past decade has seen significant advances in the genetic study of LAB, resulting in the development of a great number of genetic techniques, transformation protocols and sophisticated vector, integration and amplification systems. In addition, several food grade selection systems have been generated that are sophisticated and sustainable (Rodriquez et al., 2002).

Essential for further advances in this research field is the modulated expression of genes in a variety of combinations, as well as the delivery of their gene products. It is for this purpose that a range of gene expression and protein targeting systems has been developed that can operate in various LABs.

There are three main purposes for engaging in heterologous expression systems:

1. To assist in unravelling the function of recombinant proteins and peptides;
2. To facilitate the transcriptional/translational control of recombinant gene expression; and
3. To enhance production levels in comparison to those of native sources (Makrides, 1996).

There are various factors that can influence the effectiveness of the production of bacteriocins by LAB, especially in the food industry. Heterologous expression systems provide attractive means by which these adverse situations can be avoided when it comes to the limitation of bacteriocin production by LAB. These limitations include:

- Narrow activity spectrum;
- Unprompted loss of bacteriocinogenicity;
- Poor adaptation of the natural host to the food environment;
- Unpleasant sensorial effects caused by growth of the natural host in food; and
- The emergence of bacteriocin-resistant bacteria (Daeschel, 1993; Holzapfel et al., 1995; Schillinger et al., 1996).

Heterologous production of LAB bacteriocins may also be used to construct multi-bacteriocinogenic strains or to confer antimicrobial properties to strains of technological interest, such as starter cultures. The design of an efficient expression

system for the production of recombinant proteins or peptides, such as bacteriocins, is dependent on many variable factors, which all need to be taken into account. These factors include cell growth characteristics, expression levels, location of the gene product and post-translational modifications, or regulatory issues (Makrides, 1996).

Taking into account that expression systems comprise of host cells, genetic elements such as transcription and translation signals, regulatory factors, genes and plasmids, the choice of each component can be critical for the successful expression of the genes of interest.

Based on the available knowledge and consideration of the limiting factors and variables, a variety of constitutive or inducible gene expression systems, as well as protein targeting systems, have been developed.

2.2.4 CONSTITUTIVE EXPRESSION SYSTEMS

Transcriptional initiation plays an important role in the efficiency and control of gene expression. Consequently, emphasis has been placed on the process of transcriptional initiation in both *Lactococcus* and *Lactobacillus*, in which constitutive expression systems are involved.

Based on the analysis of large numbers of promoters, a consensus lactococcal promoter can be deduced that includes several conserved sequences, such as the transcriptional initiation site found in *E. coli* and *B. subtilis* (De Vos, 1999).

There are three different strategies that can be followed in order to isolate promoters from suitable organisms such as *Lact. lactis* and other LAB. The first strategy is based on screening vectors including both plasmids and transposons, carrying promoter-less reporter genes, such as those encoding chloramphenicol resistance or β-galactosidase or β-glucoronidase. This strategy yielded 10 promoters that differed greatly in efficiency. Other useful lactococcal promoters are still being discovered by means of this method.

The second strategy capitalises on the number of genes that have been studied and has led to the identification of promoters, notably those from native genes, that have been shown to be strong as well as constitutive. This strategy yielded several controlled promoters that show immense promise (Pouwels and Leer, 1993; De Vos and Simmons, 1994; Mercenier et al., 1994).

The third strategy is an exciting novel approach that comprises the construction and screening of synthetic promoters obtained from consensus *Lact. lactis* promoters in which the sequences of separating spacer regions were randomised. This strategy yielded a set of 38 constitutive promoters that differed in strength by three to four logs of activity. Both the strength differences and the fact that they are all constitutive render this set of promoters specifically suitable for metabolic engineering studies in which gene expression can be modulated in a constitutive way (Jensen and Hammer, 1998).

2.2.5 CONTROLLED EXPRESSION SYSTEMS

In the development of controlled expression systems, a distinction is made between promoters based on their susceptibility to control their transcriptional efficiency and limitations in their application potential. The controlled expression systems developed for LAB are divided into two groups: the first consists of the 'sugar-inducible expression systems' and the second of the 'other gene expression systems'.

2.2.5.1 Sugar-inducible expression systems

Most genes involved in sugar transportation and catabolism are organised into operons that are strongly expressed and controlled at the level of transcriptional initiation. Most systems are subject to repression by the CcpA-dependent catabolite, but many are specifically controlled by dedicated regulators.

These sugar-inducible expression systems vary in efficiency, gene location and inducing sugars. Although most promoters are subject to repression, it appears that the *Strept. thermophilus lac* promoter is under the control of a transcriptional activator. This system, in contrast to other systems, creates an ideal setting, since it is located on a high copy number plasmid but provides for the induction of single copy genes inserted in the chromosomal *lac* region by gene replacement.

Of the several sugar-inducible promoters, the best characterised is the *Lact. lactis* operon. The autoregulated *LacR* repressor controls the *lacA* promoter. With the induction of lactose, the intermediate tagatose-6-phosphate inactivitates the *LacR* repressor that results in gene expression. This expression system, in which lactose is the sugar responsible for the expression of the *luxAB* genes, has been reported to have the highest efficiency of all the sugar-inducible systems (De Vos, 1999).

2.2.5.2 Other gene expression systems

These expression systems were developed to provide a system that was controlled better than the sugar-inducible systems. The most recent developments in gene expression systems are based on the molecular analysis of genetic regulatory circuits and can each be induced 100- to 10000-fold (De Vos, 1999).

ϕ 31-Induced explosive expression system

This system was developed on the basis of one of the promoters of the lactococcal bacteriophage ϕ 31 that is induced during infection by ϕ 31. The infection not only causes the activation of the middle promoter, P8625, but also results in the synthesis of the Tac transcriptional activator **(Fig. 2.4a)**. This results in protein overproduction and lysis of the expression host (O'Sullivan et al., 1996).

Nisin-controlled expression system

The nisin-controlled system is also known as the NICE system and was derived from the molecular characterisation of the production of nisin by several strains of

Lact. lactis. In this system, the presence of nisin in the extracellular media activates a response from the regulatory system, which consists of a response regulator, *nisR*, as well as the sensor kinase, *nisK* **(Fig. 2.4b).** This two-component regulatory system then regulates the induction of gene expression via the *nisA* promoter (Kuipers et al., 1995, 1998).

Thermo-induction system

This system is based on the design of a thermolabile variant of the Rro repressor, Rro12, that, upon shifting from a permissive growth temperature of 24°C to 42°C, resulted in a 500-fold induction of gene expression **(Fig. 2.4c)**. A drawback of this system, however, is that the thermo-induction is not easy to apply on an industrial scale and may activate various heat shock genes (Nauta et al., 1997).

pH-dependent system

Several screening approaches have uncovered pH-dependent promoters, due to the fact that acidification is one of the properties of LAB. The system is based on environmentally regulated control circuits that can induce more than 1000-fold expression in the presence of 0.5 M NaCl. An example of this is the glutamate-dependent acid stress resistance process, in which the induction of the *gad* promoter is induced by chloride or acid **(Fig. 2.4d)**. This induction results in the expression of the gene regulated by the *gad* promoter at a high efficiency rate (Sanders et al., 1997, 1998).

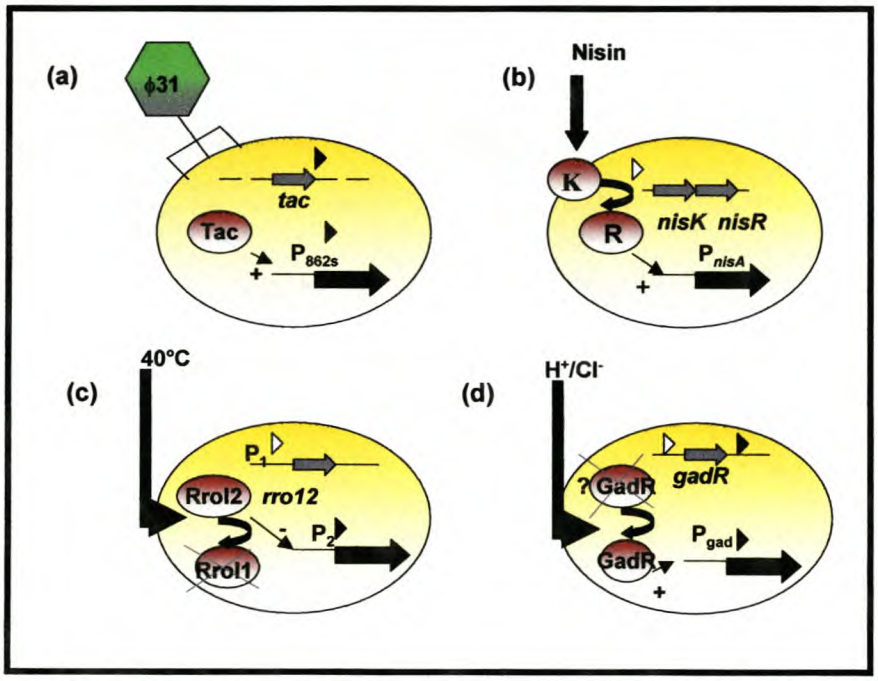

Fig. 2.4. General outline of the molecular architecture of the inducible gene expression systems developed for *L. lactis* and other LAB. (a)= ϕ31-induced explosive expression system; (b)= Nisin-inducible (NICE) system; (c)= Temperature-inducible system and (d)= pH-inducible system (De Vos, 1999).

2.2.5.3 Protein-targeting systems

Once the protein or peptide has been produced, the next step is to ensure that the molecule is properly folded, targeted and, if and when appropriate, recovered. This sets the scene for studies that aid the development of protein-targeting systems. Recent studies resulted in the export of novel peptides, such as colicin V, mesentericin and pediocin, through the use of dedicated, non-sec-dependent secretion via ABC exporters (Van Belkum et al, 1997; Horn et al., 1998a; Biet et al., 1998).

An emerging field that is being focussed on is the delivery of proteins to the cell envelope in order to facilitate protein recovery, immobilisation of cells or to present bioactive molecules such as antigens. Another lysis-specific system has been designed on the basis of the inducible expression signals that were coupled to lysine and/or holin genes from bacteriophages or to the lactococcal autolysis gene *acmA*. In all the cases presented, the lysis was achieved successfully in *Lact. lactis* and resulted in the release of intracellular enzymes. These systems show immense industrial potential, as can be seen in the NICE system, which already has been applied in cheese production (Wells et al., 1996; De Ruyter et al., 1997).

All the expression systems described above play an instrumental role in the understanding of gene function and expression in LAB. In the future, they may provide valuable information and could even be implemented as tools in other Gram-positive and Gram-negative bacteria. These systems may also be used to improve or develop new food applications for LAB and can aid in promoting the *in situ* production of compounds, either in food or in the intestinal tract. There are, however, several drawbacks to these systems, including low production levels and the fact that not all systems are suitable for all species and strains. Significant research efforts are required to develop systems that are optimal for specific conditions and applications. A new field, which may face opposition from consumers and Green Peace activists, is the use of genetically modified organisms for the production of bacteriocins. This can only be feasible if the opposition can be convinced of the advantages and that the application in the food and beverage industry will be safe. The future of these systems relies on the innovation and exploration of the researcher and has immense potential in the industry. These sophisticated expression systems show enormous value and promising prospects (De Vos, 1999).

2.2.6 EXAMPLES OF CLONED BACTERIOCINS

To date there have been several successful attempts to clone bacteriocin genes and to secrete functional bacteriocins in a variety of host cells. One of the well-known genes that has been cloned and sequenced is nisin (Kalette and Entian, 1989; Buchman et al., 1998). The cloning and heterologous expression of this peptide resulted in a 200-fold decrease in antimicrobial activity with respect to the naturally-produced nisin.

This led to an investigation into the development of a protein-engineered strategy to generate specific nisin variants. The expression system involves a lactococcal host strain that cannot produce nisin. The advantage of such a system is that it allows for the exclusive production of nisin variants and hence the assessment of the effect of a particular mutation on the biological and antimicrobial activity (Dodd et al., 1992).

The continuous development of new and improved biotechnological tools, as well as the knowledge gained by researchers on the genetics of LAB, has favoured the increasing number of studies dealing with heterologous gene expression by LAB. Another example of a bacteriocin that has been cloned and is currently being applied in the industry is helveticin J. This bacteriocin is produced by *Lb. helviticus* 481 and was expressed in *Lb. johnsonii.* Helveticin J has a narrow inhibition spectrum and its heat instability limits its use as a food preservative. The reason for its importance is that this bacteriocin may contribute to the host's ability to compete in complex dairy environments. The expression of Helveticin J was achieved by introducing the pTRK 135 plasmid containing the helviticin J structural gene into *Lb. johnsonii* NCK 64, a non-bacteriocinogenic derivative of the lactocin F-producer *Lb. johnsonii* VPI 11088. The result was a helveticin-producing strain, *Lb. johnsonni* NCK 249, the first reported case of the heterologous production of an LAB bacteriocin (Joerger and Klaenhammer, 1986).

Another example is lactocin S. This bacteriocin is a lantibiotic produced by *Lb. sakei* L45 (Mortvedt and Nes, 1990) that is encoded by a complex gene cluster located on plasmid pCIM 1 (Nes et al., 1994). Lactocin S biosynthesis was achieved in *Lb. sakei* 970 and *Lb. plantarum* NC 8 by sub-cloning the needed fragments into the shuttle vector, pELS 100 (Skaugen et al., 1999).

