Screening, identification and characterisation of bacteriocins produced by the wine isolated LAB

by

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DECLARATION

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SUMMARY

Lactic acid bacteria (LAB) play a vital role in reducing wine acidity and also contributing to its aroma and flavour. However, they can also be responsible for many wine spoilage problems that compromise the quality and value of wine. While *Oenococcus oeni* contributes positive characteristics to the sensory properties of wine, certain species of the genera, *Lactobacillus* and *Pediococcus* can affect the wholesomeness of wine by producing undesirable compounds, such as biogenic amines and ethyl carbamate.

Chemical preservatives like sulphur dioxide (SO_2) are used to prevent the growth of spoilage micro-organisms during the winemaking process. SO_2 also acts as a reducing agent and maintains the benefits of antioxidant properties of the polyphenols of wine. However, there is a worldwide demand to reduce SO_2 levels due to the increasing health related risks and other factors.

All these considerations have increased the interest in research to look for new preservation strategies, and LAB-produced bacteriocins seem to be a potential alternative that has been explored in the last decade. Various types of bacteriocins have been identified and characterized. However, there are few reports on bacteriocins produced by LAB of oenological origin or on bacteriocins present in the finished wine.

The present study screened 155 LAB isolates from the IWBT culture collection for bacteriocin production. The isolates originated from South African red wines undergoing spontenous malolactic fermentation (MLF). Eight strains (5%) were identified to be producers, as evidenced by strong inhibition zones formed against sensitive organisms on agar plates. The producers demonstrated a broad spectrum of antimicrobial activity by inhibiting *Lactobacillus* spp., *Leuconostoc mesenteroides*, *Listeria monocytogenes* and *Pediococcus pentosaceus* strains. Some of these bacterial genera are important in winemaking since they are potential wine spoilage bacteria. Hence these strains and/or the bacteriocins they produce could possibly find application in the food fermentation industry.

The physiological results, biochemical tests and sugar fermentation profiles all gave the same results for the seven isolates, which were indicative of enterococci. The identification through 16S rRNA gene sequencing revealed that the seven tested isolates were all *Enterococccus faecium*. RAPD-PCR fingerprinting gave the same profile for the seven strains confirming that they were all identical on genetic level. Determining the molecular weight using SDS-PAGE showed the peptides to be below 4.6 kDa in size. PCR amplification of the enterocin P gene, sequencing and BLAST search results confirmed that all eight strains contained the enterocin P gene from *Ent. faecium*.

The enterocin tested in this study was heat stable at 100°C (30 min), but lost 50% of its activity at 121°C (15 min). Factors such as bacteriocin production and heat resistance are among many that enable enterococci to be dominant in fermented products such as dairy foods or meat. Therefore, enterococci producing bacteriocins have potential applications in various foods and fermented products. The pH tests showed enterocin to be active over a broad pH range (2-10). Enterocin activity over a wide pH range

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make them potentially more suitable as natural preservatives of foods and fermented products where products are acidified or pH decreases due to natural LAB present. They also have potential applications in oenological process where pH levels are as low as 3 and 4.

Proteolytic enzyme treatments with lysozyme, lipase, lyticase and catalase could not inhibit enterocin activity. This indicated that their antimicrobial activity was independent of lipid or carbohydrate moieties or hydrogen peroxide. α-Chymotrypsin and proteinase K inactivated enterocin, which indicated that the compound was proteinaceous in nature.

Bacteriocin production tested in two of the isolates, #16.3 and 128.1, coincided with the exponential growth phase which occurred after 6 hours of incubation at 30°C, which was an indication of primary metabolite kinetics. The highest production of 400 AU/ml was observed after eight hours and was maintained for several hours (46 hours) in the stationery phase. The bactericidal effect of the cell free supernatants from #16.3 and 128.1 against the sensitive culture of *Lactobacillus pentosus* DSM 20314 was clearly demonstrated by complete inhibition of growth for most of the experimental period, while the control increased exponentially throughout the experiment.

In conclusion, this study has confirmed the isolation and identification of *Ent. faecium* strains from wine, a genus that is rarely found in the wine environment. Although one can speculate on the origin of this bacterium in the wine e.g. human handling and contaminated water, these bacterial isolates produced enterocin P which have antimicrobial action against wine-related LAB genera and therefore have a potential role in wine spoilage control.

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OPSOMMING

Melksuurbakterieë (MSB) speel ‘n belangrike rol in die redusering van die suurgehalte van wyn en dra ook by tot die aroma en smaak daarvan. Hulle kan egter ook verantwoordelik wees vir vele wynbederfprobleme wat die gehalte en waarde van wyn negatief beïnvloed. Hoewel *Oenococcus oeni* positiewe karaktertrekke aan die sensoriese eienskappe van wyn verleen, kan sekere spesies van die genus, *Lactobacillus* en *Pediococcus,* die heilsaamheid van wyn beïnvloed deur ongewenste verbindings, soos biogeniese amienes en etielkarbamaat, te produseer.

Chemiese preserveermiddels, soos swaweldioksied (SO_2), word gebruik om die groei van bederfmikro-organismes tydens die wynbereidingsproses te voorkom. SO_2 fungeer ook as ‘n reduseermiddel en onderhou die voordele van die antioksidant eienskappe van die poli-fenole van wyn. Daar is egter ‘n wêreldwye vraag na die redusering van SO_2-vlakke as gevolg van die toename in gesondheidsverwante risiko’s en ander faktore.

Al hierdie oorwegings het belangstelling in die navorsing van nuwe preserveringstrategieë laat toeneem en MSB-geproduseerde bakteriosiene lyk na ‘n potensiële alternatief wat in die laaste dekade ondersoek word. Verskeie tipes bakteriosiene is geïdentifiseer en getipeer. Daar is egter nog weinig gerapporteer oor bakteriosiene wat deur MSB van wynkundige oorsprong geproduseer is of oor bakteriosiene wat in afgeronde wyn teenwoordig is.

Die huidige studie het 155 MSB isolate van die Instituut vir Wynbiotegnologie se kultuurversameling vir bakteriosien-produsering gegradeer. Agt stamme (5%) is as produseerders geïdentifiseer, soos gestaaf is deur sterk inhibisiesones wat teen sensitiewe organismes op agarplate gevorm het. Die produseerders het ‘n breë spektrum van antimikrobiese aktiwiteit by inhiberende *Lactobacillus* spp., *Leuconostoc mesenteroides*, *Listeria monocytogenes* en *Pediococcus pentosaceus* stamme gedemonstreer. Sommige van hierdie bakteriese genera is belangrik in wynbereiding, omdat dit potensiële wynbederfbakterieë is. Hierdie isolate en/of die bakteriosiene wat dit produseer, kan dus moontlik toepassing in die voedselfermentasiebedryf vind.

Die fisiologiese resultate, biochemiese toetse en suikerfermentasieprofiele het almal dieselfde resultate vir die sewe isolate, wat indikatief van enterococci was, gelewer. Die identifisering deur 16S rRNA-basispaaropeenvolging het onthul dat die sewe getoetste isolate almal *Enterococccus faecium* was.

RAPD-PKR-vingerafdrukke het dieselfde profiel vir die sewe rasse gelewer, wat bevestig dat die rasse almal identies op genetiese vlak was. Deur die molekulêre gewig vas te stel deur middel van SDS-PAGE, het dit getoon dat die peptiede kleiner as 4.6 kDa in grootte is. PKR-amplifikasie van die enterosien-P geen, die bepaling van basispaaropeenvolging en BLAST-soekresultate het bevestig dat al agt rasse die enterosien-Pgeen van *Ent. faecium* bevat.

Die enterosien wat in hierdie studie getoets is, was hitte-stabiel teen 100°C (30 min), maar het 50% van sy aktiwiteit teen 121°C (15 min) verloor. Faktore soos bakteriosienproduksie en hittebestandheid, is van die vele faktore wat enterococci in staat stel om dominant in gefermenteerde

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produkte, soos suiwelprodukte of vleis te wees. Enterococci wat bakteriosiene produseer het dus potensiële toepassings in verskeie kossoorte en gefermenteerde produkte. Die pH-toetse het getoon dat enterosien-P oor 'n breë pH spektrum (2-10) aktief was. Enterosienaktiwiteit oor 'n wye pH spektrum maak dit potensieel meer geskik as natuurlike preserveermiddels vir kossoorte en gefermenteerde produkte waar produkte versuur word of die pH afneem as gevolg van natuurlike MSB wat teenwoordig is. Dit het ook potensiële toepassings in enologiese prosessering waar pH-vlakke so laag as 3 en 4 is.

Proteolitiese ensiembehandelings met lisosiem, lipase, litikase en katalase kon nie enterosienaktiwiteit inhibeer nie. Daar is getoon dat hul antimikrobiese aktiwiteit onafhanklik was van lipiede, koolhidraatdele óf waterstofperoksied. α-Chymotripsien en proteïenase-K het enterosien onaktief gemaak, wat getoon het dat die samestelling proteïenagtig van nature is.

Bakteriosienproduksie wat in twee van die stamme #16.3 en 128.1 getoets is, het ooreengestem met die eksponensiële groeifase wat na 6 ure van inkubasie teen 30°C plaasgevind het, en wat 'n aanduiding is van primêre metabolitiese kinetika. Die hoogste produksie van 400 AU/ml is na agt ure waargeneem en is vir etlike ure (46 uur) in die stasionêre fase gehandhaaf. Die bakterie-dodende effek van die selvrye supernatant van #16.3 en 128.1 teenoor die sensitiewe kultuur van *Lactobacillus pentosus* DSM 20314 is duidelik gedemonstreer deur totale inhibisie van groei vir die grootste deel van die eksperimentele periode, terwyl die kontrole eksponensieel deur die hele eksperiment toegeneem het.

Hierdie studie het dus die isolering en identifisering van *Ent. faecium*-stamme, 'n genus wat baie selde gevind word in 'n wynomgewing, vanuit wyn bevestig Alhoewel daar gespekuleer kan word oor die oorsprong van hierdie bakterie in wyn bv. menslike hantering en besmette water, het hierdie rasse wel enterosien geproduseer en daarom die potensiaal om 'n rol te speel in beheer teen verskeie bederf-MSB-genera.