Pediocins are bacteriocins produced by *Pediococcus* species. The pediococci organisms are usually associated with vegetable and meat products, but are poorly adapted to growing in a dairy environment (Chikindas et al., 1995; Rodriguez et al., 2002). Pediocin is effective against a wide range of organisms, particularly *List. monocytogenes.* This organism is a food-borne pathogen of major concern to the dairy industry in particular, as it can proliferate in various dairy products at low pH and temperatures (Faber and Peterkin, 1991). Due to the strong antimicrobial activity of pediocin against this pathogen, many research projects focussed specifically on the production of pediocin in a dairy-adopted organism. *Strept. thermophilus* is an attractive host for this purpose, seeing that it is a food-grade microorganism used as a starter culture in the production of yoghurt and Italian and Swiss-style cheeses (Coderre and Somkuti, 1999).

Pediocin has been expressed in a vast number of different hosts, resulting in various production and expression efficiencies. One of the first successes was seen in the work of Marugg et al. (1992), who expressed pediocin PA-1 in both *E. coli* and a sensitive *Ped. pentosaceus* PPE1.2 strain. This was achieved by subcloning the pediocin PA-1 operon under the control of its native promoter into the new host. In 1995, Chikindas and co-workers demonstrated the production and secretion of

pediocin PA-1 by *Ped. pentosaceus* PPE1.2 that had been transformed with a plasmid (pMC117) containing the ped operon under the control of the lactococcal promoter P32. Pediocin production levels were up to four-fold higher compared to production by the native producer, *Ped. acidilactici* PAC1.0. This work demonstrated that alternative hosts could produce bacteriocins. Coderre and Samkuti (1991) also worked on the cloning and expression of pediocin PA-1. They cloned the pediocin operon from *Ped. acidilactici* into the shuttle vector pPC 418, which was introduced into *E. coli,* and also into shuttle vector pPC318, which was introduced into *Strept. thermophilus, Lact. lactis* subsp. *lactis,* as well as other types of LAB used in dairy fermentations. Future studies on the use of pediocin will concentrate on the construction of pediocin-producing LAB strains that will qualify for GRAS (Generally Regarded As Safe) status, thereby automatically permitting the specific application of these bio-protective cultures in foods.

In the beverage industry, there has been some success in the cloning and expression of bacteriocins in wine-related yeast strains. Schoeman et al. (1999) expressed the bacteriocin, pediocin PA-1, in a laboratory strain (Y294) of *S. cerevisiae*. Pediocin activity was detected readily. This research proves that developing bactericidal yeast strains, such as an *S. cerevisiae* starter culture that not only conducts the primary fermentation in the wine but also acts as a biological control agent, will allow the winemaker to reduce the use of chemical preservatives. This could set the scene for radical innovation and consumer satisfaction in the years to come. The biggest challenge that the industry will have to face is to ensure that production remains cost-effective and economically sound, a goal that is not easily reached (Bisson et al., 2002, Pretorius and Bauer, 2002).

These bacteriocins represent only a few of the successful expression experiments performed over the last few years. In addition to the heterologous production and cloning of LAB bacteriocins, there are numerous other strategies that have been followed in this research field. These include heterologous production of bacteriocins by exchanging leader peptides and/or dedicated ABC secretion and processing systems, as well as the heterologous production of bacteriocins by adding signal peptides recognised by general secretory pathways.

Tables 2.2 and **2.3** represent the vast number of bacteriocins heterologously produced by LAB using these ABC-dedicated transport systems and the general secretory pathways, respectively.

Table 2.2

Bacteriocins heterologously produced by LAB using an ABC-dedicated transport system (Cleveland et al., 2001)

Bacteriocin	Native host	Heterologous host	Vector	Reference
Acidocin A	*Lb. acidophilus* TK9201	*Lb. casei* TK9008	pESX72	Kanatani et al. (1995)
Acidocin B	*Lb. acidophilus* M46	*Lb. plantarum* 80	PCV461	Van der Vossen et al. (1994)
	Lb. acidophilus M46	*Lb. plantarum* 80	PCV61-3	Leer et al. (1995)
	Lb. acidophilus M46	*Lb. plantarum* 80	pLPE24M	Leer et al. (1995)
Carnobacteriocin B2	*C. piscicola* LV61	*Lb. sakei* Lb79 (pSAK20)	PSC1	Axelsson et al. (1998)
	C. piscicola LV61	*Lb. sakei* Lb79 (pSAK20)	PSC2	Axelsson et al. (1998)
Divergicin A	*C. divergens* LV13	*Leuc. gelidum* UAL 187-22	pLED1	Van Belkum et al. (1997)
	C. divergens LV13	*Leuc. gelidum* UAL 187-22	pLAD6	Van Belkum et al. (1997)
	C. divergens LV13	*Leuc. gelidum* UAL 187-22	pCOD1	Van Belkum et al. (1997)
	C. divergens LV13	*Lact. lactis* IL1403 (pMB500)	pLAD6	Van Belkum et al. (1997)
Enterocin A	*Ent. faecium* DPC1146	*Ent. faecalis* OG1X	pENT02	O'Keeffe et al. (1999)
	Ent. faecium DPC1146	*Ent. faecalis* OG1X	pENT03	O'Keeffe et al. (1999)
	Ent. faecium DPC1146	*Lact. lactis* IL1403	pENT02	O'Keeffe et al. (1999)
	Ent. faecium T136	*Lact. lactis* IL1403	PHB04	Martinez et al. (2000)
	Ent. faecium T136	*Lact. lactis* IL1403	PJM04	Martinez et al. (2000)
Helveticin J	*Lb. helveticus* NCDO 481	*Lb. johnsonii* NCK249	pGK12	Fremaux and Klaenhammer (1994)
Lactacin F	*Lb. johnsonii* VPI 11088	*C. piscicola* LV17	pTRK386	Allison et al. (1995)
	Lb. johnsonii VPI 11088	*Leuc. gelidum* UAL 187-22	pTRK204	Allison et al. (1995)
Lacticin 3147	*Lact. lactis* DPC3147	*Ent. faecalis* FA2-2	pOM02	Ryan et al. (1999)
Lactocin S	*Lb. sakei* L45	*Lb. plantarum* NC8	pELS163	Skaugen et al. (1999)
Lactococcin A	*Lact. lactis* WM4	*Leuc. gelidum* UAL 187-22	pMB553	Van Belkum and Stiles (1995)
	Lact. lactis WM4	*Ped. acidilactici* PAC1.0	PKV4	Chikindas et al. (1995)
Leucocin A	*Leuc. gelidum* UAL187	*Lact. lactis* IL1403 (pMB500)	PMJ6	Van Belkum and Stiles (1995)
	Leuc. mesenteriodes Y105	*Lact. cremoris* LC	pFBYC04	Biet et al. (1998)
	Ped. acidilactici PAC1.0	*Ped. pentosaceus* PPE1.2	pMC117	Chikindas et al. (1995)
	Ped. acidilactici PAC1.0	*Ped. pentosaceus* PPE1.2	pSRQ220	Venema et al. (1995)
	Ped. acidilactici PAC1.0	*Ped. pentosaceus* PPE1.2	pMC117	Venema et al. (1995)
Pediocin PA-1	*Ped. acidilactici* PAC1.0	*Lact. lactis* IL1403	pMC117	Chikindas et al. (1995)
	Ped. acidilactici PAC1.0	*Lact. lactis* LL108	pMC117	Chikindas et al. (1995)
	Ped. acidilactici PAC1.0	*Lact. lactis* MM210	pMC117	Buyong et al. (1998)
	Ped. acidilactici PAC1.0	*Lb. sakei* Lb790 (pSAK20)	pPED1	Axelsson et al. (1998)
	Ped. acidilactici PAC1.0	*Lb. sakei* Lb790 (pSAK20)	pPED2	Axelsson et al. (1998)
	Ped. acidilactici 347	*Lact. lactis* IL1403	PFI2126	Horn et al. (1998b)

Table 2.2

(continued)

Bacteriocin	Native host	Heterologous host	Vector	Reference
Pediocin PA-1 (continue)	*Ped. acidilactici* 347	*Lact. lactis* IL1403	pFI2126,pFI2148	Horn et al. (1999)
	Ped. acidilactici 347	*Lact. lactis* IL1403	PFI2160	Horn et al. (1999)
	Ped. acidilactici 347	*Lact. lactis* MG1614	pFI2126,pFI2148	Horn et al. (1999)
	Ped. acidilactici 347	*Lact. lactis* MG1614	PFI2160	Horn et al. (1999)
	Ped. acidilactici 347	*Lact. lactis* FI5876	pFI2126,pFI2148	Horn et al. (1999)
	Ped. acidilactici 347	*Lact. lactis* FI5876	PFI2160	Horn et al. (1999)
	Ped. acidilactic F	*Strept. thermophilus* ST128	PPC418	Coderre and Somkuti, (1999)
	Ped. acidilactici F	*Lact. lactis* ML3	PPC418	Coderre and Somkuti, (1999)
	Ped. acidilactici F	*Lact. lactis* SLA1.1	PPC418	Coderre and Somkuti, (1999)
	Ped. acidilactici F	*Ent. faecalis* DL3	PPC318	Coderre and Somkuti, (1999)
	Ped. acidilactici 347	*Lact. lactis* IL1403	PJM03	Martinez et al. (2000)
	Ped. acidilactici 347	*Lact. lactis* IL1403	PJM04	Martinez et al. (2000)

Table 2.3

Bacteriocins heterologously produced by LAB using a general secretory pathway (Cleveland et al., 2001)

Bacteriocin	Native host	Heterologous host	Vector	Reference
Brochochin C	*B. campestris* ATCC 43754	*C. piscicola* LV17C	pVB-2	Bohaychuk et al. (1999)
	B. campestris ATCC 43754	*C. piscicola* UAL 26	pVB-2	Bohaychuk et al. (1999)
	B. campestris ATCC 43754	*C. divergens* UAL278	pVB-2	Bohaychuk et al. (1999)
Carnobacteriocin B2	*C. piscicola* LV17	*C. divergens* LV13	pJKM14	McCormick et al. (1996)
Colicin V	*E. coli*	*C. piscicola* UAL 26	pJKM37	McCormick et al. (1996)
	E. coli	*C. piscicola* LV17C	pJKM37	McCormick et al. (1996)
	E. coli	*C. divergens* LV13	pJKM37	McCormick et al. (1996)
	E. coli	*Lact. lactis* IL1403	pJKM37	McCormick et al. (1996)
Divergicin A	*C.divergens* LV13	*C. piscicola* UAL 26	pCD4.4	Worobo et al. (1995)
	C.divergens LV13	*C. piscicola* LV17A,B and C	pCD4.4	Worobo et al. (1995)
	C.divergens LV13	*C. piscicola* LV17C	pRW5.6	Worobo et al. (1995)
	C.divergens LV13	*Lact. lactis* MG1363	pRW5.6	Worobo et al. (1995)
	C.divergens LV13	*Lact. lactis* IL1403	pRW5.6	Worobo et al. (1995)
Enterocin B	*Ent. faecium* BFE 900	*Ent. faecalis* ATCC 19433	pCMAP03	Franz et al. (1999)
	Ent. faecium BFE 900	*C. piscicola* LV17A	pCMAP04	Franz et al. (1999)
MBP-pediocin PA-1	*Ped. acidilactici* LB42-923	*E. coli* E609L	pPR6821	Miller et al. (1998)
Mesentericin Y105	*Leuc. Mesenteriodes* Y105	*E. coli* DH5α	pFBYC02	Biet et al. (1998)
	Leuc. Mesenteriodes Y105	*Lact. cremoris* LC	pFBYC07	Biet et al. (1998)

2.2.7 APPLICATIONS

2.2.7.1 Possible uses in food biopreservation

Customers expect modern food processing to produce foods and beverages with prolonged shelf life and increased safety. This poses a dilemma, as the consumers also demand minimally processed food products that are free of chemical preservatives. Consequently, the food and beverage industries employed the so-called 'green technologies' to provide possible solutions and novel approaches to how to minimise the processing of products. This has led to the exploitation of microbial metabolites, such as bacteriocins, for their use as biopreservatives (Ross et al., 2002).

Over the past few years, encouraging results have been obtained not only from the practical studies of bacteriocins, but also from their commercial application in the food and beverage industry. The main focus of the research is a bacteriocin called nisin. Nisin is produced commercially in the fermentation of milk by lactococci and is sold under the tradename Nisaplin, a product that contains 2.5% (w/w) nisin A. Nisin was approved by the World Health Organisation for use in foods in 1968 and it gained approval from the US Food and Drug Administration in 1988 (Hill, 1995; Delves-Broughton et al., 1996).

Nisin has found its application as a prolonger of shelf life in a range of products, which include cottage cheese, dairy desserts and liquid egg (De Vuyst and Vandamme, 1994). Nisin-producing strains have also been shown to improve vegetable fermentations and are found to be an effective inhibitor of spoilage bacteria during the fermentation of beer and wine. Other commodities that benefit from the use of nisin include milk products, canned foods, fish and meat products (Ross et al., 2002).

The principal application of nisin-producing strains is as a preservative in processed cheese products. The pathogen of primary concern from a food safety point of view is *List. monocytogenes*, which is found in various cheese products. This pathogen is capable of surviving refrigeration temperatures, acidic conditions and increase in pH. It has been known to survive the manufacturing process of cottage cheese, camembert and cheddar (Seelinger and Jones, 1986; Doyle et al., 1987; Faber and Peterkin, 1991). Studies have shown that, with the addition of nisin-producing strains or Nisaplin to the manufacturing process, *List. monocytogenes* was effectively inhibited (Maisnier-Patin et al., 1992; Ferreira and Lund, 1996).

Lacticin 3147 is another bacteriocin that has been demonstrated to be an effective biopreservative with various possibilities in food application. A major advantage of lacticin 3147 is that it can be produced by various transconjugants and that the performance of the generated strain is not compromised during food fermentations (Ross et al., 1999).

During the cheese production process, the use of lacticin 3147 starter cultures reduces the occurence of other non-starter LAB during cheese ripening. The LAB are

associated mostly with crystal formation and flavour and slit defects that influence the quality of the product. By using lacticin 3147-producing starter cultures, control over the product quality can be improved (Ryan et al., 1996).

Nisin and lacticin 3147 demonstrate the vast possibilities that lie within the application of bacteriocins as natural food preservatives. The following tables represent the bacteriocins isolated from foods and beverages (**Table 2.4**) and those that are being applied under patent rights in the food industry (**Table 2.5**).

2.2.7.2 Possible use of bacteriocins as biocontrol agents in beverages

The malolactic fermentation (MLF) is performed by lactic acid bacteria and plays a key role during the secondary fermentation of wine. In many cases, these bacteria can also be detrimental to the quality of the wine, especially if proliferation of certain LAB occurs at an inappropriate time while the wine fermentation is in progress (Kunkee, 1984).

LAB are found on the grapes, in the must and on cellar equipment and can survive the stresses of wine conditions. They have adapted to low pH ranges, low temperatures, low levels of SO_2, nutrient deficiencies and increasing ethanol levels. During the fermentation process, a number of LAB are suppressed by yeast interactions, rising ethanol levels and the production of bacteriocins by other LAB (Lonvaud-Funel, 1999). The unfavourable effect on the quality of the wine occurs after the alcoholic fermentation, when the LAB start to grow again, and may subsequently result in spoilage in the wine.

Research in Europe has demonstrated that nisin has potential to control spoilage LAB in beer and wine. These studies indicated that only the LAB, such as lactobacilli, were sensitive to nisin; the yeast was unaffected and there was no effect on the composition and flavour of the product (Delves-Broughton et al., 1996).

The management of MLF is often difficult when it is needed most. It is here that nisin might play an important role in the control of spoilage organisms present in wine and other alcoholic beverages. Nisin can be used to promote the quality of both red and white wines. In the case of wine that needs to undergo MLF, nisin and nisin-resistant *Oenococcus oeni* strains can be added to the wine after the yeast fermentation. The nisin will prevent the growth of LAB and only the resistant strains will grow and ensure the onset of MLF. In cases in which MLF is undesirable, nisin alone can be added, thus ensuring the absence of MLF (Radler, 1990; Daeschel et al., 1991; Hill, 1995; Delves-Broughton et al., 1996).

Similar results have been shown in the beer industry, with nisin preventing the growth of spoilage bacteria such as pediococci and lactococci that are present in the beer fermentation process. Another field in which the addition of nisin showed positive results was that of distilled alcohol production. The elimination of LAB due to added nisin resulted in less competition for yeast growth and thus allowing an increase in alcohol yield (Hill, 1995; Delves-Broughton et al., 1996).

Although bacteriocins provide the wine industry with a safe alternative to the use of chemical preservatives, their cost is high and their efficiency is limited. Currently, there studies are underway that focus on the development of wine yeast strains with antimicrobial activity. This is being done by using recombinant DNA technology to transfer the genes encoding for antimicrobial enzymes and peptides to *S. cerevisiae* yeast strains. This process will allow the winemakers to reduce the use of chemical preservatives, since the yeast will have the ability to secrete these novel biological preservatives while fermenting.

Table 2.4
Bacteriocins isolated from foods and beverages (adapted from Cleveland et al., 2001)

Source	Strain	Active against	References
Commercial probiotic product	*Streptococcus* sp. CNCM I-841	*Clostridium* spp., *List. monocytogenes*	Gomez et al. (1997)
Bulgarian yellow cheese	*Lb. delbrueckii* sp.	*List. monocytogenes*, *S. aureus*, *Ent. faecalis*, *E. coli*, *Yersinia enterocolitica*, *Y. pseudotuberculosis*	Miteva et al. (1998)
Vegetables	*Ent. mundtii*	*List. monocytogenes*, *C. botulinum*	Bennik et al. (1998)
Radish	*Lact. lactis* subsp. *cremoris* R	*Clostridium*, *Staphylococcus*, *Listeria* and *List. monocytogenes*	Yildirim and Johnson (1998)
"Waldorf" salad	*Lb. plantarum* BFE905	*List. monocytogenes*	Franz et al. (1998)
French mold-ripened soft cheese	*Carnobacterium piscicola* CP5	*Carnobacterium*, *Listeria* and *Enterococcus* spp.	Herbin et al. (1997)
Bean sprouts	*Lact. lactis* subsp. *lactis* (NisZ)	*List. monocytogenes Scott A*	Cai et al. (1997)
Munster cheese	*Lb. plantarum* WHE92 (PedAcH)	*List. monocytogenes*	Ennahar et al. (1996)
Spoiled ham	*C. piscicola* JG126	*List. monocytogenes*	Jack et al. (1996)
Traditional French cheese	*Ent. faecalis* EFS2	*L. inocua*	Maisnier-Patin et al. (1996)
Dry sausage	*Lb. plantarum* UG1	*List. monocytogenes*, *Bacillus cereus*, *C. perfringens*, *C. sporogenes*	Enan et al. (1996)
Irish kefir grain	*Lact. lactis* DPC3147	*Clostridium*, *Enterococcus*, *Listeria*, *Leuconostoc* spp.	Ryan et al. (1996)
Dry fermented sausage	*Lact. lactis* (NisA)	*List. monocytogenes*	Rodriguez et al. (1995)
Fermented sausage	*Lb. plantarum* SA6	*Lactobacillus* spp.	Rekhif et al. (1995)
Red smear cheese	*Brevibacterium lines* M18	*Listeria* and *Corinebacterium* spp.	Valdes-Stauber and Scherer (1994)
Meat	*Leuc. carnosum* Ta11A (LeuA)	*List. monocytogenes*	Felix et al. (1994)
Sour doughs	*Lb. bavaricus* (bavA)	*List. monocytogenes*	Larsen et al. (1993)
Whey	*Ent. faecalis* 226	*List. monocytogenes*	Villani et al. (1993)
Goat's milk	*Leuc. mesenteroides* Y105	*List. monocytogenes*	Héchard et al. (1992)
Sauerkraut	*Lact. lactis* subsp. *lactis* (Nis)	*List. monocytogenes*	Harris et al. (1992)
Beer	*Ped. damnosus* and *Ped. pentosaceus*	*Salmonella*	Skytta et al. (1993)
Wine	*Ped. pentosaceus* N4p	*Ped. pentosaceus* N5p	Strasser de Saad and Manca d Nadra (1993)
Rioja red wine	*Lb. plantarum* J-51	*Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*	Navarro et al. (2000)

Table 2.5
Examples of patented food applications of bacteriocins (Cleveland et al., 2001)

Author	US Patent	Patent Title	Use
Vandenburgh et al.	5,817,362 (10.06.98)	Method for inhibiting bacteria using a novel lactococcal bacteriocin	A method for inhibiting Gram-positive bacteria in foods by using a novel bacteriocin produced by *Lact. lactis* NRRL-B-18535
Blackburn et al.	5,753,614 (05.19.98)	Nisin compositions for use as enhanced, broad-range bactericides	Combination of nisin, a chelating agent and a surfactant to inhibit both Gram-positive and Gram-negative organisms in meat, eggs, cheese and fish; use as food preservative
Wilhoit	5,573,801 (11.12.96)	Surface treatment of foodstuffs with antimicrobial compositions	Use of Streptococcus-derived or Pediococcus-derived bacteriocins in combination with a chelating agent to protect food against *Listeria*
Vedamuthu	5,445,835 (08.29.95)	Method of producing a yoghurt product containing bacteriocin PA-1	A yoghurt product with increased shelf life containing a bacteriocin derived from *Ped. acidilactici*
Boudreaux et al.	5,219,603 (06.15.93)	Composition for extending the shelf life of processed meats	Use of a bacteriocin from *Ped. acidilactici* and a propionate salt to inhibit bacterial growth and to extend shelf life of raw and processed meat
Hutkins et al.	5,186,962 (02.16.93)	Composition and method for inhibiting pathogens and spoilage organisms in foods	Use of bacteriocin-producing lactic acid bacteria to inhibit growth of food-borne pathogens
Collison et al.	5,015,487 (05.14.91)	Use of lanthionines for control of post-processing contamination in processed meats	Inhibiting the contamination of processed meat products by pathogenic or spoilage microorganisms by treating the surface of the meat product with a lantibiotic
Vandenburgh et al.	4,929,445 (05.29.90)	Method for inhibiting *List. monocytogenes* using a bacteriocin	Inhibition of *List. monocytogenes* by a bacteriocin produced by *Ped. Acidilactici*
Gonzalez	4,883,673 (11.28.89)	Method for inhibiting bacterial spoilage and resulting compositions	Inhibition of food spoilage microorganisms in salads and salad dressing by a bacteriocin from *Ped. Acidilactici*
Matrozza et al.	4,790,994 (12.13.88)	Method for inhibiting psychotrophic bacteria in milk and cream	Inhibition of bacterial growth in cottage cheese by a bacteriocin-producing *Ped. pentosaceus* cells

Preliminary studies have indicated their potential usefulness, but the use of natural alternatives to chemical preservatives in wine production is largely dependent on their cost-effectiveness (Du Toit and Pretorius, 2000). Further research is needed to overcome these obstacles before the technology can be effectively applied in the wine industry.

2.3 FUTURE PROSPECTS

Enormous reseach efforts have led to a new, knowledge-based approach to microbiology. Modern technologies have led to a wealth of information on various microorganisms, including LAB. The genomes of many microorganisms used for food production have already been sequenced and their molecular mechanisms unravelled. In the field of biotechnology, the potential of genetic engineering has been demonstrated with the enchanced stability and activity of subtilin. The ongoing development of genetic recombination techniques and new expression systems accelerates the knowledge and understanding of various biological activities associated with LAB. These studies may soon lead to new possibilities to design

novel antimicrobials with specific target ranges, or to combine bacteriocin characteristics to enchance the culture performance (McAuliffe et al., 2001; Ross et al., 2002).

New research projects are aiming to improve the safety and quality of food supplies and are focussing on heterologous over- and multi-expression of bacteriocins, the genetic engineering of bacteriocins and the multi-hurdle concept, which involves the work of bacteriocins in combination (Ennahar et al., 2000; Du Toit, 2002; Ross et al., 2002).

2.3.1 HETEROLOGOUS AND MULTI-EXPRESSION

The production of bacteriocins has limited efficiency in fermented foods and can be ascribed to factors such as regulatory systems, occurrence of resistance amongst target bacteria and genetic instability. The recent trends in bacteriocin research may provide excellent tools to overcome these obstacles. Amongst these trends are the cloning and expression of bacteriocin genes in new hosts that allow constitutive production and overexpression of bacteriocins, therefore overcoming the limitations of the regulatory system (Chikindas et al., 1995; Fremaux et al., 1995; McCormick et al., 1996). Another use of heterologous expression systems is in the development of strains with multiple bacteriocin-production characteristics, resulting in specific target ranges that may also enhance the overall antimicrobial efficiency of LAB in food. As can be seen from these possibilities, heterologous expression may create interesting solutions and possibilities for future developments and for the extension of bacteriocin applications to the food and beverage industries as biopreservatives.

2.3.2 GENETIC ENGINEERING

The main objective of the application of genetic engineering is to improve existing strains or to develop novel strains for effective fermentation purposes. As can be seen from the research presented, the knowledge gained regarding the genetics and physiology of LAB is accumulating and it is becoming possible to genetically construct strains with desired characteristics for specific purposes (McKay and Baldwin, 1990).

LAB have an essential role in the majority of food fermentation processes and present a significant part of the food and beverage processing industry. One of the major advantages of these LAB is the production of antimicrobial peptides (McKay and Baldwin, 1990).

The new trend in research is based on the genetic engineering of organisms in order to produce superior strains. The engineering of bacteriocins through genetic or chemical modifications offers the possibility of developing new and improved biologically important peptides that may show large improvements in activity levels and stability. Interesting developments in this field have already begun with the study by Miller, which showed that a significant increase in pediocin PA-1 activity was

possible by substituting the Lys 11 residue with Glu (Miller et al., 1998; Ennahar et al., 2000).

2.3.3 MULTI-HURDLE APPROACH

The multi-hurdle concept refers to the use of bacteriocins in combination with one another in order to enhance the overall effectiveness against the target organisms. The use of bacteriocins in multi-hurdle food preservation systems may result in additive or synergistic effects that can improve preventative efforts or delay undesirable microbial activity. Due to their diverse spectra of activity, the class II bacteriocins are most suited for the multi-hurdle food preservation approach. This allows for many different combinations of bacteriocins that are adapted for each product to effectively eliminate pathogenic and/or spoilage organisms.

All of the above systems are in line with current and future consumer and industry trends, which are demanding minimally processed, safe foods of adequate shelf-life and convenience, as well as with the global need for a rapid increase in food supplies (Ennahar et al., 2000).

The industries mentioned above are only a few of those whose futures are being researched and in which new technologies are being considered. The following table provides examples of suggested applications in which these systems and organisms show immense potential and in which they may be applied successfully (**Table 2.6**).

2.4 CONCLUSION

It is evident that LAB play a pivotal role in our daily lives. They are present in the food we eat, the beverages we consume and even in our intestinal tracts. LAB are part of our past, our present and most certainly our future.

Since their initial discovery in 1873, they have come a long way towards becoming essential components of the food and beverage industry. They have been exploited as starter cultures, as aroma enhancers in cheese and milk, as an acidification aid, as well as for the production of several inhibitory compounds (Mäyrä-Mäkinen and Bigret, 1998). The most emphasis has been placed on the production of bacteriocins that may act as biopreservatives in both the food and beverage industries. These antimicrobial compounds have been studied intensively over the past decade, they have proven to be of fundamental importance and may lead the way to a healthier and safer environment. The emphasis is now on the optimisation and innovation of current and possible developments of these systems and their possible uses (Pretorius and Bauer, 2002).