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BIOGRAPHICAL SKETCH

Buyani Ndlovu was born in KwaZulu Natal, South Africa on the 20th February 1984. He matriculated at Sakhelwe High School, South Africa in 2002.

He obtained a BSc (Biological Sciences) and HonsBSc (Cellular Biology) at the University of KwaZulu Natal, South Africa in 2007 and 2008, respectively. In 2010, he enrolled for an MSc-degree in Wine Biotechnology at Stellenbosch University.

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DEDICATION

This thesis is dedicated to my mother

Tholakele Mashabalala Ndlovu

Hierdie proefskrif is opgedra aan my moeder

Tholakele Mashabalala Ndlovu

Lolu cwaningo-mbhalo lubhekiswe kuMama wami

uTholakele Mashabalala Ndlovu

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PREFACE

This dissertation is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the International Journal of Food Microbiology.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

An overview on the use of bacteriocins produced bylactic acid bacteria (LAB), as well as other natural, chemical, and novel technologies in food and wine preservation.

Chapter 3 **Research Results**

Screening, identification and characterization of bacteriocins produced by wine-isolated LAB strains

Chapter 4 **General Discussion and Conclusions**

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CHAPTER 1

General Introduction & Project Aims

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CHAPTER 1

1.1 Introduction

Wine is a microbially diverse environment that harbours microorganisms such as yeasts and bacteria in varying degrees at different oenological stages. The four most relevant oenological genera of lactic acid bacteria (LAB) are *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Bae et al., 2006; Dicks et al., 2009; Ruiz-Larrea, 2010). Freshly extracted juice or must that is produced under commercial conditions generally contains various species of *Lactobacillus*, *Pediococcus* and *Leuconostoc* at a population range of 10^2 – 10^4 colony forming units (CFU) per ml (Bae at al., 2006; Ruiz-Larrea, 2010). These bacteria are thought to be originating from the surface of the grapes or contaminants of winery equipment that is used to process the juice or must. There is no record of the presence of *Oenococcus oeni* on the surfaces of immature, mature or damaged grape berries (Bae et al., 2006). However, *O. oeni* starts to appear and predominates during and after alcoholic fermentation. This is because oenococci have been long reported as the most resistant LAB to harsh wine conditions such as low pH, high sulphur dioxide (SO_2), alcohol content, limited nutrient availability and the presence of inhibitory compounds such as polyphenolics (Versari et al., 1999; Bartowsky, 2005; Mills et al., 2005; Ruiz-Larrea, 2010). However, other strains of LAB such as *Enterococcus* (Capozzi et al., 2011), *Lactobacillus*, *Pediococcus* and *Leuconostoc* can also survive and have been isolated from finished wines (Ruiz-Larrea, 2010).

Depending on various factors such as the species, the strain, population, and on the moment they multiply, LAB growth may be beneficial or detrimental to wine quality. Therefore, the control of bacterial growth during winemaking is necessary. Some wine LAB are the main cause of spoilage problems such as ropiness, excess acetic acid and production of off-flavours, which all compromise the quality of wine (Daeschel et al., 1991; Smit et al., 2008; Lerm et al., 2010; Ruiz-Larrea, 2010). Wine spoilage LAB can be controlled by maintaining a low pH (<3.2), a high alcoholic content (> 14%) and a high SO_2 level (>50 mg/ml). However, these conditions are desirable but not always feasible for the majority of wines (Daeschel et al., 1991). So far, adding acceptable doses of SO_2 in fermenting grape juice remains the only commercially recognised way of dealing with wine spoilage problems.

SO_2 is a universal chemical preservative that is commonly used to inhibit spoilage bacteria and yeasts in wines. It may cause allergic reactions to sensitive individuals or can be a source of hydrogen sulphide formation. The antimicrobial activity of SO_2 decreases with an increase in pH and is also known to vary in its activity against different bacterial strains (Gerbaux et al., 1997).

The public concern on the use of chemical preservatives and the subsequent demand for foods that are less processed has prompted research interest on natural preservatives. Over the past few decades the focus has been on antimicrobials such as lysozyme and bacteriocins, amongst others, as potential food and wine biopreservatives. Lysozyme is an enzyme present in the hen egg white and it has been reported to have lytic activity against most LAB (Gerbaux et al., 1997). In contrast to SO_2, the antimicrobial activity of

lysozyme increases with an increase in pH. However, similar to many antimicrobials, it has also been observed that some bacteria are more resistant to lysozyme activity (Gerbaux et al., 1997; Bartowsky, 2009). Bacteriocins are antimicrobial proteinaceous substances that are produced by some bacteria against other bacteria that are closely related to the producing organism growing in the same medium (Klaenhammer, 1988; Navarro et al., 2000; Cleveland et al., 2001; Hernández et al., 2005; Ruiz-Larrea et al., 2007). Four classes of LAB bacteriocins have been identified on the basis of biochemical and genetic characterization. These are: lantibiotics (class I), small, heat-stable nonlanthionine peptides (class II), large heat-labile proteins (class III) and complex bacteriocins containing chemical moieties such as lipids and carbohydrates (class IV) (Klaenhammer, 1993; Hernández et al. 2005).

Many bacteriocins produced by LAB are capable of inhibiting the growth of many food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (Hernández et al., 2005; Ruiz-Larrea et al., 2007). Antimicrobial activity of bacteriocins such as leucocin from *Leuconostoc mesenteroides* (Du Toit et al., 2002), nisin from *Lactococcus lactis* (Ogden, 1986; Radler, 1990a, b), pediocin PA-1 from *Pediococcus acidilactici* and plantaricin from *Lactobacillus plantarum* have been studied in wine applications individually and in synergy with other antimicrobials such as SO_2 (Du Toit et al., 2002; Rojo-Bezares et al., 2007b; Knoll et al., 2008). These bacteriocins are reported to inhibit most wine spoilage LAB such as *Lb. plantarum, Lb. paracasei*, *Lb. brevis*, *Lb. hilgardii, Lb. pentosus, Leuc. mesenteroides,* and *Ped. pentosaceus* (Du Toit et al., 2002; Rojo-Bezares et al., 2007b; Knoll et al., 2008). However, there are very few reports on wine isolated LAB that produce bacteriocins or on the presence of bacteriocins from finished wine (Lonvaud-Funel and Joyeux 1993; Strasser de Saad and Manca de Nadra 1993; Navarro et al., 2000; Rojo-Bezares et al., 2007a; Knoll et al., 2008; Saenz et al., 2009).

1.2 Research Aims

This study forms part of the biopreservation projects of the Institute of Wine Biotechnology (IWBT) where the focus is on researching alternative preservation methods to reduce the use of SO_2 in wine. Previously, a major focus was on bacteriocin-producing *Lb. plantarum* wine isolates. Therefore, the main objective of this study was to assess the bacteriocin producing ability of other genera and species associated with winemaking.

1.2.1 Specific Aims:

- Screening of LAB isolates from spontaneous malolactic fermentation wines (IWBT culture collection) for the production of bacteriocins;
- Identification of bacteriocin-producing LAB isolates;
- Determining the proteinaceous nature of the antimicrobial compounds from these isolates using proteolytic enzymes;
- Identification of the putative bacteriocins and determining their size;

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- Evaluating the impact of pH and temperature on the antimicrobial activity of the putative bacteriocins;
- Determining the growth and bacteriocin production kinetics of the producer isolates; and
- Assessing the mode of action of the antimicrobial peptides.

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CHAPTER 2

Literature Review

An overview on the use of bacteriocins produced by lactic acid bacteria (LAB), as well as other natural, chemical, and novel technologies in food and wine preservation

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CHAPTER 2

2.1. General introduction

Modern food processing is currently facing a challenge in that it aims to extend shelf-life and safety of foods and beverages by chemical means, while on the other hand consumers prefer foods that are minimally processed and free from chemical preservatives. This has aroused great interest in so-called "green technologies" including new strategies to minimally process and exploit microbial metabolites for biopreservation (Cleveland et al., 2001; Ross et al., 2002; Du Toit et al., 2002; Bauer et al., 2003; Martínez and Rodríguez 2005). Consequently, natural antimicrobial compounds derived from plants (e.g. phytoalexins, low-molecular-weight components of herbs and spices; phenolics such as oleuropein; and essential oils), animals (e.g. enzymes such as lysozyme and lactoperoxidase; other proteins such as lactoferrin, lactoferricin, ovotransferrin, and serum transferrins; small peptides such as histatins and magainins; and the immune system), and microorganisms (e.g. bacteriocins such as nisin and pediocin) have been the focus of recent research (Bauer et al., 2003; Gould, 1996).

Lactic acid bacteria (LAB) have been used in the production of a variety of dairy, vegetables and meat fermented foods for many centuries. Besides their contribution to the sensory characteristics of these food products (Fox and Wallace, 1997; Hernandez et al., 2005), LAB exhibits a strong inhibitory activity against many food pathogenic and spoilage microorganisms. This result from a wide range of antimicrobial substances they produce, including organic acids, hydrogen peroxide, diacetyl, acetoin, reuterin, reutericyclin, antifungal peptides, inhibitory enzymes and bacteriocins (Piard and Desmazeaud, 1992; Hernandez et al., 2005; Gálvez et al., 2007). The production of bacteriocins is widely reported among some LAB, which has GRAS (Generally Regarded as Safe) status because of their wide application as starters in food production. Bacteriocins have a great potential as food-grade antimicrobials. Nisin is a good example since it is currently used as a biopreservative in many countries (Martínez and Rodríguez 2005).

The current review will discuss many aspects of bacteriocins, including their long history of consumption as LAB metabolites, screening methods, purification and characterisation, classification, mode of action, spectra of activity, and resistance by target microorganisms. This will also include the general application of bacteriocins in food and beverage products, as well as the preservation techniques used in the wine industry. Moreover, the safety of bacteriocin consumption and their regulatory considerations will also be addressed.