Table 2.6

Examples of suggested applications of bacteriocins (Cleveland et al., 2001)

Bacteriocin	Application	Conclusion	References
Nisin A	Incorporation of nisin into a meat-binding system (Fibrimex)	Addition of nisin can reduce undesirable bacteria in restructured meat products	Cutter and Siragusa (1998)
Pediacin AcH	Use of a pediocin AcH producer, *Lb. plantarum* WHE 92 to spray on the surface of Munster cheese at the beginning of the ripening period	Spray prevents outgrowth of *List. monocytogenes* and can be used as an antilisterial treatment	Ennahar et al. (1996)
Enterocin 4	Use of an enterocin producer, *Ent. faecalis* INIA4, as a starter culture for the production of Manchego cheese	Use of an *Ent. faecalis* INIA4 starter inhibits*List. monocytogenes* Ohio, but not *List. monocytogenes* ScottA	Nunez et al. (1997)
Linocin M-18	Use of *Bre. lines* as a starter culture for the production of red smear cheese	Causes 2 log reduction of *L. ivanovi* and *List. monocytogenes*	Eppert et al. (1997)
Nisin A	Use of nisin to control *List. monocytogenes* in ricotta cheese	Nisin effectively inhibits *List. monocytogenes* for 8 weeks	Davies et al. (1997)
Piscicolin 126	Use of piscicolin 126 to control *List. monocytogenes* in devilled ham paste	More effective than commercially available bacteriocins	Jack et al. (1996)
Leucocin A	Use of leucocine-producing *Leuc. gelidum* UAL187 to control meat spoilage	Inoculation of vacuum-packed beef with the bacteriocin producer delays spoilage by *Lb. sake* for up to 8 weeks	Leisner et al. (1996)
Lactocin 705	Use of lactocin 705 to reduce the growth of *List. monocytogenes* in ground beef	Lactocin 705 inhibits growth of *List. monocytogenes* in ground beef	Vignolo et al. (1996)
Pediocin AcH	Use of the pediocin producer, *Ped. acidilactici,* to inhibit *List. monocytogenes*	*Ped. acidilactici* (Ped^+) starter culture contributes to the effective reduction of *List. monocytogenes* during manufacture of chicken summer sausage	Baccus-Taylor et al. (1993)
Pediocin	Expression of the pediocin operon in *S. cerevisiae*	Potential application in preserving wine and bakery products	Schoeman et al. (1999)
Pediocin AcH	Addition of pediocin preparation to raw chicken	Controlled growth of *List. monocytogenes* at 5°C for 28 days	Goff et al. (1996)
Pediocin PA-1	Use of a *Ped. acidilactici* (Ped^+) strain as a starter culture in sausage fermentation	Pediocin effectively contributes to inhibition of *List. monocytogenes*	Foegeding et al. (1992)
Enterocin	Addition of enterocin to inoculated ham, pork, chicken breast, paté, sausage	Controlled growth of *List. monocytogenes* under several conditions	Aymerich et al. (2000a, b)

Several advances have been made in the study of LAB. The improvement of gene transfer techniques, the construction of cloning vectors and the development of heterologous expression systems suited for most LAB offer valuable tools for future experiments. However there still are LAB, such as *Oenococcus oeni,* that are not suited for these systems. These bacteria play an important role in the wine industry in particular, where they are responsible for the onset of MLF in certain wines. The ability to transform LAB into bacteriocin-producing strains or the selection of bacteriocin-producing native *O. oeni* may hold the key to the production of a natural, healty wine that is, free of chemical preservatives.

It is evident that the knowledge gained by researchers has opened up several possibilities. The prospective applications of LAB are overwhelming and promise to deliver a bright future for the food and beverage industries.

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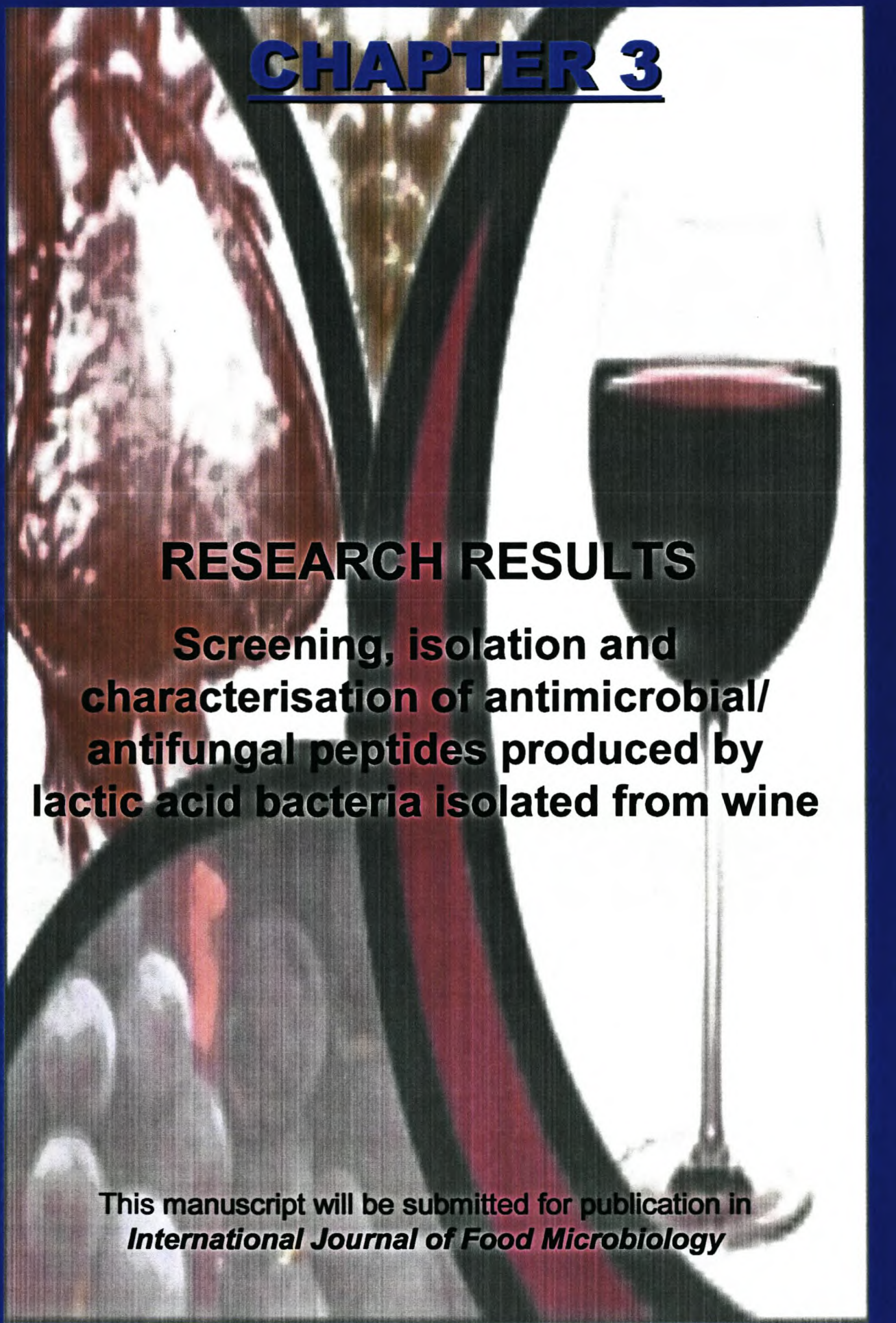

CHAPTER 3

RESEARCH RESULTS

Screening, isolation and characterisation of antimicrobial/ antifungal peptides produced by lactic acid bacteria isolated from wine

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3. RESEARCH RESULTS

Screening, isolation and characterisation of antimicrobial/antifungal peptides produced by lactic acid bacteria isolated from wine

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ABSTRACT

A total of 170 lactic acid bacteria (LAB) were isolated from Pinotage, Merlot and Cabernet Sauvignon cultivars in the Western Cape region. Samples were collected from grapes and during different stages of the winemaking process and screened for antimicrobial activity. Of the total isolates, 25 showed activity predominantly towards *Lactobacillus*- and *Pediococcus*-sensitive strains. Two isolates were selected for further characterisation due to their stability in bacteriocin production and constant high activity against the indicator organism, *Lactobacillus plantarum* LMG 13556. The two bacteriocin-producing strains were identified as *Lactobacillus paracasei* #77 and *Lactobacillus brevis* #81.1. The bacteriocins were inactivated by proteinase K, α-chymotrypsin and lysozyme, but not by catalase. The bacteriocins were heat stable and displayed the highest activity from pH 3 to pH 7. The selected isolates were found both to be bacteriostatic in their mode of action. The highest production of bacteriocin occurred after approximately 16 h of growth at 30°C. The molecular weight of the bacteriocins, as determined by tricine SDS-PAGE, was between 6.5 and 14.0 kDa. The LAB isolates were also tested for antifungal activity against *Botrytis cinerea*. The most predominant activity was seen after 24 h of incubation with germinating spores. This study shows that LAB found in South African vineyards and in the winemaking process produce antimicrobial substances that have the potential to inhibit or out-compete other non-producing or sensitive LAB naturally present in the wine and also to influence the proliferation of fungal spores.

3.1 INTRODUCTION

Many lactic acid bacteria (LAB) secrete small ribosomally-synthesised antimicrobial peptides, which are referred to as bacteriocins (De Vuyst and Vandamme, 1994). These substances have a narrow activity spectrum and prevent the growth of closely related bacteria. The producer strain has a degree of specific protection against its own secreted bacteriocin and is described as the immunity protein. Bacteriocins are divided into three main classes on the basis of their size: I) the lantibiotics are small

membrane-active peptides that contain the unusual amino acid, lanthionine, and β-methyllantionine and undergo extensive post-translational modification; II) the non-lanthionine peptides undergo minimal post-translational modification and have diverse chemical and genetic characteristics; they are small (<10 kDa), heat-stable, non-lanthionine-containing peptides; and III) the non-lantibiotic proteins are large (>30 kDa), heat labile and relatively uncommon amongst the antibacterial compounds of LAB (Nes et al., 1996; Van Belkum and Stiles, 2000).

Bacteriocins constitute only part of the inhibitors produced by lactic acid bacteria; other inhibitory substances include hydrogen peroxide, diacetyl, acetoin, formic and benzoic acids (Delgado et al., 2001). Some strains also produce low molecular weight metabolites, such as reuterin and pyroglutamic acid (Ouwehand, 1998).

LAB play an important role in the food fermentation process and are well known for their positive contribution towards the aroma, texture and flavour of the product, as well as towards the extension of shelf life and product safety (Lindgren and Dobrogosz, 1990; Stiles, 1996). In recent years, LAB and/or their antimicrobial compounds have received considerable attention due to their possible uses as control agents in the food industry. The production of bacteriocins shows immense potential in improving product quality and preservation and offers a healthier alternative to the frequent use of chemical preservatives in the food and beverage industries (Martínez et al., 1996; Van Reenen et al., 1998; Pretorius, 2000; Pretorius and Bauer, 2002).

LAB are also found in the wine environment and play a pivotal role during the malolactic fermentation (MLF). MLF plays an important role in governing the acidity and sensory characteristics of certain wines. Deacidification is caused by the conversion of malic acid to lactic acid, resulting in an increase in pH (Daeschel et al., 1990). Depending on the style and the composition of the wine, MLF may be advantageous or detrimental. The growth of indigenous LAB in certain wines may be the cause of spoilage characteristics, such as ropiness and the formation of excess acetic acid. MLF is difficult to manage in low pH wines, in which it is needed, whereas MLF is unwanted in high pH wines, in which it cannot always easily be prevented. The inoculation of selected LAB strains can promote consistency, but naturally occurring LAB still pose a threat (Kunkee, 1967; Radler, 1990).

Various methods to prevent MLF are used in the industry, such as maintaining a low pH (<3.2), a high alcohol content (>14%) and a high sulphur dioxide level (>50%). These conditions are not ideal for the majority of wines, however, and are not considered to be a healthy alternative (Daeschel et al., 1991).

It has been shown in the literature that the bacteriocin nisin, which is produced by *Lactococcus lactis* subsp. *lactis*, can inhibit the growth of certain LAB (Radler, 1990). Nisin does not affect the sensory characteristics or the yeast fermentation process. In a previous study (Daeschel et al., 1990), the inclusion of nisin was shown

to either prevent the onset of MLF, or to promote MLF by the addition of nisin together with the inoculation of nisin-resistant mutants of *Oenococcus oeni*.

Several bacteriocin-producing strains have been isolated from South African beverages such as sorghum beer and have shown inhibition against a number of spoilage bacteria (Van Reenen et al., 1998). Few bacteriocin-producing LAB from wine have been investigated and noted thus far. A study undertaken by Strasser de Saad and Manca de Nadra (1993) revealed the bacteriocin pediocin N5p, which is produced by *Pediococcus pentosaceus* isolated from Argentinean wines. Another example is *Lactobacillus plantarum* J-51, which was found to produce antimicrobial substances and were isolated from Rioja red wines in Spain (Navarro et al., 2000). However, in the South African wine industry, few reports have been found on either the production of bacteriocins by wine-associated LAB or by bacteriocins present in a finished wine. Eleven species of the genus *Lactobacillus* are associated with the winemaking process. These include *Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus fructivorans, Lactobacillus hilgardii, Lactobacillus nagelii, Lactobacillus kunkeei, Lactobacillus jensenii, Lb. plantarum* and *Lactobacillus sakei* (Du Plessis, 2002). Of these *Lactobacillus* strains, only *Lb. plantarum* J-51, isolated from red wine, showed antimicrobial activity (Navarro et al., 2000). No antimicrobial substances produced by *Lactobacillus* species or any of the other wine-associated LAB, which include *Pediococcus, Leuconostoc* and *Oenococcus*, have thus far been found in the South African wine environment.

Few reports have been found on the antifungal properties of these antimicrobial substances. Studies indicate that LAB are capable of exerting activity towards fungi produced mainly by *Lactobacillus* and *Lactococcus* species, although no reports indicate any antifungal properties of LAB isolated from a wine environment (El-Gendy and Marth, 1981; Vandenbergh, 1993; Gourama and Bullerman, 1995; Corsetti et al., 1998; Niku-Paavola et al., 1999; Okkers et al., 1999; Lavermicocca et al., 2000; Magnusson and Schnürer, 2001).

This study is an investigation of the possible production of bacteriocins by wine-associated LAB, to determine their spectrum of inhibition and to characterise the bacteriocins. The study also included the screening of the LAB isolates for antagonism against the fungus *Botrytis cinerea*.

3.2 MATERIALS AND METHODS

3.2.1 Media and culture conditions

MRS broth and MRS agar (Biolab, Merck, South Africa) were used for the cultivation of the LAB reference strains and isolates used in this study. Tomato juice agar (10 g

peptone, 10 g peptonised milk, 400 ml diluted and filtered tomato puree, 2.5% (w/w) bacteriological agar; pH 6) pour plates were used for the isolation of *Oenococcus*. The bacterial strains used in this study to determine the inhibition spectra of the antibacterial substances are listed in **Table 3.1**. Throughout the experiments, the strains were sub-cultured twice in MRS broth for 24 h at 30°C before continuing. The exception was *Listeria monocytogenes* B73 (Dykes and Hastings, 1998), which was grown in BHI broth at 37°C (Biolab, Merck, South Africa).

3.2.2 Isolation of lactic acid bacteria from grape berries and fermentations

LAB isolates were obtained from grape berries and fermentation tanks during various stages of the red winemaking process. The strains were isolated from Pinotage, Merlot and Cabernet Sauvignon cultivars in the Western Cape region of South Africa. Grapes were crushed in sealed plastic bags, a serial ten-fold dilution of the grape juice was made in Ringer solution (Merck, Germany) and 100 µl of each dilution was spread or pour plated out on MRS agar (Biolab, Merck, South Africa), supplemented with 25 mg/l kanamycin sulphate (Roche, Germany) and 50 mg/l Delvocid (Gist-Brocades, Netherlands) to prevent acetic acid bacteria and yeast growth respectively. The plates were incubated at 30°C under aerobic conditions. Colonies from the highest dilution showing growth were isolated and restreaked onto MRS agar twice to ensure purity. The purified isolates were maintained at -80°C in 20% (v/v) glycerol.

3.2.3 Screening for bacteriocin-producing strains

All Gram-positive, catalase-negative isolates were preliminary taken as LAB and screened for antimicrobial activity using the spot-on-lawn method (De Vuyst et al., 1996). The isolates were grown in 10 ml MRS broth for 18-24 h and 10 µl of the culture was spotted onto buffered bacteriocin-screening media (BSM) (Tichaczek et al., 1992) agar plates (without the catalase) and grown at 30°C for 24 h. The isolates were all tested against a wide variety of LAB and *List. monocytogenes* B73. The plates were examined for zone formation after 18-24 h. Isolates that displayed activity against more than one of the indicator strains were selected for further investigations.

3.2.4 Identification of bacteriocin-producing lactobacilli

The *Lactobacillus* isolates #77 and #81.1 were identified with species-specific PCR primers in a study conducted by Krieling (2003).

3.2.5 Activity assay and spectrum of inhibition

The cell-free, neutralised culture supernatants (CFNS) were obtained from isolates that were grown in MRS broth for 24 h at 30°C. The cultures were centrifuged at 12

000 rpm for 5 min at the pH of the supernatant, which was adjusted to pH 6-7 with 0.2 N NaOH solution. The supernatant was heated at 100°C for 3 min. The CFNSs were then spotted (10 µl) onto BSM agar plates according to the agar spot test method of Uhlman et al. (1992). All the isolates were tested against wine-associated LAB, such as *Lb. plantarum* K57, *Lb. brevis* W32, *Lb. vermiforme* NCDO 962, *Leuc. mesenteroides* DIII M:1, *Leuc. mesenteroides* EIII M:3(D):4, *Lb. plantarum* DM 3:1, *Lb. vermiforme* W16, *Lb. paracasei* DSM 5622, *Lb. pentosus* DSM 203154, *O. oeni* Q29, *O. oeni* B69 and *Lb. paracasei* L43.

3.2.6 Bacteriocin preparation

Three steps were used to partially purify the bacteriocins for further use. The methods firstly entailed the preparation of the CFNS of a 500 ml overnight culture grown in MRS broth at 30°C, then subsequent concentration by means of ammonium sulphate precipitation at 85% (w/w) saturation (Bollag and Edelstein, 1991), followed by chloroform/methanol precipitation (De Vuyst et al., 1996; Callewaert et al., 1999; Zamfir et al., 1999).

3.2.7 Characterisation of antimicrobial peptides

3.2.7.1 Sensitivity to enzymes

CFNS (3 200 AU/ml) was treated with the following enzymes according to the method of Franz et al. (1996): proteinase K (pH 7, Sigma, Germany), α-chymotrypsin (pH 7, Sigma, Germany), lysozyme (pH 7, Sigma, Germany) and catalase (pH 7, Sigma, Germany). The effect of the enzyme treatments on the antimicrobial activity was assayed using the spot-on-lawn method, with *Lb. plantarum* LMG13556 was used as indicator organism in all the following experiments, unless otherwise stated.

3.2.7.2 Storage and filtration stability

CFNS (3 200 AU/ml) was stored at -20°C, 4°C and 25°C for 24 h, after which activity assays were performed by means of the spot-on-lawn method, with *Lb. plantarum* LMG 13556 as indicator organism. The strains were also filtered through CAMEO 25As acetate filters (Osmonics) with a pore size of 0.22 and 0.45 µm and again assayed for activity by using the spot-on-lawn method.

3.2.7.3 Stability of bacteriocins at various temperatures and pH values

The neutralised cell-free supernatant (3 200 AU/ml) of isolates #77 and #81.1 underwent heat treatment at 25°C for 6 h, 65°C for 30 min, 80°C for 30 min and was autoclaved (121°C, 15 min) in separate treatments. Each of the samples was assayed for activity. The effect on antimicrobial activity was also determined at pH values ranging from pH 2-10 (intervals of 1.0). The adjustments were made with 0.2 N NaOH and 0.2 N HCl solutions. The samples were incubated for 10 min at 30°C, whereafter the effect of the pH values of the samples were evaluated by means of the spot-on-lawn test.

3.2.7.4 Ion exchange experiments with antimicrobial peptides

In an effort to determine if the antimicrobial activities of isolates #77 and #81.1 were due to the presence of a single or a heterogenic mixture of antimicrobial peptides, an ion exchange approach was followed. The CFNS of an overnight culture of the isolates grown in MRS broth at 30°C was adjusted to 50 mM MES at pH 6 (4-morpholine-ethane-sulfonic acid) by addition of an equal volume of 100 mM MES at pH 6 to the culture supernatant. SP and DEAE sepharose matrixes (500 µl) were equilibrated in 50 mM MES at pH 6, and these were, in turn, added to the sample mixture. The proteins present in the CFNS were allowed to bind to the respective matrix for 10 min while shaking at 150 rpm. The matrix was pelleted by centrifugation at 9 000 rpm, with subsequent resuspention in 1 ml 50 mM MES at pH 6. The resulting slurry was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 5 min at 9 000 rpm. The supernatant was removed and 1 ml of 1M NaCl, dissolved in 50 mM MES at pH 6, was added to the matrix pellet to disassociate the proteins from the matrix. The resulting sample, containing the proteins (10 µl), was assayed by means of the spot-on-lawn method to determine activity. The CFNS acted as a positive control, whereas the 1 M NaCl/MES (pH 6) acted as the negative control.

3.2.8 Growth and bacteriocin production kinetics of isolates #77 and #81.1

Each bacteriocin-producing strain was inoculated at 10^6 cfu/ml and incubated at 30°C in 50 ml MRS broth (pH 6.5). Bacteriocin activity was determined over a period of 48 h. Readings were taken every 2 h for the first 24 h and every 4 h for the remaining 24 h. Bacteriocin activity was determined by using the critical dilution method described by Schillinger et al. (1993). The activity was measured in arbitrary units (AU), according to which one unit was defined as the reciprocal of the highest dilution showing a clear zone of inhibition. The activity was then multiplied by a factor of 100 to obtain AU/ml. Viable cell counts were monitored by spread plating onto MRS agar plates at various dilutions.

3.2.9 Mode of action

The mode of action of the isolates was measured according to the method described by Franz et al. (1996). The indicator organism was grown in MRS broth (pH 6.5) for 24 h at 30°C. A 1 ml sample of the suitable diluted culture was inoculated into a 9 ml MRS broth solution that contained 1 ml of CFNS (5 120 AU/ml). The bacteriocin was concentrated (51 200 AU/ml) by means of the ammonium sulphate precipitation method, described by Bollag and Edelstein (1991). The control contained 9 ml of MRS broth solution and 1 ml of diluted indicator organism with no concentrated bacteriocin added. The samples were incubated at 30°C and cell counts were determined by spread plating onto MRS agar over a 10 h period.

3.2.10 Protein size determination

To determine the molecular size of the bacteriocins, tricine SDS-PAGE was used in accordance with the method described by Schägger and Von Jagow (1987). The CFNS of isolates #77 and #81.1 was partially purified and concentrated by centrifugation through a 3 000 Da column (Micon, USA) at 12 000 rpm for 1 h. The >3 000 Da fraction was used for the analysis. The gel was divided into two parts; one half was used for molecular weight determination and was fixed and stained with silver according to the method of Swain and Ross (1995). The other half of the gel was fixed in a 20% (v/v) iso-propanol and 10% (v/v) acetic acid solution for 15 min. The fixed gel was then rinsed with distilled water for 90 min at 30 min intervals, whereafter it was overlayed with MRS soft agar (0.7% w/v) that was inoculated with the sensitive organism at 10^7cfu/ml. The gel was incubated at 30°C overnight. An inhibition zone that surrounded the peptide indicated the position of the active bacteriocin.

3.2.11 Screening of LAB isolates for antifungal activity

3.2.11.1 Preparation of fungal inoculants

Botrytis cinerea spores were inoculated onto apricot halves (Natural Lite canned apricots), washed with sterile water to remove the syrup. The inoculants were incubated at 25°C for 10 days or until sporulation. *Aspergillus niger* was maintained on Potato Dextrose Agar (PDA) plates at 25°C until sporulation.

Fungal spores were harvested in 3 µl Tween-dH_2O (distilled water with three drops of Tween 20) using a glass hockey stick to remove spores from the hyphae. Spore concentrations were determined with a haemocytometer.

3.2.11.2 Antifungal activity

All the experiments assaying the inhibitory activity of the isolates were performed in triplicate. Antifungal activity was determined by two different assays, namely the overlay method and the microtitre plate well assay, described by Broekaert et al. (1990). For the overlay method, LAB colonies were spotted (10 µl) onto BSM agar plates and grown at 30°C. After 24 h, the plates were overlayed with 10 ml of soft agar (0.7% w/v) PDA containing 10^4 *B. cinerea* or *A. niger* spores per ml. The plates were then incubated at 25°C for 48 h and examined for clear inhibition zones around the colonies. In the microtitre plate assay, each well contained 50 µl of sample and 50 µl of PDA broth containing 2000 *Botrytis* spores. The plates were incubated at 25°C for 48 h. Fungal growth was measured at an optical density of A_{595nm} in a Powerwave X microtitre plate reader (Bio-Tek Instruments Inc.). The inhibition caused by peptide activity was expressed in terms of growth inhibition, which was defined as 100x the ratio of the corrected A_{595nm} of the control minus the corrected A_{595} nm of the sample over the corrected A_{595nm} of the control. The results were plotted against each other in a graph using Excel (Microsoft).

3.3 RESULTS

3.3.1 Identification of bacteriocin-producing LAB wine isolates

One hundred and seventy LAB strains of oenological origin from the Stellenbosch region were screened for the production of antimicrobial substances. Nine LAB strains and one *Listeria monocytogenes* strain were used as indicators for bacteriocin production. From the 170 strains isolated, 25 showed antimicrobial activity against various indicator organisms, including *Lb. nageli*, *Lb. plantarum*, *Leuc. mesenteroides*, *O. oeni* and *Lb. fermentum*, in the spot-on-lawn test done with the CFNS (**Table 3.1**).

Out of the total isolates, 14.7% showed positive results, with 88% of the positive isolates showing activity against *Lb. plantarum*. Of all the positive isolates that produced antimicrobial activity, only two were selected for further studies, namely isolates #81.1 and #77. Both isolates showed strong, consistent activity against the indicator strain *Lb. plantarum* LMG 13556 (**Fig. 3.1**). Isolate #77 was isolated from a Merlot fermentation tank on a renowned wine farm in the Stellenbosch region. The isolate was identified as *Lb. paracasei*. Isolate #81.1 was isolated from Merlot grape must from a wine farm in close proximity to the Stellenbosch region. The isolate was identified as *Lb. brevis* (Krieling, 2003).

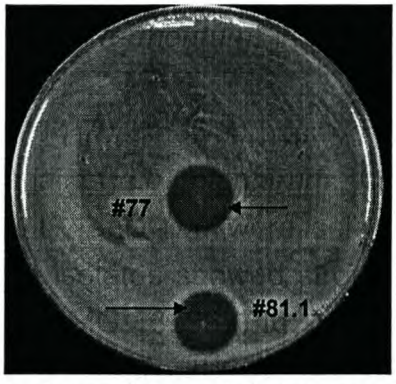

Fig. 3.1. Inhibition zones of isolates #77 and #81.1 against *Lb. plantarum* LMG 13556 by means of the spot-on-lawn test (Uhlman et al., 1992).