2.2. History of LAB and bacteriocins

Classification of LAB was initiated in 1919 by Orla-Jensen (Orla-Jansen, 1919; Savadogo et al., 2006). However the classification criteria were only limited to morphological, metabolic and physiological criteria (Savadogo et al., 2006; Khalid, 2011). LAB form a diverse group of Gram-positive, non-spore-forming, non-motile rods, cocci or coccobacilli-shaped, catalase-lacking organisms. They are chemo-organotrophic and only grow in complex media. Fermentable carbohydrates and higher alcohols are used as

energy sources to form mainly lactic acid. LAB degrade hexoses to lactate (homofermentatives) or lactate and additional products like acetate, ethanol, CO_2, formate, or succinate (heterofermentatives). They exist in diverse ecosystems and are commonly found in foods (dairy products, fermented meats and vegetables, sourdough, silage, beverages), sewage, on plants but also in genital, intestinal and respiratory tracts of humans and animals (Savadogo et al., 2006). According to recent taxonomic revisions, there are 15 LAB genera of which *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form the core group (Khalid, 2011). Out of 15 genera, there is only four that are of oenological importance, namely *Lactobacillus, Leuconostoc, Pediococcus* and *Oenococcus* (Dicks et al., 2009; Ruiz-Larrea, 2010).

Bacteriocins are antimicrobial peptides that are produced by some bacteria to inhibit other micro-organisms which are closely related to the producer (Klaenhammer, 1988; Hernandez et al., 2005). These compounds are produced by both Gram-negative and Gram-positive organisms, including LAB (Franz et al., 1996). They inhibit a wide range of both Gram-negative and Gram-positive bacteria, and have a narrower spectrum of activity than antibiotics (Navarro et al., 2000; Hernandez et al., 2005). In England, Rogers and Whittier (1928) made the first observation that resulted in the discovery of bacteriocins. This was based on the discovery that certain lactococcal strains have an inhibitory effect on the growth of other LAB. Similar observations were made in 1933 by researchers in New Zealand who observed inhibition of cheese starter cultures. They isolated and identified the active antimicrobial compound as being proteinaceous in nature. Since the producer strains were identified as lactic streptococci of the serological group N, the bacteriocin was called nisin or group N inhibitory substance. The bacteriocin was first marketed in England in 1953 and since then approved for use in over 48 countries (Ross et al., 2002; Ruiz-Larrea, 2010). Bacteriocin production have been observed among lactobacilli, lactococci, enterococci, leuconostocs, pediococci, staphylococci and carnobacteria (Franz et al., 1996; Callewaert et al., 1999; Bauer et al., 2005; Hernández et al., 2005; Ferreira et al., 2007; Rojo-Bezares et al., 2007; Chaimanee et al., 2009). Most bacteriocins inhibit food-borne pathogens, like *Listeria monocytogens*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus*; hence they are considered as potential natural food preservatives (Franz et al., 1996; Cleveland et al., 2001).

2.3. Classification of Bacteriocins

Bacteriocins are classified into four distinct classes based on biochemical and genetic characterization. These are lantibiotics (class I), small heat stable non-lanthionine peptides (class II), large heat labile proteins (class III) and complex bacteriocins containing chemical moieties such as lipids and carbohydrates (class IV) (Klaenhammer 1993; Hernandez et al., 2005; Savadogo et al., 2006). However some authors only recognize three classes (class I-III) of bacteriocins as being defined on sound scientific basis (Martínez and Rodríguez, 2005; Savadogo et al., 2006). Some sub-divide class I and class II into sub-class Ia and Ib, and sub-class IIa and IIb, with sub-class IIc being eradicated due to its close similarity to sub-class IIa, both being secreted by the (*sec*)-system (Cintas et al., 1997; Cleveland et al., 2001). To date there has been no class IV bacteriocins purified. This strengthens the belief that this bacteriocin type is an artifact

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due to the cationic and hydrophobic properties of bacteriocins, which combine with other macromolecules in the crude extract to form complex molecules. Plantaricin S is a bacteriocin that has demonstrated a similar phenomenon. It was initially known to be a large complex molecule, but later declared to be a small peptide with no similarity to any of the purified LAB bacteriocins (Jimenez-Diaz et al., 1995; Cleveland et al., 2001). Bacteriocins produced by LAB usually range from 3-6 kDa (Savadogo et al., 2006), however, there are exceptions (Martínez and Rodríguez, 2005).

Bacteriocins of class I and class II are best understood and have great potential for food applications due to their target specificity and and strong antimicrobial activity (Cleveland et al., 2001). Class I bacteriocins have drawn much attention due to *L. lactis* produced nisin, which is the most widely studied bacteriocin and used as food preservative in several countries (Kawamotto et al., 2002; Ferreira et al., 2007). Pediocin PA-1, produced by *Ped. acidilactici* PAC 1.0 is an example of a well known class II bacteriocin (Marugg et al., 1992). Hence, in trying to elucidate the protein structure of bacteriocins, the focus will be on those well known such as nisin and pediocin from Class I and Class II, respectively.

2.3.1. Protein structure of nisin and pediocin

In 1971 nisin A was characterised as belonging to class I. It is a small thermoresistant peptide consisting of only 34 amino acids. Production of nisin A is quite complex. It requires a number of transcription, translation and post-translational modification processes, secretion and cellular signalling mechanisms (Engelke et al., 1992; Montville and Chen, 1998; Ruiz-Larrea et al., 2007). Nisin A is a polycyclic peptide comprising dehydrated residues (dehydroalanine and dehydrobutyrine) and five lanthionine rings and /or β-methyllanthionine containing disulphur bridges (Rollema et al., 1996; Cotter et al., 2005; Cheigh and Pyun, 2005; Ruiz-Larrea et al., 2007). It has a cationic nature and is an amphipathic molecule with a hydrophobic N-terminal region. The C-terminal region contains most of its charged and hydrophilic residues and this domain has a hydrophobic and hydrophilic side (Mol et al., 1999; Ruiz-Larrea et al., 2007). There are two types of nisin: nisin A is the most active type and nisin Z only differs in residue 27, being Asn (asparagine) (Figure 2.1) (Ruiz-Larrea et al., 2007).

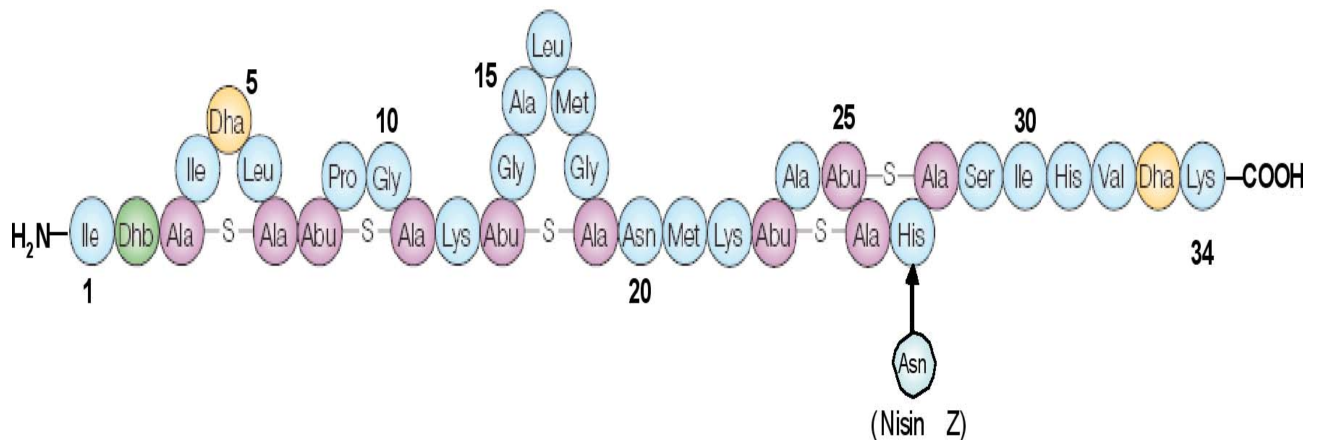

Figure 2.1: Primary structure of nisin A. Ala-S-Ala represents the lanthionine ring. Abu-S-Ala represents the β-methyllanthionine ring. Dehydrated amino acids: Dha (dehydroalanine) and Dhb (dehydrobutyrine). The arrow shows the amino acid substitution His27 to Asn27, which converts nisin A into nisin Z (Ruiz-Larrea et al., 2007).

Pediocin PA-1 is a class IIa bacteriocin which do not possess modified amino acids. It is a small thermoresistant peptide consisting of 44 amino acids and has a strong cationic and hydrophobic character. Pediocin, like all class II bacteriocins, have a primary structure that contains a consensus sequence: YGNGVXC. This common sequence has been proposed to be associated with strong activity against listeria (Ennaher et al., 2000). The predicted secondary structure of the C-terminal region forms an α-helix (Figure 2.2), which is considered to be the trans-membrane segment that is inserted into the cellular membrane of the inhibited cell (Ruiz-Larrea et al., 2007).

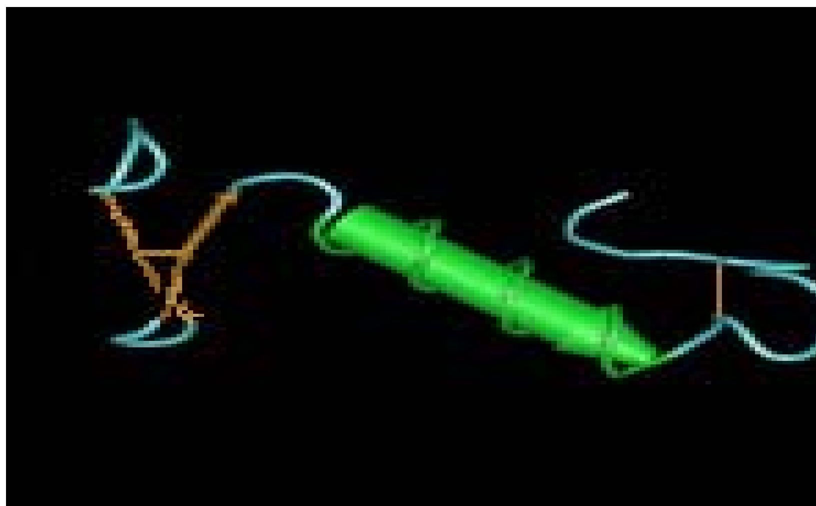

Figure 2.2: Tridimensional structure proposed for pediocin embedded in lipidic micelles. Blue segments represent loops, the green arrow represents the α-helix, and brown segments represent β-strands and two disulphur bridges (PDB 1OHN at http://www.ncbi.nlm.nih.gov/Structure).

2.4. Mode of Action: Class I and Class II bacteriocins

Most bacteriocins are bactericidal in their mode of action (Schillinger and Lücke, 1989; Schillinger, 1990; Holzapfel et al., 1995; Cleveland et al., 2001; Savadogo et al., 2006). However, there are a few

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exceptions which demonstrate bacteriostatic mode of action, e.g. leuconocin S (Lewus et al., 1992; Holzapfel et al., 1995) and leucocin A-UAL (Hastings et al., 1991; Holzapfel et al., 1995). Most bacteriocins weaken the overall structural integrity of the cytoplasmic membrane by pore formation (Jack et al., 1995; Martínez and Rodríguez, 2005; Savadogo et al., 2006).

Gram-positive bacteria are well known to have a high anionic lipid content in their membranes. The bacteriocin's mode of action results from its initial binding to the bacterial membrane of the target. Nisin activity has been demonstrated to act on energized membrane vesicles to disrupt the proton motive force, inhibit amino acid uptake and cause release of accumulated amino acids (Jung and Sahl, 1991; Abe et al., 1995). The mechanism is driven by the electrostatic forces between negatively charged bacterial membrane lipids and the positive charges of bacteriocins, which are localized in either the C- or N- terminal region (C-terminal in the case of nisin, and N-terminal of pediocin). This is followed by the bacteriocin insertion into the lipid bilayer (lipid II), which involves the N-terminal region in the case of nisin (Moll et al., 1999; Ruiz-Larrea et al., 2007), and the transmembrane α-helix of the C-terminal region in case of pediocin (Ennhar et al., 2000; Ruiz-Larrea et al., 2007). This result in pore formation through the bacterial membrane which becomes permeabilized (Figure 2.3). The cell loses ions and metabolites which are essential for survival and subsequently leads to bacterial death (Ruiz-Larrea et al., 2007).

Even though lipid II seems to play a vital role in pore formation (particularly in the case of nisin) by acting as a docking station, pore formation does not necessarily require the presence of lipid II. For instance, at high concentrations of nisin, pore formation can occur in the absence of lipid II if a cell membrane contains at least 50% of negatively charged phospholipids. Under these conditions the positively charged C-terminus of nisin is essential for initial binding and antimicrobial activity (Wiedemann et al., 2001; Bauer et al., 2005).

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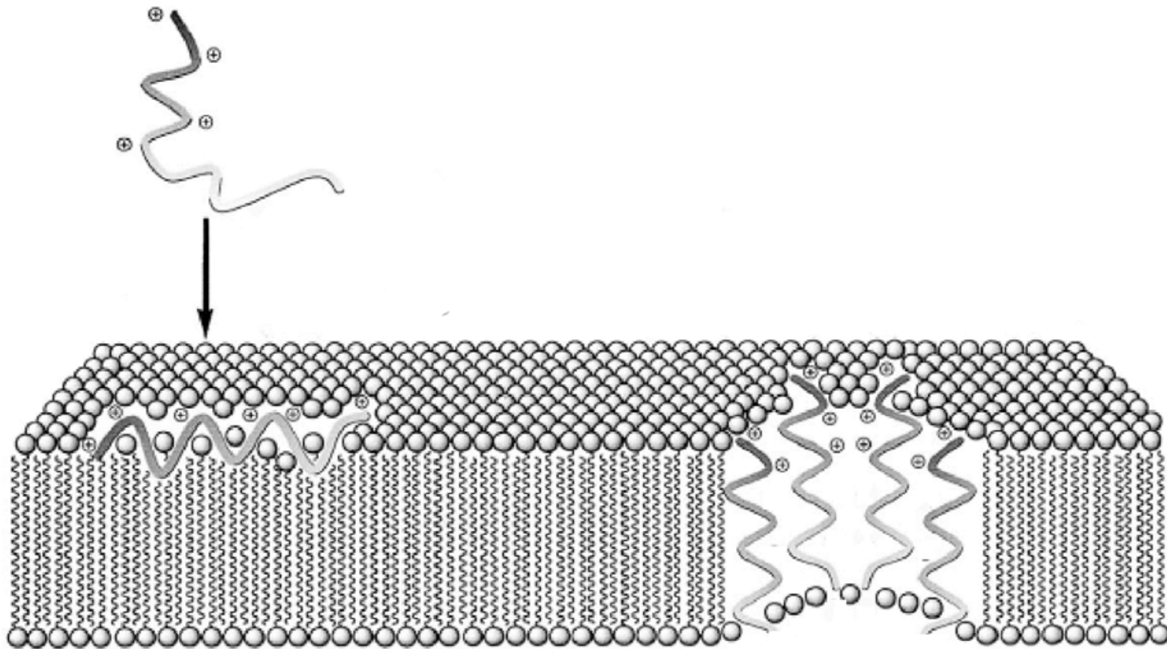

Figure 2.3: Mechanism of action of bacteriocins by pore formation in the bacterial membrane (Ruiz-Larrea et al., 2007).

Exposure of *List. monocytogenes* to nisin Z resulted in immediate loss of cellular potassium ions, depolarization of the cytoplasmic membrane, hydrolysis and partial efflux of cellular ATP (Abee et al., 1994), showing that the primary target of nisin Z in this species is the cytoplasmic membrane. Nisin A and Z were reported (Gao et al., 1991; Garcia-Garcera et al., 1993; Abee et al.,1994) to demonstrate elevated activity levels at acidic pH values and thus permeabilize membranes at very low to completely absent membrane potentials (Abee et al., 1995). Nisin A can form transient multistate pores that have diameters ranging from 0.2 – 1.2 nm in lipid membranes when trans-negative potentials are applied. These pores would allow hydrophilic solutes with a molecular size of up to 0.5 kDa to pass through. Both nisin A and nisin Z have been demonstrated to induce the leakage of ATP from target cells (Jung and Sahl, 1991; Abee et al., 1994).

Hurst (1981) demonstrated nisin to have activity against *Clostridium* and *Bacillus* spores with the mechanism of action not clearly elucidated. Jung and Sahl (1991), and Ray and Daeschel (1992) reported that sublethal heat treatment of spores caused sufficient injury to induce sensitivity to nisin (Abee et al., 1995).

Pediocin PA-1 acts on the cytoplasmic membrane where it dissipates ion gradients and inhibits transport of amino acids in sensitive cells. This bacteriocin contains two disulfide bonds, with the bond between cysteine residues at positions 24 and 44 being essential for activity. Earlier studies on the membrane permeabilizing effects of several pediocin-like bacteriocins have shown that sakacin A and P (Chikindas et al., 1993), leucocin and carnobacteriocin B2 and BM1 might induce similar effects to pediocin PA-1 (Abee et al., 1995). The conclusion of those studies was that pediocin PA-1 forms hydrophilic pores in the

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cytoplasmic membrane of target cells in a protein receptor-mediated, voltage-independent manner, similarly to the action of lactococcin A (Lcn A), a bacteriocin produced by *Lact. lactis* (Van Belkum et al., 1991). Lcn A is a small (54 amino acids) hydrophobic peptide which shows inhibitory activity against other *Lact. lactis* subspecies. In its purified form, Lcn A demonstrated effects on whole lactococcal cells and membrane vesicles, which is an indication that it increases the permeability of the cytoplasmic membrane of sensitive lactococci in a voltage-independent manner. The specificity of this bacteriocin for lactococci is thought to originate from the fact that it recognizes a *Lactococcus*-specific membrane receptor. The receptor protein is thought to play a role in pore formation since Lcn A could not permeabilize liposomes only composed of phospholipids obtained from sensitive lactococcal cell protein (Van Belkum et al., 1991). Furthermore, when Lcn A was treated with proteinase K, the membrane vesicles were no longer sensitive to the bacteriocin, which was apparently due to proteolytic digestion of the bacteriocin receptor (Venema et al., 1994).

The inhibitory activity of bacteriocins is limited to Gram-positive bacteria. The inhibition on Gram-negative bacteria has not been reported yet. This can be better explained by a detailed analysis and comparison of the cell wall composition of both Gram-positive and Gram-negative bacteria (Figure 2.4). In both bacterial types, there is a cytoplasmic membrane which forms a separating border between the cytoplasm and the external environment. This separating border is surrounded by a peptidoglycan layer which is significantly thinner in Gram-negative bacteria. Gram-negative bacteria have an additional outer membrane which is impermeable to most molecules and composed of phospholipids, proteins and lipopolysaccharides. However, this outer membrane possesses porins which allow free diffusion of molecules below 600 Da (Abee et al., 1995). The smallest bacteriocins produced by LAB are approximated to be 3 kDa and thus too large to reach the cytoplasmic membrane, which is their target (Stiles and Hastings, 1991; Klaenhammer, 1993). However, Abee et al. (1995) reported that some authors (Stevens et al., 1991; Ray, 1993) have shown that *Salmonella* species and some Gram-negative bacteria became sensitive to nisin after being exposed to treatments that change the properties of the permeability barrier of the outer membrane. Furthermore, Svetoch et al. (2011) has recently isolated *Lb. salivarius* 1077 from poultry intestinal material and characterized its bacteriocin. Bacteriocin L-1077 has a molecular weight of 3454 Da, contains 37 amino acids and contains the Class IIa bacteriocin signature N-terminal sequence, YGNGV. The purified bacteriocin demonstrated a wide spectrum of activity as it showed activity against 33 Gram-negative and Gram-positive pathogenic bacterial isolates.