In addition to the activity test against the selected *Listeria monocytogenes* B73 and LAB reference strains listed in **Table 3.1**, the two selected isolates, #77 and #81.1. were also tested against a panel specifically selected to give a wide representation of species associated and isolated from the wine environment in South Africa (**Table 3.2**). Both isolate #77 and #81.1 showed activity against four of the indicator strains, namely *Lb. plantarum* K52, *Lb. vermiforme* U75, *Lb. vermiforme* W16 and *O. oeni* B69.

Table 3.1

Inhibitory spectrum of the pH-neutralised, cell-free supernatant of LAB strains isolated from grape berries and red wine fermentation tanks

Inhibitory activity$^{\Delta}$									
	Sensitive organisms								
*Isolates	A$^{\Omega}$	B$^{\Omega}$	C$^{\Omega}$	D$^{\Omega}$	E$^{\Omega}$	F$^{\Omega}$	G$^{\Omega}$	H$^{\Omega}$	I$^{\Omega}$
3	-	++	+	-	-	-	++	-	++
4	-	++	++	-	-	-	++	++	-
21.1	-	-	++	-	-	-	-	-	-
30	-	+	+	-	-	-	++	++	-
31		++	++				++	++	++
31.2	-	++	++	-	-	-	++	++	-
32	-	+	++	-	-	-	-	++	++
32.1	-	++	++	-	-	-	++	++	++
32.2	-	+	++	-	-	-	+	++	++
32.3	-	-	-	-	-	-	-	++	++
33.1	-	+	++	-	-	-	+	++	++
33.5	-	+	-	-	-	-	-	++	++
35.1	-	-	++	-	-	-	++	-	-
36	-	++	++	-	-	-	++	++	++
36.2	-	+	++	-	-	-	-	++	++
42	-	++	++	-	-	-	++	++	++
42.1	-	++	++	-	-	-	++	++	++
44	-	++	++	-	-	-	+	++	++
76.1	-	-	-	-	-	-	-	-	
77	-	-	++	-	-	-	-	-	-
79.2	-	+	++	-	-	-	-	-	-
81.1	-	-	++	-	-	-	-	-	-
89.1	-	-	++	-	-	-	-	-	-
89.2	-	++	+	-	-	-	+	-	+
91	-	++	++	-	-	-	++	++	-

*Only isolates that displayed activity against at least one sensitive organism are included.

$^{\Delta}$- = no zone/ + = 1-10 mm / ++ = >10 mm;

$^{\Omega}$ **A** = *Lb. kunkeei* DSM[1] 12361; **B** = *Lb. nagelii* ATCC[2] 700692 ; **C** = *Lb. plantarum* LMG[3] 13556; **D** = *Lb. sakei* LMG 13558;

E = *Ped. acidilactici* ATCC 12697; **F** = *Ped. pentosaceus* LMG 13561; **G** = *Leuc. mesenteroides* Ta33a; **H** = *O. oeni* MLF starter culture (Viniflora oenos, Christian Hansen, denmark); **I** = *Lb. fermentum* DSM13554.

[1]DSM = Deutsche Sammlung von Microorganismen, Braunschweig, F.R.G. [2]ATCC = American Type Culture Collection, USA. [3]LMG = Culture Collection Laboratorium Microbiologie, Gent, Belgium.

Table 3.2

The inhibitory spectrum of the CFNS of isolates #77 and #81.1 against wine-isolated LAB and indicator strains

Sensitive organism	Size of zone diameter	
	#77	#81.1
Lb. Plantarum K57	8 mm	8 mm
Lb. brevis W32	nz[a]	nz
Lb. vermiforme NCDO 962	8 mm	7 mm
Leuc. mesenteriodes DIII M:1	nz	nz
Leuc. mesenteriodes EIII M:3(D):4	nz	nz
Lb. Plantarum DM 3:1	nz	nz
Lb. vermiforme W16	13 mm	13 mm
Lb. Paracasei DSM 5622	nz	nz
Lb. Pentosus DSM 203154	nz	nz
O. oeni Q29	nz	nz
O. oeni B69	7 mm	10 mm
Lb. Paracasei L43	nz	nz

nz[a] = no zone

3.3.2 Effect of enzymes, pH and temperature on the CFNS

In order to increase the activity of the bacteriocins for further characterisation, the isolates were partially purified and concentrated by means of ammonium sulphate and the chloroform/methanol precipitation methods. **Table 3.3** illustrates the increase in AU/ml values for each of the preperation protocols.

Table 3.3

Influence of purification steps on the increase or decrease in Arbitrary Units (AU/ml) for the bacteriocin activity of each of the isolates

Purification method	Bacteriocin activity (AU/ml)		Increase or Decrease	
	#77	#81.1	#77	#81.1
Unpurified supernatant (CFNS)	6 400	6 400	Control	Control
Ammonium sulphate precipitation	51 200	51 200	Increase	Increase
Chloroform/methanol precipitation	25 600	51 200	Increase	Increase

The antimicrobial activity of the isolates #77 and #81.1 was stable after filtration and storage at -20°C and +4°C. After treatment with enzymes (proteinase K, lysozyme, catalase and α-chymotrypsin), the antimicrobial activities of these strains

were completely lost, except for in the catalase treatment (**Table 3.4**). This demonstrates that the inhibitory activity was due to a proteinaceous molecule.

Table 3.4

Effect of enzyme treatment on the inhibitory activity of the CFNS of isolates #77 and #81.1

Treatment	Activity	
	#77	#81.1
Proteinase K	-	-
α-Chymotrypsin	-	-
Lysozyme	-	-
Catalase	+	+

- = no zone / + = zone

The antimicrobial activities of isolates #77 and #81.1 were evaluated at different pH values and various temperatures at variable intervals. The inhibitory activity of isolate #77 remained active over a wide pH range, from pH 2.0 to pH 10.0. The activity was the highest from pH 3.0 to pH 5.0, ranging from 12 800 AU/ml to 51200 AU/ml. Isolate #81.1 also remained active over a pH range of pH 2.0 to pH 10.0. The activity was the highest from pH 2.0 to pH 7.0, ranging from 12 800 AU/ml to 25 600 AU/ml (**Table 3.5**). The tested temperature variants ranged from 4°C overnight to 121°C for 15 min, with both isolates maintaining half of their activity in all the conditions tested, except for the 15 min at 121°C, which caused a severe reduction in activity (**Table 3.6**).

Table 3.5

The effect of pH on the bacteriocin activity of isolates #77 and #81.1

pH values	AU/ml	
	#77	#81.1
2	6 400	6 400
3	12 800	12 800
4	12 800	12 800
5	51 200	12 800
6	6 400	25 600
7	3 200	12 800
8	3 200	6 400
9	3 200	6 400
10	1 600	3 200

Table 3.6

Effect of heat treatment on inhibitory activity of isolates #77 and #81.1

Temperature	AU/ml	
	#77	#81.1
Control	**51 200**	**51 200**
121°C for 15 min	800	1 600
80°C for 30 min	25 600	25 600
65°C for 30 min	25 600	25 600
25°C for 6 h	25 600	51 200
4°C overnight	51 200	51 200

3.3.3 Bacteriocin production kinetics

The growth kinetics and bacteriocin production of isolates #77 and #81.1 are shown in **Fig. 3.2** and **Fig. 3.3** respectively. The cell counts of the isolate #77 culture increased from 10^6 to 10^{10} within 28 h at 30°C. For this isolate, bacteriocin production started to increase after 12 h, from 400 AU/ml up to 3 200 AU/ml at 20 h, when maximum production was reached. Bacteriocin production decreased rapidly after 26 h and activity could be detected for up to 34 h. The cell density counts remained constant at 10^9 to 10^{10} from 28 h onwards. For isolate #81.1, bacteriocin production started to increase after 10 h, from 400 AU/ml up to 3 200 AU/ml after 16 h, when maximum production was reached. Bacteriocin production decreased dramatically after 18 h and activity could be detected for up to 30 h. The cell density remained constant at 10^{10} from 24 h onwards

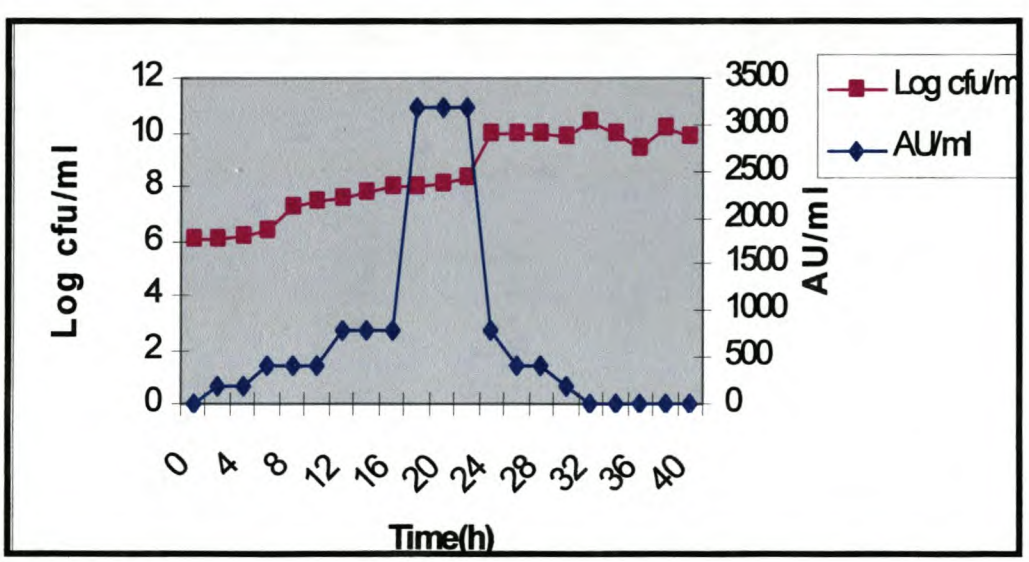

Fig. 3.2. Growth kinetics and bacteriocin production curve of isolate #77. Measured in log cfu/ml for cell viability and AU/ml for bacteriocin activity over time (h).

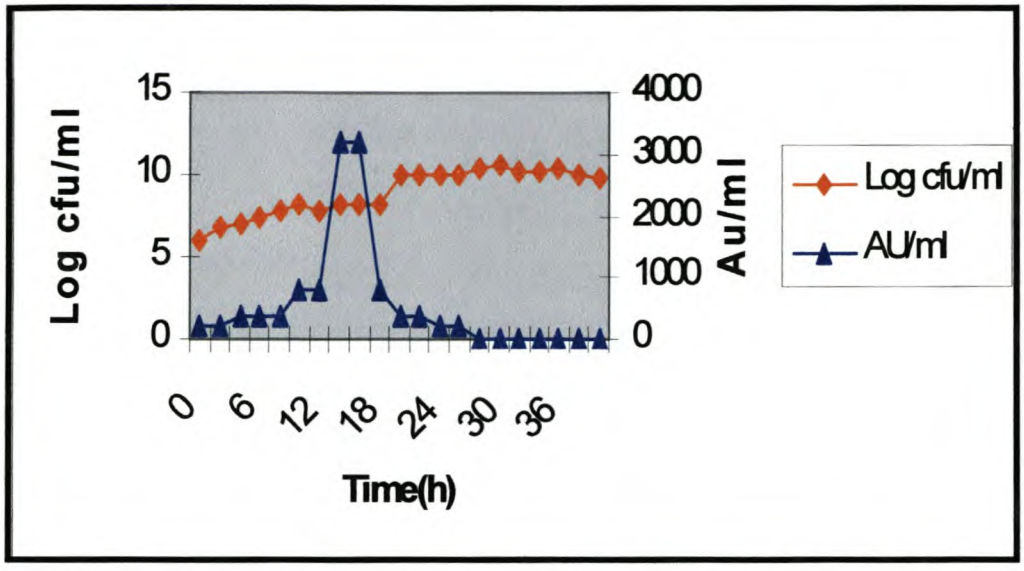

Fig. 3.3. Growth kinetics and bacteriocin production curve of isolate #81.1. Measured in log cfu/ml fro cell viability and AU/ml for bacteriocin activity over time (h).

3.3.4 Mode of action

The effect of the bacteriocins of isolate #77 and #81.1 on the growth of *Lb. plantarum* LMG 13556 is shown in **Table 3.7**. The bacteriocin produced by isolate #77 was added to an overnight culture of *Lb. plantarum* (10^6 cfu/ml) at 320 AU/ml. The addition of bacteriocin inhibited further growth of the indicator organism and cell counts remained relatively constant. The cell density over 9 h at 30°C ranged from 10^7 to 10^6. For isolate #81.1, the addition of the bacteriocin to *Lb. plantarum* (10^6 cfu/ml) at 320 AU/ml caused a slight increase, whereafter a decrease in cell density, ranging from 10^8 to 10^7, occurred. The control culture increased in cell density from 10^6 to 10^9 within 9 h at 30°C.

Table 3.7

Bacteriostatic effect of the bacteriocins produced by isolates #77 and #81.1 against *Lb. plantarum* LMG 13556 in MRS broth at 30°C

Cell viability of *Lb. plantarum* (log cfu/ml)			
Time (h)	**Control***	**#77**	**#81.1**
0	6.74	7.44	7.47
1	7.68	7.58	7.60
3	7.82	7.35	8.15
5	7.95	6.80	8.38
7	8.00	6.63	8.24
9	9.54	6.85	7.43

*MRS broth with no bacteriocin added

3.3.5 Partial purification and molecular size determination

The ion exchange, homo/heterogenic activity tests showed positive results for both isolates #77 and #81.1. In both these isolates, activity was observed through clearing zones on the agar plate assays. Activity was seen with the SP and DEAE sepharose matrix for both isolates, which indicates that active proteins were present on the cation as well as on the anion matrix (**Fig. 3.4**).