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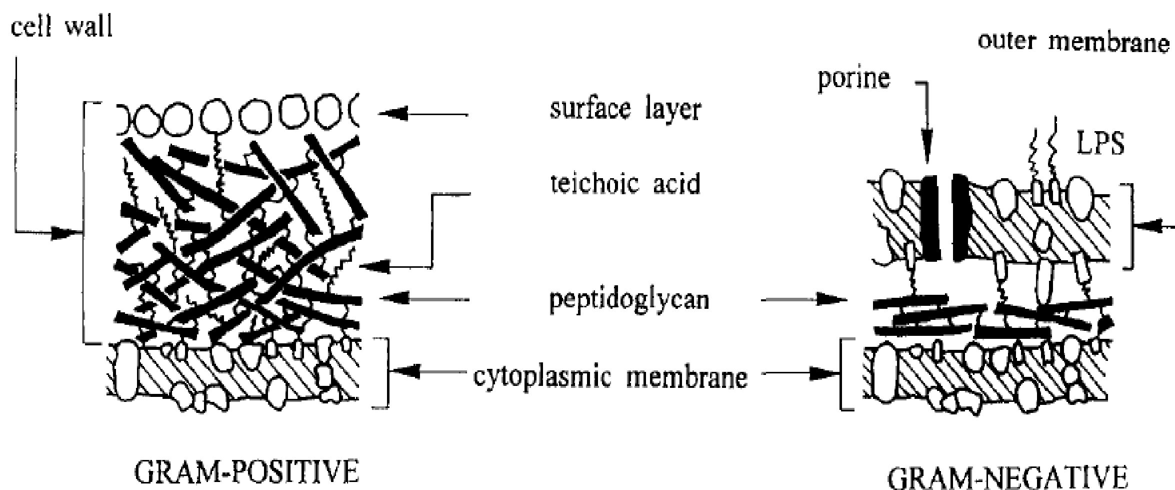

Figure 2.4: Schematic presentation of the cell envelope of Gram-positive and Gram-negative bacteria. LPS, lipopolysaccharide (Abee et al., 1995).

2.5 Resistance to Bacteriocins

The occurrence of antimicrobial peptide (AMP) resistance in sensitive bacterial strains has been deemed impossible, since these peptides manipulate the basic features of the bacterial cell. AMPs have been employed for thousands of years without losing their antimicrobial activity (Zasloff, 2002; Maróti et al., 2011). However, pathogenic bacteria have co-evolved in order to counter the efficiency of AMPs (Peschel and Sahl, 2006). Evolutionary models have created doubts around a claim that co-evolution of AMP resistance with the therapeutic use of AMPs is improbable (Bell and Gouyon, 2003; Buckling and Brockhurst, 2005). Experimental evolution has successfully showed that bacterial populations are capable of developing resistance to AMP drugs (Perron et al., 2006). This raises serious concerns for AMP antibiotics, but more importantly because antibiotics are fundamentally important for our own immune system (Maróti et al., 2011).

Target microorganisms may to some level develop resistance to the pore-forming bacteriocins. The resistance can be either transient or remain stable (Martínez and Rodríguez, 2005). A number of nisin resistance mechanisms have been described (Abee et al., 1995). Many Gram-positive bacteria are resistant to nisin because of their ability to synthesize nisinase, an enzyme that could inactivate nisin (Jarvis, 1967; Abee et al., 1995). The enzyme was isolated from several *Bacillus* spp. and was demonstrated to be a dehydropeptide reductase since it particularly reduced the C-terminal dehydroalanyllysine of nisin to alanyllysine (Hurst, 1981). Adaptation of cells to high concentrations of bacteriocins is another resistance mechanism (Abee et al., 1995). Nisin resistance mechanisms are marked by the changes in the membrane composition and fluidity and polysaccharide production (Jarvis, 1967; Breuer and Radler, 1996). Nisin-resistant *List. monocytogenes* has been found to possess reduced amounts of phosphatidylglycerol, diphosphatidylglycerol and bisphosphatidylglyceryl phosphate (Ming and Daeschel, 1993). Moreover, an altered gene expression was detected in *List. monocytogenes* mutants that were nisin resistant (Gravesen et

al., 2004). The resistance of microorganisms to class II bacteriocins has also been linked to the absence of one of the components of the mannose transport system (Hechard et al., 2001; Gravesen et al., 2002).

Bacteriocin resistance can present difficulty in further bacteriocin applications (Martínez and Rodríguez, 2005). This issue has only been addressed by a few studies in recent years (Crandall and Montville, 1998; Martínez and Rodríguez, 2005). There is a huge concern regarding the intensive use of bacteriocins in food due to a potential cross-resistance in food pathogens towards clinically used antibiotics. Microorganisms are already showing resistance to several antibiotics and the transferral of resistance between organisms has been reported (Cleveland et al., 2001). Even though bacteriocins are different to antibiotics, there is a concern that exposure to bacteriocins may result in cells that are more resistant to antibiotics (Cleveland et al., 2001).

Since nisin and antibiotics have different mechanisms of action, it has been demonstrated that nisin exposure has no effect on the frequency of resistance of *List. monocytogenes* Scott A to ampicillin and chloramphenicol (Tchikindas et al., 2000). In their study, Martínez and Rodríguez (2005) also concluded that nisin resistance in dairy *List. monocytogenes* would not hinder the use of antibiotics since no cross-resistance to the most clinically used antibiotics was observed. However, they argued that it may affect the future applications of antimicrobial peptides which employ the membrane permeability as a mechanism of action. They also argued that several nisin resistant phenotypes can be envisaged depending on the strains (Martínez and Rodríguez, 2005). Thus, they suggested that a survey be extended to a larger number of *List. monocytogenes* isolated from several sources, especially foods that are preserved with nisin and also susceptible to *List. monocytogenes* contamination. It is however worth noticing that nisin and vancomycin, which is an antibiotic, act on the same lipid II target (Martínez and Rodríguez, 2005). The Europeon Union (EU) has already considered reviewing the use of nisin (E-234) in food. This was one of the key actions within its strategy to counter the problem of bacteriocin resistance (Martínez and Rodríguez, 2005). However, the European Food Safety Authority (EFSA) later confirmed that the development of antibiotic resistance should not be expected from the use of nisin in food (EFSA, 2006; Commission Directive, 2010).

When bacteria exhibiting resistance to several drugs were subjected up to 400 IU/ml nisin, they remained sensitive to nisin (Severina et al., 1998). In another study, where cross-resistance between nisin and 33 other antimicrobials was investigated, *S. aureus* which is resistant to penicillin was 50 times more sensitive to nisin (Szybalski, 1953). There are also other cationic peptides which show activity against organisms that are known to be resistant to antibiotics such as methicillin-resistant *S. aureus* and vancomycin-resistant *S. haemolyticus* (Friedrich et al., 2000).

The bacteria that are resistant to nisin do not necessary show cross-resistance with antibiotics, however it is crucial to understand the mechanism of resistance in order to prevent it. Antibiotic resistance can be determined on the genetic level which is a driving force behind the transfer of resistance between cells, strains and species (Cleveland et al., 2001). On the other hand, bacteriocin resistance can also be caused by a physiological change in the target cell membrane (Ming and Daeschel, 1993; Mazzotta et al., 1997; Crandall and Montville, 1998). *List. monocytogenes* has a rigid membrane with a lower C15: C17 ratio

which enables the increased nisin tolerance (Mazotta et al., 1997). Another study shows that *List. monocytogenes* mutants resistant to pediocin PA-1 exhibit high expression of gene fragments encoding for β-glucoside-specific phosphoenolpyruvate-dependent phosphotransferase systems (PTS). However, the mechanism underlying the interaction of β-glucoside-specific PTS with pediocin to confer resistance still needs to be explained (Gravesen et al., 2000).

The antibiotic resistance of 166 environmental strains isolated from surface and waste-waters and belonging to seven *Enterococcus* species was studied against 11 antibiotics. Only two strains (*Ent. faecium* DR25 and *Ent. casseliflavus* S34) were multiresistant to five or more antibiotics and two strains (*Ent. hirae* and *Ent. casseliflavus* S34) were resistant to vancomycin (Pangallo et al., 2008). In a similar study, seventy two strains from different origins (food and feed, animal isolates, clinical and nonclinical human isolates), belonging to bacteriocin-producing *Ent. faecium* and *Ent. faecalis* were screened for the presence of hemolysin/cytolysin activity and vancomycin resistance (*vanA*) genes. The incidence of the *vanA* gene among the *Ent. faecium* strains was low (11.1%) and only occurred in one food isolate (De Vuyst et al., 2003).

In the investigations on bacteriocins from different classes, there is usually contradicting data on cross-resistance irrespective of whether the resistance is genetically encoded or due to adaptation (Mazzotta et al., 1997; Song and Richard, 1997; Crandall and Montville, 1998; Rasch and Knochel, 1998).

2.6 Bacteriocin Screening Methods

Bacteriocin antimicrobial activity can be detected using many microbial techniques. Among them includes: critical dilution assays (Parente et al., 1995), a flip-streak method (Spelhaugh and Herlander, 1989; Lewus and Montville, 1991), a well diffusion assay (Kivanç, 1990; Stecchini et al., 1992; Sarkar and Banerjee, 1996), a disc diffusion assay (Fleming et al., 1975; Pulusani et al.,1979), the spot-on-lawn method (Schillinger and Lucke 1989; Çon et al., 2001) and many more (Çadirci and Çitak, 2005). Some of these techniques rely on dilution of the antimicrobial agent in broth while others rely on diffusion of solid or semi-solid culture media to inhibit the growth of indicator organisms (Çadirci and Çitak, 2005). The most commonly used of these techniques are the well diffusion and spot-on-lawn methods.

A well diffusion assay involves overlaying an agar plate with a soft agar that is inoculated with a sensitive organism and allowed to cool. The wells are formed by cutting the cooled soft agar seeded with a sensitive organism followed by the addition of supernatants of putative bacteriocin producers into the wells. Plates are then incubated at optimal growth conditions. Clear zones of inhibition will indicate that the strains in question are producers of bacteriocins (Cintas et al., 1995; Cintas et al., 1997).

The spot-on-lawn method involves the spotting of potential bacteriocin producing strains onto agar plates and incubation overnight in semi-anaerobic conditions. The plates are then overlaid with a soft agar that is seeded with an indicator organism, followed by 24 h incubation. A clear zone will form around a spot of a producer strain, indicating inhibitory activity (Schillinger and Lucke, 1989).

Çadirci and Çitak (2005) compared the disc diffusion assay and the spot-on-lawn method in order to establish the most effective method for determining the antimicrobial activity. Their results showed that the inhibitory activity observed with the spot-on-lawn method gave clearer inhibition zones. The authors described the spot-on-lawn method as a practical and suitable technique in antimicrobial activity research.

Recently, some authors have developed many web-based devices that can be used to identify putative structural bacteriocin genes and/or AMPs. These include: BAGEL (de Jong et al., 2006), BAGEL2 (de Jong et al., 2010), and CAMP (Collection of Antimicrobial Peptides) (Thomas et al., 2009). These tools consist of databases of previously known information on identified bacteriocin sequences and/or AMPs from published literature (patented and non-patented). Such tools allow the detection of open reading frames (ORFs), including the very small, poorly conserved ORFs of bacteriocins that are difficult to detect (de Jong et al., 2006).

2.7 Purification of Bacteriocins

Different protocols and chromatographic methods have been suggested for the purification of LAB-produced bacteriocins. Various combinations of chromatography usually follow after the concentration step by either salt precipitation (Muriana and Klaenhammer, 1991; Callewaert et al., 1999) or acid extraction (Yang et al., 1992). Different chromatographic methods may include ion exchange chromatography (IEC), hydrophobic interaction, gel filtration, reverse-phase C18 solid phase extraction, adsorption-desorption (AD), reverse-phase high-perfomance liquid chromatography (RP-HPLC), and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) (Hastings et al., 1991; Nissen-Meyer., 1992; Pengitore et al., 2007). Several authors have reported simple methods for bacteriocin purification (Callewaert et al., 1999). These methods involve adsorption based on hydrophobic or electrostatic interactions (Stoffels et al., 1993), acid extraction and RP-HPLC (Daba et al., 1994), ammonium sulphate precipitation and reversed-phase chromatography (Joosten et al., 1996), pH-mediated cell adsorption-desorption and semi-preparative RP-HPLC (Elegado et al., 1997), and ethanol precipitation, preparative isoelectric focusing and ultrafiltration (Venema et al., 1997).

Homogenous purification of bacteriocins is a requirement for their characterization. To elucidate the biochemical structure of bacteriocins requires a pure, homogenous peptide, and adequate yield of protein (Pengitore et al., 2007). Due to the diverse nature of bacteriocins there is not one general purification method or protocol that has been identified. There is instead a wide range of purification methods for LAB bacteriocins and those of related microorganisms (Pingitore et al., 2007).

Characterizing a new antimicrobial peptide relies heavily on the purification strategy as they are produced in low amounts and therefore requires a few steps that concentrate the substance. To serve this purpose, protocols such as ammonium sulphate concentration, adsorption-desorption and organic solvent extraction have been implemented for partial purification of bacteriocin extracts (Pingitore et al., 2007). The selection of an appropriate purification technique(s) depends on the knowledge available on the peptide characteristics (Pingitore et al., 2007). Métivier et al. (2000) describes a phase-partitioning technique that

consists of two successive steps, phase-partitioning in Triton-X-114, a non-ionic detergent and cation-exchange chromatography (CEC). This technique excludes the common salting-out step that involves ammonium sulphate precipitation. It could be used as a large-scale procedure to fulfil the quantities required to validate the bacteriocin efficiency in food products.

Over the past two decades, there have been many publications describing the characterisation of newly found LAB bacteriocins or other Gram-positive bacteriocins. Therefore to avoid characterising and purifying bacteriocins that have been previously described, a good approach would be to first screen the producer strain by PCR using "bacteriocin-specific" primers that are available in the literature or design them using the sequences available in Genebank (http://www.ncbi.nlm.nih.gov/Genbank/) (Pengitore et al., 2007).

2.8 Bacteriocin Characterisation

Bacteriocin-characterisation assays provide the necessary information about the newly identified bacteriocin. This information also helps to establish optimal conditions for applications of bacteriocin (s). Characterisation can be divided into biochemical and physiological characteristics. Regarding biochemical characteristics many authors focus on factors like heat resistance, pH sensitivity, enzyme treatment, organic solvents, synergistic effects, molecular weight, storage conditions, and many more (Callewaert et al., 1999; Chamainee et al., 2009; Bauer et al., 2005; Franz et al., 1996; Ferreira et al., 2007; Knoll et al., 2008; Navarro et al., 2000). Physiological characteristics normally involve the bacteriocin mode of action, growth and bacteriocin production kinetics (Franz et al., 1996; Knoll et al., 2008).

2.9. General Applications of Bacteriocins in Food Products

Bacteriocins used in the food industry should meet some of the following requirements: the producing strain should preferably have QPS (qualified presumption of safety) status, should be heat stable, active against pathogenic or spoilage bacteria and pose no health risks. If added in food products, it should demonstrate beneficial impacts such as improved safety, quality or flavour, and should not be added in higher concentrations than those found in its natural source. Lastly, it should have high specific activity and should only inhibit pathogenic and spoilage organisms. Nisin and pediocin, the current biopreservatives used in the food industry, have demonstrated all of the above-mentioned characteristics. These bacteriocins are used individually in food systems and for experimental purposes where they are used in combination (Ross et al., 2002) with other preservation techniques like: heat treatment, high pressure or modified atmosphere packaging (Allende et al. 2006).

Two main strategies exist for bacteriocin application in food systems. One is the inoculation of food with LAB that produces bacteriocins, while the other strategy is the direct addition of a purified or semi-purified bacteriocin (Lloyd and Drake, 1975; Ross et al., 2002; Bauer et al., 2003).

Pediocin PA-1 has gained wide applications in the food industry as a biopreservative. It was formerly known as Pediocin AcH and is produced by some strains of the following species: *Ped. acidilactici* of meat origin, *Ped. parvulus* of vegetable origin, and one *Lb. plantarum* isolated from cheese (Cintas et al.,

2001). Pediocin is currently used commercially and is covered by several U.S. and European patents (Cheng and Hoover, 2003). The LAB fermentate that contains pediocin is named Alta ™ (Kerry Bio-Science) and it is used as a preservative for ready-to-eat meat products, salads and sauces (Ruiz-Larrea et al. 2007). Nisin prevents clostridial spoilage of processed and natural cheeses, inhibits the growth of some psychrotropic bacteria in cottage cheese, extends the shelf life of milk in warm countries, prevents the growth of spoilage lactobacilli in beer and wine fermentations and provides additional protection against *Bacillus* and clostridial spores in canned foods. It is a permitted food additive in more than 50 countries including the US and Europe under the trade name Nisaplin but not yet permitted in wine (Vandenberg, 1993; Delves-Broughton et al., 1996).

Butyric acid bacteria such as *Clostridium tyrobutyricum* pose a serious problem in cheese production (Hurst, 1981). Nitrate addition to cheese to prevent outgrowth of clostridia spores is a common dairy practice. However, Nisin A can effectively replace this chemical. The outgrowth of *C. tyrobutyricum* spores in Gouda cheese without nitrate treatment were completely inhibited when a nisin A producing strain was added to the starter culture (Hugenholtz and de Veer, 1991). Nisin A also prevented the growth of *List. monocytogenes* in camembert (Maisnier-Patin et al., 1992) and in cottage cheese, both at 4°C and 37°C (Benkerroum and Sandine, 1988). However, the application of nisin in dairy foods, which require LAB as starter cultures, is a challenge due to the wide spectrum of activity demonstrated by nisin. Thus an alternative approach that could help to control specific pathogens or spoilage organisms in dairy foods is that of applying bacteriocins that have a highly specific activity range. Pediocin PA-1 presents those features as it demonstrates high activity against *Listeria* and yet shows no activity against many other LAB strains (Ruiz-Larrea et al., 2007). Another pediocin-like, heat stable bacteriocin is enterocin 1146 which is produced by *Ent. faecium* DPC1146 and shows extremely high activity against *List. monocytogenes* at levels which does not affect lactococcal starters (Parente and Hill, 1992a).

Vegetable products are usually contaminated with high numbers of microorganisms and may also contain pathogenic microorganisms such as *List. monocytogenes*, *B. cereus* and *S. aureus* (Uhlman et al., 1992; Carlin and Nguyen-The, 1994). There is an increasing diversity of minimally processed vegetable products that are available on the European market, and ready-to-eat types of products are gaining popularity with consumers. Since these products may present a potential health risk, LAB cultures or purified bacteriocins may be used to improve their safety and hygiene levels (Franz et al., 1996).

LAB produced bacteriocins also find application in accelerating the ripening period of fermented foods. This is achieved by lysing the LAB starter which results in the release of intracellular enzymes and substrates for reactions leading to flavour development (Oumer et al., 1999; Morgan et al., 2002).

Natural production of bacteriocins offers several advantages compared to synthetic means regarding both the legal aspects and cost. Reducing the costs of biopreservation processes may be highly attractive, especially for small economies and developing countries where food safety may be seriously compromised (Holzapfel, 2002; Gálvez et al., 2007). In that regard, several studies have been done to evaluate effectiveness of bacteriocin producing LAB as starter (bacteriocinogenic) cultures in food fermentations as

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opposed to non-producing commercial starters. Some of the natural bacteriocin producers used in the studies are: *Lb. plantarum*, *Ped. acidilactici* and *Ent. faecalis* (Campanini et al., 1993; Nunez et al., 1997). Nunez et al. (1997) found that counts of *List. monocytogenes* Ohio in Manchego cheese inoculated with a producer culture of *Ent. faecalis* were reduced by 6 logs in 7 days, whereas the surviving cells in cheese inoculated with commercial starter culture were not affected. Similar results were obtained by Campanini et al. (1993) where *List. monocytogenes* cells found in naturally contaminated salami sausage decreased when the product was inoculated with *Lb. plantarum* MCS1, a bacteriocin producer.

The use of bacteriocinogenic cultures requires careful selection of strains that are well adapted to the particular food environment in which they will be used, able to grow under the food processing and/or storage conditions and to produce enough bacteriocin amounts as to inhibit the target pathogenic or spoilage bacteria. Therefore, it is necessary to implement the right experimental approaches to select bacteriocin-producing strains that are suitable for use in food production (Galvez et al., 2007).