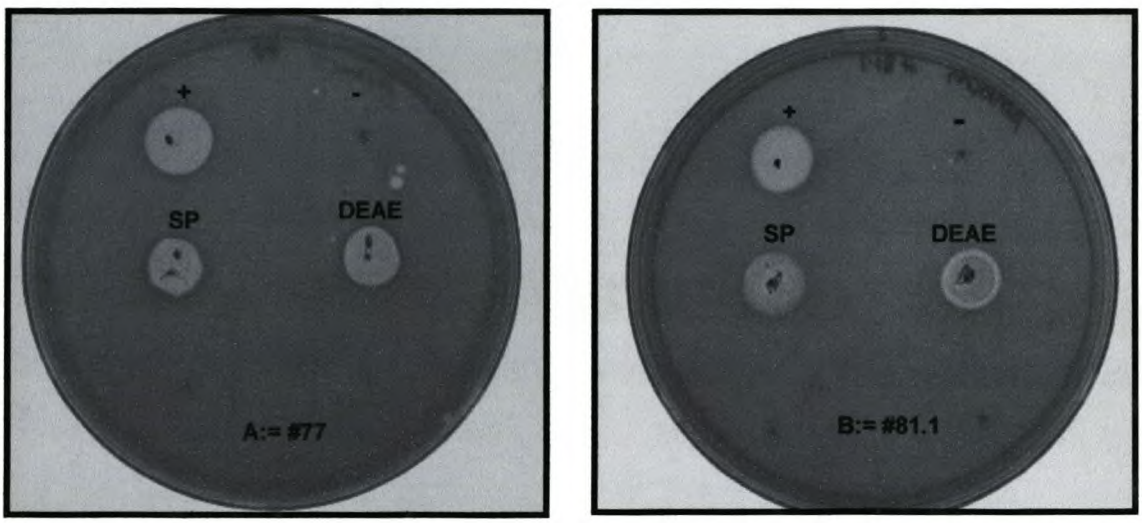

Fig. 3.4. Spot-on-lawn plates assays of ion exchange on SP and DEAE sepharose columns of isolate A:=#77 and B:=#81.1, respectively against the indicator organism *Lb. plantarum* LMG 13556.

The tricine SDS-PAGE showed peptide bands for the partially purified CFNS bacteriocin extracts. The protein bands responsible for the antibacterial activity could be observed on the overlay plates, where clear zones surrounded the active band at a size of between 6.5 and 14.0 kDa (**Fig. 3.5** and **Fig. 3.6**).

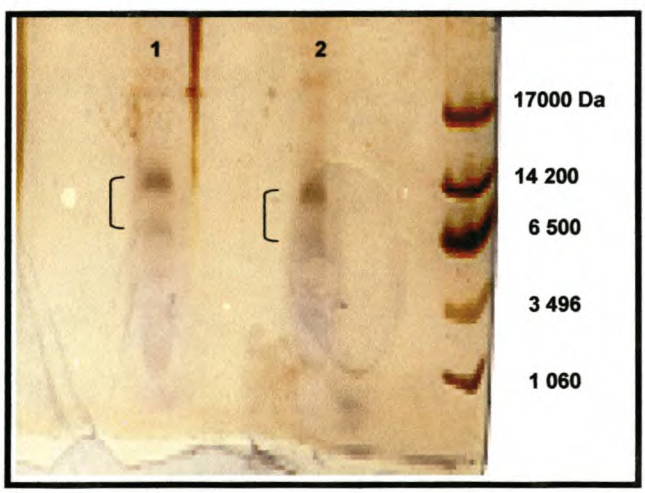

Fig. 3.5. Silver-stained tricine SDS-PAGE of the crude and ammonium sulphate precipitated bacteriocins produced by isolates #77, in lane 1 and #81.1 in lane 2.

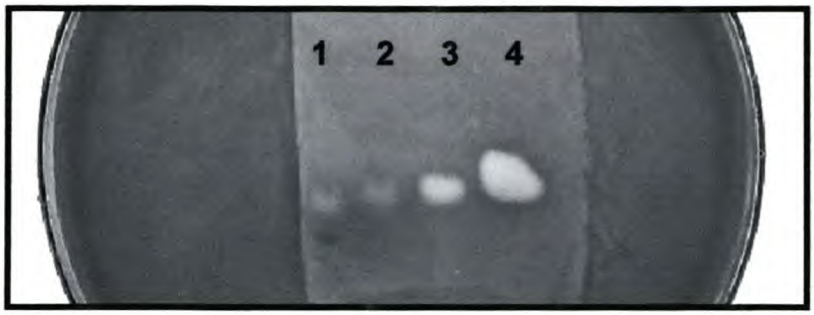

Fig. 3.6. Tricine SDS-PAGE overlay of crude protein extracts of isolates #77 and #81.1 with *Lb. plantarum* LMG 13556 as indicator organism showing zones of inhibition. Lane 1 represents the CFNS from isolate #81.1, while lane 2 represents the CFNS from isolate #77. Lanes 3 and 4 contain the ammonium sulphate precipitated protein of isolates #81.1 and #77 respectively.

3.3.6 Antifungal activity assay

Low activity was observed for the colony as well as for the microtitre plate assay methods. The colony assay showed no definite positive results and no zones could be detected around colony growth. The activity obtained with the microtitre plate assay ranged between 0 to 30% inhibition over the 48 h period. Inhibition of 20% or more was considered to be of significance. Isolates #80.4 and #91 showed the highest antifungal activity against *B. cinerea* of 28% and 26% respectively after 24 h. The rest of the isolates did not show significant inhibition and ranged between 0 to 18% inhibition. The most inhibition from all the isolates tested were seen after 24 h, when 91.43% of the isolates showed some inhibition, compared to the 8.57% of isolates that showed inhibition after 48 h (**Fig. 3.7**).

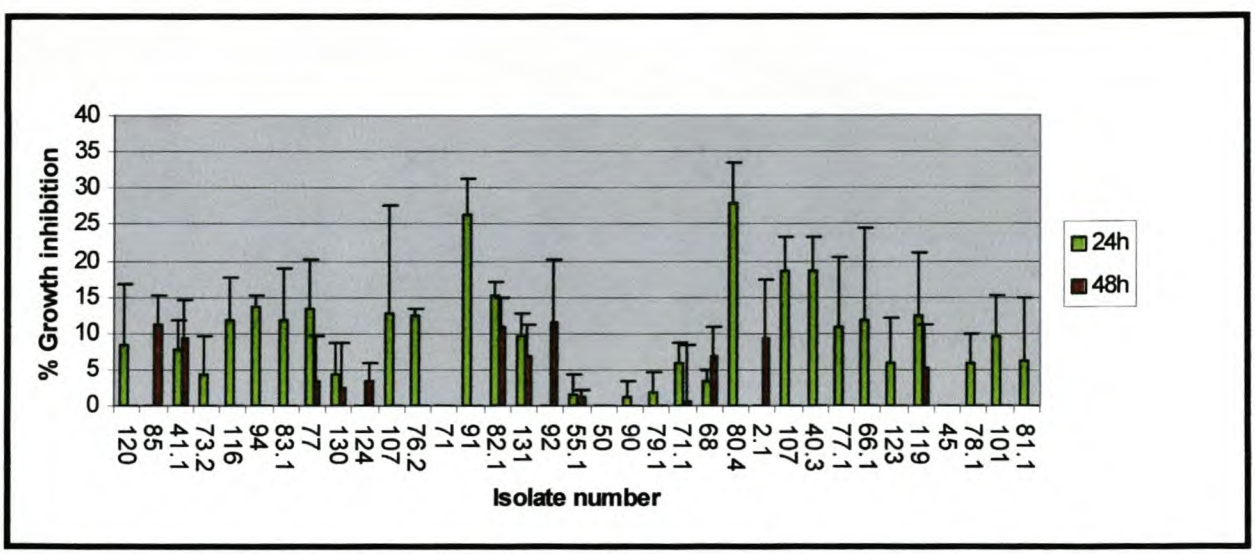

Fig. 3.7. The antifungal activity of the CFNS of *Lactobacillus* isolates against 2000 *B. cinerea* spores, which was assayed after incubation at 25°C for 48 h. The A_{595nm} (after 48 h) was used to determine the percentage of growth inhibition normalised against the negative control (50 µl PDA broth). The values were corrected by subtracting the time zero measurements at A_{595nm}.

3.4 DISCUSSION

Bacteriocin production was observed in 25 of the 170 evaluated LAB wine isolates. The isolates tested belonged to the four genera mainly associated with the wine environment, namely *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus*. The 25 isolates that showed bacteriocin activity belonged to the genera *Pediococcus, Lactobacillus* and *Leuconostoc*.

These results show that South African LAB wine isolates are more varied, as all the isolates in the study conducted on bacteriocin-producing LAB from Rioja red wines belonged to the genus *Lactobacillus* (Navarro et al., 2000). Strasser de Saad and Manca de Nadra (1993) reported one *Ped. pentosaceus* strain from Argentinean wines that showed inhibitory activity against other LAB strains, including *Pediococcus, Leuconostoc* and *Lactobacillus.* The CFNS of the 25 isolates displayed activity against *Leuconostoc*, *Pediococcus*, *Oenococcus* and *Lactobacillus* species, which is similar to, the activity of the *Lb. plantarum* isolated from Rioja and the *Ped. pentosaceus* isolated in Argentina.

The antimicrobial activities of only two of the 25 isolates, namely isolates #77 and #81.1, were studied further. It was observed that the antimicrobial activities were lost after treatment with the proteolytic enzymes lysozyme, proteinase K and α-chymotrypsin. This demonstrated that the inhibitory activities were due to proteinaceous molecules.

It was also found that the isolates were stable after filtration and storage at -20°C and +4°C. This suggests the absence of lipophilic structures in the molecules (Strasser de Saad and Manca de Nadra, 1993; Navarro et al., 2000). The peptides were stable over various temperatures over variable time intervals; this thermostability is characteristic of other bacteriocins produced by *Lb. casei* and *Lb. brevis*. Lactocin 705 produced by *Lb. casei* CRL 705, brevecin 37 produced by *Lb. brevis* B 37, caseicin A produced by *Lb. casei* OGM 12, brevicin 286 produced by *Lb. brevis* VB 286 and brevicin 27 produced by *Lb. brevis* SB 27 are all examples of heat stable bacteriocins that have similar characteristics to the results shown in this study (Rammelsberg and Radler, 1990; Olasupo et al., 1995; Coventry et al., 1996; Palacios et al., 1999).

The thermostability of the peptides produced by isolates #77 and #81.1 can be due to a small structure with low complexity or to a compact globular structure that is stabilised by covalent bonds (De Vuyst and Vandamme, 1994). The antimicrobial activity of isolates #77 and #81.1 remained active over a wide pH range, from pH 2.0 to pH 11.0. Growth and bacteriocin production at low pH levels may be advantageous for their use as possible biopreservatives in wine products, which in South Africa have a variable pH ranging from pH 3.3 to pH 4.0. The observed pH stability corresponds to the antimicrobial activity detected in LAB from Rioja red wines from Spain, whereas the study done on LAB isolated from Argentinean wines yielded different results (Strasser de Saad and Manca de Nadra, 1993; Navarro et al., 2000).

The growth kinetics and bacteriocin production of isolates #77 and #81.1 were similar. Both showed the higest cell counts after 24 h, with bacteriocin production being the highest towards the end of the logarithmic growth phase. The sudden decrease in bacteriocin production after 20-26 h may be due to the degradation by culture proteases released into the media after prolonged incubation or to the decrease in pH (Parente and Hill, 1992; Parente and Ricciardi, 1994). The highest bacteriocin production during the exponential growth phase was also detected for lactocin 705 and brevicin 286, which are produced by *Lb. casei* CRL 705 and *Lb. brevis* VB 286, respectively (Coventry et al., 1996; Palacios et al., 1999).

The bacteriostatic activity of the bacteriocins produced by isolates #77 and #81.1 was indicated by the inhibition of further growth of the indicator strain *Lb. plantarum* LMG 13556. The cell numbers stayed relatively constant after the addition of the bacteriocin. This type of inhibition kinetics is comparable to that found in similar studies conducted by Vignola et al. (1993) on the effect of lactocin 705 on the growth cycle. The addition of concentrated and neutralised supernatant containing lactocin 705 to a fresh culture of sensitive cells produced a rapid inactivation of growth.

The ion exchange experiments indicated activity by both the SP and the DEAE sepharose matrix. This could be due to more than one bacteriocin being present in the supernatant, or might indicate that the active bacteriocins fall in the binding range that overlaps both these sepharose matrixes. Further research is needed to determine the nature of the activity shown.

The approximate sizes as determined by tricine-SDS-PAGE analysis of the bacteriocins produced by isolates #77 and #81.1 were shown to be between 6.5 and 14.0 kDa. This is in the range of most of the class II bacteriocins that show heat stability, are small in size and consist of non-lanthionine-containing membrane active peptides. Class II also includes other bacteriocins produced by *Lb. brevis* that are similar to isolate #81.1 (Ouwehand, 1998).

Few reports have been published on the production of specific antifungal substances produced by LAB when compared to the reports on antibacterial activity of LAB. Studies indicated that LAB indeed are however capable of exerting antimicrobial activity against fungi, including mainly *Lactobacillus* and *Lactococcus* species (El-Gendy and Marth, 1981; Suzuki et al., 1991; Vandenbergh, 1993; Gourama and Bullerman, 1995; Corsetti et al., 1998; Niku-Paavola et al., 1999; Okkers et al, 1999, Lavermicocca et al., 2000; Magnusson and Schnürer, 2001).