Bacteriocinogenic strains can be used as direct starter cultures, as co-cultures in combination with a starter culture, or as protective cultures (especially in the case of non-fermented foods). When used as a starter culture, a bacteriocinogenic strain must be able to perform the fermentation process optimally apart from being able to produce enough bacteriocin for protection. In some cases, bacteriocin production capacity may serve as a complement to a starter by increasing its implantation capacity, competitiveness and stability. As co-cultures, bacteriocinogenic cultures do not have to contribute to the fermentation, but must not interfere with the fermentation role of the starter culture. Therefore bacteriocin resistance of the starter culture becomes crucial. The latter can be achieved by selection of natural resistant mutants, which can be generated by repeated subcultivation with increasing bacteriocin amounts, or by genetic modification. However, this may not always be necessary as the bacteriocin may not have any effects on the starter (a feature exhibited by many bacteriocins that have antilisterial activity), or the starter may be more tolerant to the bacteriocin than the target bacteria in the food system. Other factors like differences in inoculum density, a faster starter growth rate or delayed bacteriocin production may allow the starter to grow without any interference from a bacteriocinogenic co-culture (Gálvez et al., 2007).

Bacteriocinogenic protective cultures can have application in extending the shelf life of non-fermented foods by inhibiting the spoilage and pathogenic bacteria. The protective cultures may grow and produce bacteriocin during refrigeration storage, and/or during temperature abuse conditions. The former condition requires the protective culture growth to have neutral impact on the physicochemical and organoleptic properties of the food, while on the latter condition the protective culture may even act as a predominant spoiler in order to inhibit the growth of pathogenic bacteria and thus ensuring that a spoiled food is not consumed (Gálvez et al., 2007).

Numerous studies in recent years all point to the fact that application of bacteriocins in food preservation can offer several benefits (Thomas et al., 2000; Gálvez et al., 2007): (i), an extended shelf life of foods, (ii) provide extra protection during temperature abuse conditions, (iii) decrease the risk for transmission of foodborne pathogens through the food chain, (iv) reduce the economic losses due to food

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spoilage, (v) reduce the application of chemical preservatives, (vi) permit the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (vii), permit the marketing of "novel" foods (less acidic, with a lower salt content and with a higher water content), and (viii) they may serve to satisfy industrial and consumers demands. As a result, some of the trends in the European food industry, such as the need to eliminate the use of artificial ingredients and additives, the demand for minimally-processed and fresher foods, as well as for ready-to-eat food or the request for functional foods and nutraceuticals could be satisfied, at least in part, by application of bacteriocins (Gálvez et al., 2007).

There is a constant need to identify and characterize more bacteriocins that could be of industrial importance. From what is already known, bacteriocin efficacy in food systems seems to be greatly influenced by both chemical composition and physical conditions of food (Figure 2.5) (Cleveland et al., 2001; Gálvez et al., 2007). Some of the factors include pH (Liu and Hansen, 1990; Abee et al., 1995; Cleveland et al., 2001), cell concentration, lipid content, proteolytic enzymes, limited diffusion in solid matrixes, temperature, narrow spectrum of activity (not active against Gram-negative pathogens and spoilage bacteria), lower levels of production and bacteriocin-resistant bacteria (Abee et al., 1995; Devlieghere et al., 2004).

The complexity of the food matrix and the difficulty of quantifying bacteriocin activities in foods have prompted *in vitro* studies which are meant to simulate and study the *in situ* functionality of bacteriocinogenic starters. Mathematical modeling and positive predictive microbiology are amongst many tools employed in this endeavour (Leroy *et al*., 2002). Understanding the relationship between the food environment and kinetics of the starter culture has provided valuable information about the *in situ* production of bacteriocins and its interactions with the target strains. This will be important if bacteriocins or bacteriocin producing strains are to be increasingly used in food systems (Leroy *et al*., 2005). In particular, such information is essential when dealing with the potential problem of bacteriocin-resistant target bacteria (Khalid, 2011).

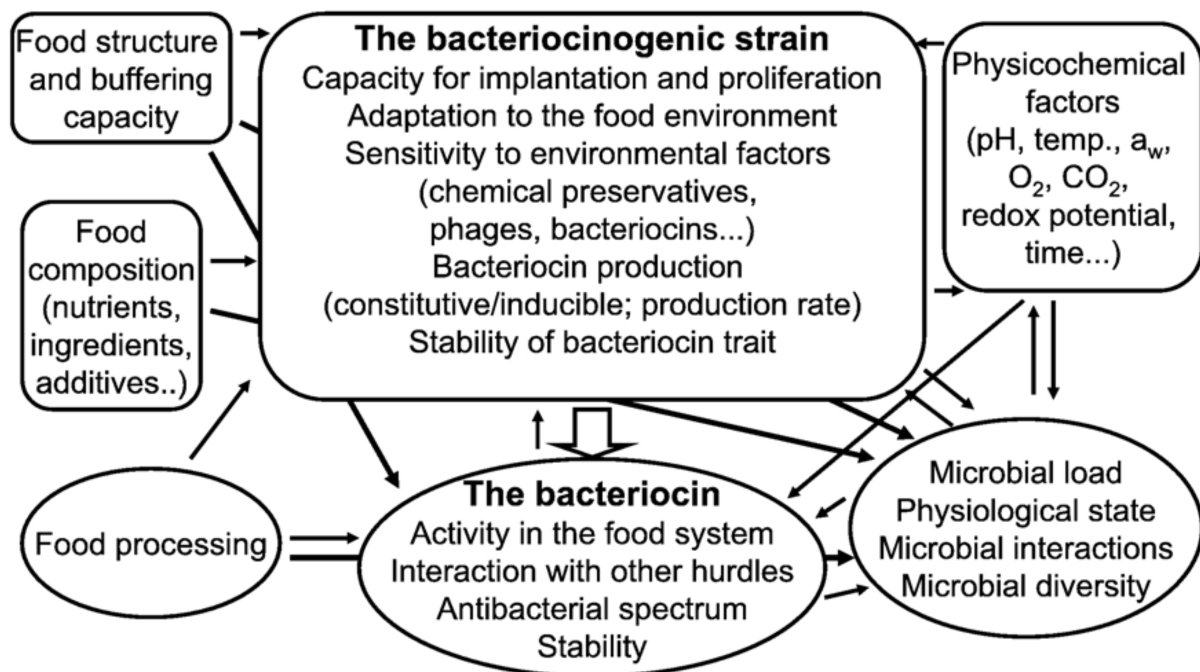

Figure 2.5: Influence of different factors on the efficacy of *in situ* bacteriocin production for biopreservation (Gálvez et al., 2007).

2.10. Preservation Using Non-bacteriogenic Cultures

Some bacterial cultures demonstrate a very competitive role in controlling spoilage and pathogenic bacteria. Examples include *Lb. alimentarius* BJ-33 (Andersen, 1995), *Lb. sakei* TH1 isolated by Bredholt et al. (2001), the *Lact. lactis* strain found by Elsser (1998) and others (Devlieghere et al., 2004). The activity of these cultures can be hypothetically explained on the basis of lactic acid production which results in acidification that causes growth arrest of the spoilage and/ pathogenic bacteria (Juven et al., 1998). However, the full explanation of their inhibitory activity could be more complex and may probably be due to the combination of effects such as production of antimicrobials and competition for nutrients or depletion of specific nutrients (Devlieghere et al., 2004).

Different types of LAB that are well characterised, psychrotrophic and mildly acidifying are well suited for biopreservation of refrigerated food products. Comparatively high levels of these cultures may be needed for the protection against some pathogens (Abee et al., 1995).

Most protective cultures are heat sensitive LAB that do not survive commercial cooking processes and thus need to be added by dipping or spraying after heat treatment. Therefore, heat resistance could be an important characteristic of protective cultures to reduce the additional cost of supplementing a product with living cells (Lemay et al., 2002).

Competitive and bacteriogenic LAB may provide an additional hurdle in food preservation (Hugas, 1998). However, preservation using protective cultures should be considered as a supplement to good manufacturing, processing, storage and distribution conditions (Holzapfel et al., 1995).

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2.11. Preservation Techniques in Winemaking

LAB occur naturally in the wine ecosystem and play an essential role in winemaking by reducing its acidity and contributing to the aroma and flavour of the wine. In contrary, they can also be the source of many unwanted wine spoilage problems which reduce the wine quality and value. For instance, unmanaged microbial growth before, during or after wine fermentation can change chemical composition and hence compromise the quality of the end-product. The three stages at which micro-organisms can enter the winemaking process and influence the quality of wine include raw material (grapes), during must fermentation, and post-fermentation. At stage three, wine spoilage can occur in the bottle or during storage in oak barrels (Du Toit et al., 2002). LAB such as *O. oeni* contribute positively to the sensory characters of wine, while species like *Lactobacillus* spp. and *Pediococcus* spp. can produce undesirable volatile compounds. Some of the negative effects that result from bacterial wine spoilage include mousy taint, volatile acidity, oily and slimy-texture, overt buttery characters, ropiness, acrolein formation, bitterness, tartaric acid degradation and geranium off-flavour (Du Toit and Pretorius, 2000). Some LAB can also influence the wholesomeness of wine by producing biogenic amines and ethyl carbamate precursors (Smit et al., 2008; Lerm et al., 2010; Du Toit et al., 2011).

Dealing with wine spoilage bacteria could be as easy as controlling wine acidity and adding sulphur dioxide (SO_2) (Bartowsky, 2009). SO_2 is a chemical preservative that is used to prevent the growth of spoilage micro-organisms in winemaking. Apart from being an antimicrobial agent, SO_2 is an efficient antioxidant agent that prevents browning and spoilage of some foods (Facio and Warner, 1990). SO_2 is added to the grapes, grape must and/or to the end-product (Oliveira et al., 2002). However, because of increasing health implications associated with SO_2, consumer preferences of natural foods as well as the stricter legislation on preservatives, there is a worldwide trend to reduce SO_2 levels (Du Toit and Pretorius, 2000; Du Toit et al., 2002; Bauer et al., 2003). Furthermore, it is a well known fact that only molecular SO_2 demonstrates antimicrobial activity. Only 5 – 10% of SO_2 are available in this form in wines with pH 3 and as little as zero in wines with pH 4. It is reported that several bacterial species (Bartowsky, 2009) and some wine spoilage yeasts may tolerate the maximum levels of SO_2 permitted for usage (Du Toit and Pretorius, 2000). Therefore, additional antimicrobial agents that could replace or supplement SO_2 are needed.