In this study it was shown that the bacteriocins produced by various LAB had a negative effect on spore germination of *B. cinerea*. This was determined by means of a quantitative microtitre plate assay that showed fungal inhibition of up to 28% for some of the isolates tested. The highest fungal inhibition towards *B. cinerea* was shown by isolates #80.4 and #91 after 24 h. The fungi were able to overcome the inhibition effect after 48 h and no significant effect on hyphal development was detected. The assays performed on colony growth plates all showed negative results for inhibition of fungal growth on both *B. cinerea* and *A. niger*. A possible explanation

for this result could be due to the low percentage of inhibition against spore germination that is easily conquered and might result in no visual detection of inhibition zones. The results obtained in this study confirm that antimicrobial peptides in general could make an important contribution towards the fight against unwanted fungal growth. Further investigation is needed of the production of antifungal peptides produced by LAB. The possibility of using these peptides as natural inhibitors of fungal spoilage and mycotoxin formation may be a promising option for both the food and beverage industries.

This study clearly demonstrates the production of bacteriocins by LAB of oenological origin that showed inhibitory activity towards other LAB found in the wine environment. These LAB are better adapted to the fermentation process and best suited for use as biopreservatives in the wine industry. These results are the first recording of *Lb. brevis* and *Lb paracasei* species isolated from a wine environment that produce bacteriocins.

Further research is needed to determine the influence of these bacteriocins on the wine ecology and vinification process. Information on gene location, expression and the immunity regulation function must be gathered for future studies that may clarify the working of these peptides.

3.5 ACKNOWLEDGEMENTS

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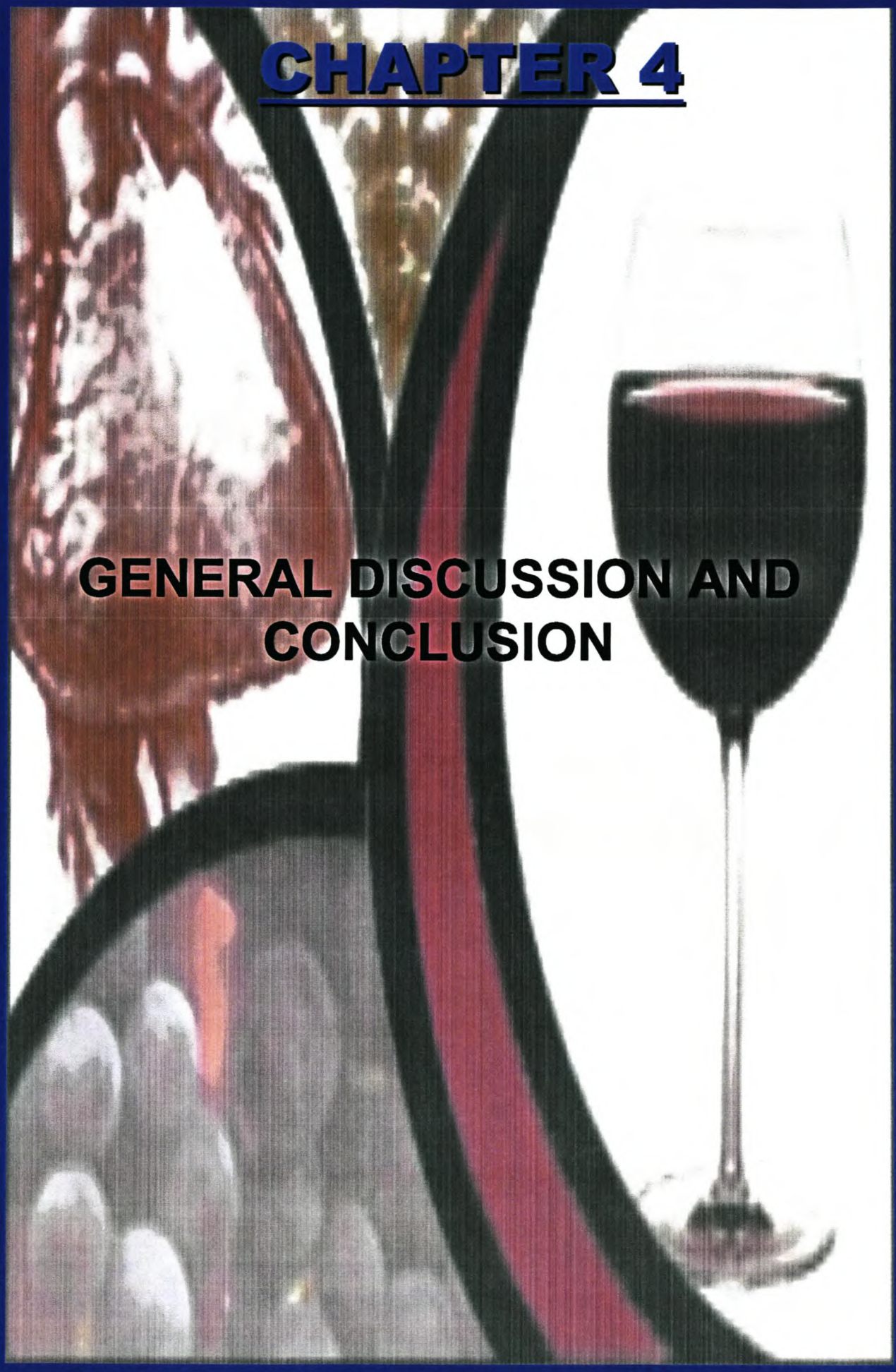
CHAPTER 4



GENERAL DISCUSSION AND CONCLUSION

4. GENERAL DISCUSSION AND CONCLUSION

4.1 CONCLUDING REMARKS

In any successful practice today, be it in an attorney's office, the local supermarket or in a wine cellar, they all listen to the same authoritive figure, the customer. Without the 'loyal customer' many practises will not stand the test of time, not even the age-old practise of winemaking. In previous years the producers defined wine quality and production trends, however today the modern consumer makes that decisions and steers the wine production wheel single handedly. In order to survive these uncertain times, the wine producers must satisfy the consumers' needs. The first step to survival is the understanding of the consumers' request, followed by the satisfaction of their needs (Bisson et al., 2002; Pretorius and Bauer, 2002; Vivier and Pretorius, 2002). In order for winemakers to keep up with the consumer trends, they have to incorporate parts of the 'scientific body of knowledge' led by innovative technological advancements into the winemaking process.

In recent years, consumers have become increasingly health conscience and aware of the importance of using environmentally friendly practices. One of the concerns is the use of chemical preservatives in the food and beverage industries. This has ensured renewed interest in novel biological preservation (biopreservation) methods, specifically the use of antagonistic microorganisms and their antimicrobial compounds. These potentially natural preservatives have the ability to control the growth of spoilage and/or pathogenic microorganisms in foods and beverages, and also to control mycotoxinogenic fungi (Karunaratne et al., 1990; Luchese and Harrigan, 1990; Schillinger et al., 1996)

The microorganisms most suited for the biopreservation research are the lactic acid bacteria (LAB). They are typically associated with the food industry and are generally regarded as safe (Schillinger et al., 1996). LAB have the ability to exert an inhibitory effect against other microorganisms by the production of bacteriocins or other inhibitory compounds, such as organic acids and hydrogen peroxide (Tagg et al., 1976).

The focus of this study was specifically on the production and characteristics of bacteriocins, produced by wine-isolated LAB. Bacteriocins are considered to be natural preservatives, better known as biopreservatives. These inhibitory substances are released extracellularly to exert either a bactericidal or bacteriostatic effect on other microorganisms that are closely related to the producer strain (Tagg et al., 1976). To date, the only bacteriocin that has found practical application is nisin. Many other bacteriocins have been characterised and have shown immense potential, but have not yet been introduced into industrially processed foods and beverages (Delves-Broughton, 1990; Buchman et al., 1998). There are several strategies for the application of bacteriocins in food preservation, including the inoculation of LAB

starter cultures that produce bacteriocins, the addition of the purified form of the desired bacteriocin, or the addition of a product that has been fermented previously with a bacteriocin-producing strain as an ingredient (Schillinger et al., 1996). Another method to introduce bacteriocins into the product would be to construct a fermentative strain, be it in yeast or bacteria, to acquire the ability to produce and secrete desired bacteriocins into the product during the fermentation process. Various transformation systems for yeast and a few for bacteria are already available for such construction and transformation experiments. Recent investigations into the genetic modification of LAB led to a sophisticated genetic analysis of gene structure and function that unravelled metabolic parthways. Protein and metabolic engineering have been applied on an experimental scale to help improve the process properties of this important group of dairy starters. However, there still is a need for more efficient gene transfer systems and for more information on gene expression and regulation. The challenge of this type of research is to modify the traditional properties of the product in an ethical and legally acceptable way (Von Wright and Sibakov, 1998).

Genetically modified microorganisms could be used as biological control agents instead of chemical preservatives to ensure the microbiological stability of fermented foods and beverages. This would not only be the healthier alternative, but would also act in an environmentally friendly manner that may partially satisfy consumer needs. However, the above-mentioned alternatives are not yet optimised or fully developed. Some may be expensive and not commercially viable, whereas others may influence the overall quality of the product (Daeschel, 1993). Others are faced by consumer resistance and are not fully understood. Including the above-mentioned obstacles, there are many more factors that may affect the bacteriocin-producing strains or the inhibitory effect of the bacteriocin itself that still need clarification. Before bacteriocins can be successfully introduced as biopreservatives in the food and beverage industries, bacteriocins need to be evaluated and a better understanding must be obtained of interactions between the bacteriocins and food components, as well as of the interactions between various microorganisms, not only towards one another, but also towards the environment to which they are subjected (Von Wright and Sibakov, 1998).

In this study the inhibitory activity of wine-isolated LAB that was found to produce antimicrobial substances were evaluated against other closely, related LAB. The best activity was observed against sensitive strains that belonged to the genera *Lactobacillus* and *Pediococcus*. The strains that showed to be sensitive are all associated with spoilage problems in the wine environment of South Africa and world-wide and may these antimicrobial substances be of vital importance when introduced as possible biopreservatives. Various *Lactobacillus* and *Pediococcus* strains form part of the spoilage microbes that affects wine quality and wholesomeness. LAB that may cause high levels of unwanted volatile acidity includes *Lactobacillus hilgardi* and *Lactobacillus fructivorans.* Another area of

concern in the winemaking process is the occurrence of ropiness and the production of carcinogenic substances such as ethylcarbamate by *Pediococcus damnosus* and *Lactobacillus hilgardi* respectively. These are only a few of the LAB that is of concern and needs to be eliminated without the aid of sulphiting. The discovery of LAB that are naturally present in the wine environment that show antibacterial activity towards these harmful LAB species may hold the key that will enable winemakers to provide a microbialy stable and healthier wines to the consumer

On further investigation into the LAB strains that produced these antimicrobial substances, the factors effecting the production, efficiency and optimal environments were determined. The antimicrobial substances isolated from the two producer strains *Lactobacillus brevis* and *Lactobacillus paracasei* were both stable and active at pH levels ranging from pH 3 to 7. This demonstrates the ability of these substances to function in the wine environment where pH levels range between pH 3 and 3.5. The temperature experiments indicated the substances use in all wine making conditions and showed no inactivation of activity through extreme temperature shifts. The evaluation continued and the best production levels occurred at a concentration of 3 200 AU/ml after 16 h to 20 h. The bacteriocin size was determined to motivate classification of bacteriocin. Most bacteriocins vary in size, ranging from 4 kDa up to 30 kDa. The approximate size of the bacteriocins investigated in this study was between 6.5 and 14.0 kDa.

In order to extent the use of these bacteriocins as biopreservatives their possible aid in the prevention of fungal spoilage was evaluated. In the wine industry their are various fungi that are responsible for poor grape and wine quality, such as *Botrytis cinerea* as well as fungi associated with cork spoilage that may introduce musty and earthy off flavours into the wine. In this study the bacteriocin activity showed some inhibition against *B. cinerea* spore germination for a short period of time. The fungi overcame the inhibitory affect within 24 h. The findings showed a glimpse of hope in the combat against fungi spoilage in wine. Their potential of these antifungal metabolites were evaluated and considered for further investigation.

The study performed provides a better understanding of the bacteriocin-production ability of LAB found the South African wine industry, their characteristics and inhibitory activity and potential uses of these bacteriocins. This study provides plausible baseline information on which to build future research projects to optimise the potential of bacteriocins as biopreservatives in the food and beverage industries. The next logical step will be to determine the gene sequences of the peptides isolated in this study. This will enable researchers to develop a cost-effective method for the introduction of these genes into wine starter cultures. The ideal setting would be to introduce these bacteriocin genes into *Saccharomyces cerevisiae* starter cultures, as demonstrated in the study done by Schoeman et al. (1998). This type of research would provide the industry with tailored wine yeast starter culture that is specifically bred for the wine environment. Their unique characteristics and desired qualities can be selected to suit the needs of the wine producers, in accordance with

the consumers' demands. The alternative is to introduce the bacteriocin genes into LAB starter cultures such as *Oenococcus oeni*. However, this type of research is in the preliminary stage and the development and optimisation of genetic tools are still needed. The proposed studies will enable yeast or LAB starter cultures to produce bacteriocins during their primary fermentation stages that will eliminate spoilage and harmful microbes in a natural and cost-effective manner.

In conclusion, the results obtained in this study are of vital importance for the development of bactericidal yeast and or LAB starter cultures. These cultures could possibly be used for the production of wine, other beverages and food products that are preservative free and can be labelled as the healthier alternative.

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