Some alternative technologies to eliminate bacteria in wine have been explored. For example, UV irradiation, pressure and electric fields have been successfully used in many beverage industries to sterilize products and recent trials in grape must or wine have been encouraging (Bartowsky, 2009; Buzrul, 2011; Fredericks et al., 2011). Physical filtration of juice or wine could be effective in removing micro-organisms, but since filtration is only applied prior bottling, it cannot remove micro-organisms during winemaking (Bartowsky, 2009).

The overall efforts to reduce the use of SO_2, and recently, the need to reduce filtration for concerns from some winemakers that it may have unfavourable impact on the wine flavour, has led to a desperate search for alternative approaches that could prevent bacterial wine spoilage (Bartowsky, 2009).

Several chemical inhibitors and natural products are possibilities that present a great potential to reduce or eliminate bacterial populations in winemaking (Table 1). However, since some of these substances are additives, a legislative approval for their use in winemaking is required (Bartowsky, 2009; Santos et al., 2012).

Table 2.1. Approaches to limit or halt bacterial growth in wine (Bartowsky, 2009).

Controlling agent	Mechanism of action
Traditional	
Sulfur dioxide	Inhibits the development of bacteria
Filtration	Physical removal of bacteria from wine
Chemical	
Dimethyl dicarbonate (DMDC)	Reacts irreversibly with the amino groups on active sites of enzymes
Natural products	
Lysozyme	Disrupts cell wall synthesis causing cell lysis
Bacteriocins	Alters cell wall components causing cell lysis
Up and coming physical technologies	
Ultrahigh pressure	Causes damage to cytoplasmic membrane and inactivates enzymes
High power ultrasound	Sound waves cause thinning of cell membranes, localized heating and production of free radicals
UV irradiation	Damages DNA
Pulsed electric fields	Dielectrical breakdown of cell membranes

Dimethyl dicarbonate (DMDC) is an important chemical preservative in winemaking. It is a microbial control agent with legal limits of 200 mg/l in wine and can be used to reduce SO_2 levels to prevent fermentation in semi-sweet wines and stop alcoholic fermentation in *Botrytis*-affected wines (Threlfall and Morris, 2002; Divol et al., 2005; Enrique et al., 2007). DMDC inhibits micro-organisms by inactivating cellular enzymes. It hydrolyses to methanol and carbon dioxide (CO_2), naturally occuring in the grape juice and wine, and therefore poses no threat to flavour and colour of the wine. DMDC has approval in most winemaking countries, but its effectiveness varies between species and strains. For example, a study by Delfini et al. (2002) showed that bacteria were more resistant to DMDC than yeasts (50 – 1000 and 150 – 400 mg/l, respectively). Another study by Costa et al. (2008) informed that the permitted amounts of DMDC (200 mg/l) do not effectively inhibit LAB or acetic acid bacteria (AAB), suggesting that DMDC may not be a choice preservative against wine spoilage bacteria. Other limitations of DMDC as a wine preservative is that it has low solubility in water and presents a toxicity risk after ingestion or inhalation during treatment of

wine (Bartowsky, 2009). Furthermore, since DMDC hydrolyses to methanol and CO_2, there may be no activity left to protect the bottled product (Terell et al., 1993).

Fumaric acid has been demonstrated to inhibit malolactic fermentation, but it is metabolized by yeast and LAB, making it unstable (Ough et al., 1974).

Phenolic compounds, which are oenological products, have been shown to have inhibitory activity against pathogenic bacteria (Papadopoulou et al., 2005; Vaquero et al., 2007). Compounds such as hydroxycinnamic and hydroxybenzoic acids can also prevent the growth of bacteria in wine (Vivas et al., 1997; Reguant et al., 2000).

Lysozyme and bacteriocins are natural products which have been used successfully to prevent the growth of bacteria. These products have been applied in various pharmaceutical and food industries for more than 50 years (Bartowsky, 2009). Lysozyme is a small single peptide with muramidase activity and has been approved by the Office International de la Vigne et du Vin (OIV) for use in winemaking at a maximum concentration of 500 mg/l (Bartowsky, 2009). However, lysozyme is not effective against eukaryotic cells and thus it cannot be used to control the growth of spoilage yeasts, like *Dekkera*/*Brettanomyces* (McKenzie and White, 1991). Lysozyme also has a limitation against AAB due to the structural differences that exist between Gram-negative and Gram-positive bacteria (Bartowsky, 2009). It is however a bacteriolytic enzyme that is active against most Gram-positive bacteria (Gould, 1996) and has effective synergistic activity with nisin under certain conditions (Monticello, 1989). Although different LAB vary in their sensitivity to lysozyme in wine (Bartowsky, 2009), it can effectively inhibit *Lactobacillus* species during alcoholic fermentation and therefore reduce the risk of increased volatile acidity, delaying or blocking the onset of malolactic fermentation, controlling LAB populations during sluggish or stuck alcoholic fermentation, and to inhibit the onset of malolactic fermentation post bottling (Gerbaux et al. 1999). Lysozyme has no effect on yeast, is not affected by alcohol, and is active in the pH range of wine (Fugelsang, 1997). Moreover, the addition of lysozyme does not have any negative impacts on the wine aroma (Bartowsky et al., 2004). However, as with all wine treatments, addition of lysozyme requires careful consideration as it is able to bind with tannins and polyphenols in red wines, resulting in slight decrease in wine colour or formation of wine haze (Gerbaux et al. 1999; Bartowsky, 2003, 2009; Bartowsky et al., 2004). The economic implications of using lysozyme are still a limiting factor (Bauer et al., 2003).

Antimicrobial action of synthetic peptides towards wine yeasts was studied by Enrique et al. (2007). The study employed nine sequence-related anti-fungal hexapeptides (PAFs), two representative lactoferricin B (LfcinB)-derived peptides and five wine yeast genera, *Cryptococcus albidus*, *Dekkera bruxellensis*, *Pichia membranifaciens*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii* and *Zygosaccharomyces bisporus*. The PAF26, PAF36 and $LfcinB_{17–31}$ peptides demonstrated high inhibitory activities against several yeasts at low micromolar concentrations, with *Z. bailii* and *Z. bisporus* being the most sensitive of the yeasts. However, only $LfcinB_{17–31}$ against *Z. bisporus* showed inhibitory and fungicidal properties in wine at the concentrations assayed. These results showed that the antimicrobial action of each peptide depends both on the food matrix and target micro-organism, as all three peptides demonstrated fungicidal activities against *Z.*

bailii, *Z. bisporus* and *S. cerevisiae* in laboratory growth medium, but not in wine. The inability of other peptides to show fungicidal properties against *Z. bailii* in wine was due to the presence of salt ions other than divalent cations. On the other hand, the presence of salts did not affect the fungicidal action of $LfcinB_{17–31}$ against *Z. bailii* (Enrique et al., 2007). The authors also highlighted the importance of evaluating the antimicrobial activities in the food matrix (for practical application) as the efficacy of antimicrobial peptides was reduced in wine as compared to laboratory growth medium. Cleveland et al. (2001) also stated that results from broth systems can show inhibition of target organisms by bacteriocins, but applied studies need to be done to confirm if the results will be the same in food systems.

Although there are numerous reports on bacteriocins produced by LAB and some thoroughly studied for application in food preservation, there are few reports on bacteriocins produced by LAB of oenological origin or on bacteriocins present in a finished wine (Joyeux 1993; Strasser de Saad and Manca de Nadra 1993; Navarro et al., 2000; Rojo-Bezares et al., 2007a; Knoll et al., 2008; Saenz et al., 2009).

Nisin is a bacteriocin isolated from *Lact. lactis* of non-oenological origin, but this bacteriocin is important in winemaking since it has been effectively used to control the growth of spoilage LAB in wine and beer (Ogden, 1986; Radler, 1990a,b ; Delves-Broughton et al., 1996). Nisin and pediocin PA-1 have been reported to inhibit LAB found in wine including, *Lb. plantarum, Lb. paracasei*, *Lb. brevis*, *Lb. hilgardii, Lb. pentosus, Leuc. mesenteroides, Ped. pentosaceus* and *O. oeni. O. oeni* strains are more sensitive to nisin than *Lactobacillus* and *Pediococcus* species (Mendes Faia and Radler 1990; Rojo-Bezares et al., 2007b), and pediocin and plantarincin have been shown to successfully kill *O. oeni* (Nel et al., 2002). Nisin and pediocin are stable in the wine environment and have no negative influence on alcoholic fermentation or the wine sensory profile (Du Toit et al., 2002; Rojo-Bezares et al., 2007; Knoll et al., 2008). Rojo-Bezares et al. (2007b) observed a synergetic effect between nisin and metabisulphite on bacterial growth. In this the effect was observed in 64 LAB strains. The authors concluded that appropriate combinations of nisin and metabisulphite could control the growth of bacteria in wine and thus help to reduce SO_2 levels in wine.

Bacteriocins offer a promising alternative to control bacterial growth during winemaking and preservation. Even though bacteriocins could help reduce SO_2 levels, its application will still have some relevance in winemaking due to its antioxidant properties (Rojo-Bezares et al., 2007b). Although the use of bacteriocins to control LAB in wine has great potential, its use has not yet been approved (Bartowsky, 2009). Cost may be a major factor that will prevent the acceptance of peptides as a preservation strategy (Bauer et al., 2003). Considering the many advantages offered by a biological system over classical chemical control methods, it can be said that when these systems have reached their full maturity, they will be regarded as healthier and ecologically friendly alternatives (Bauer et al., 2003). Literature shows strong evidence for the potential role that bacteriocins may play in wine fermentation (Navarro et al., 2000; Du Toit et al., 2002; Rojo-Bezares et al., 2007a,b; Ruiz-Larrea et al., 2007; Saenz et al., 2009).

Physical technologies such as ultra high pressure, high power ultrasound, UV irradiation and pulsed electric fields (PEF) are also promising alternatives, but their application in wine have not been fully

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explored. Fredericks et al. (2011) investigated the potential of UV-C radiation to control wine-spoilage microorganisms in grape juice and wine. Puértolas et al. (2009) has established an optimum PEF treatment (186 kJ/kg at 29 kV/cm) to reduce 99.9% of spoilage wine microorganisms in must and wine.

2.12. Optimising the Production and Effectiveness of Bacteriocins

2.12.1 Bacteriocins and Recombinant DNA Technology

The challenge of low production levels of bacteriocins and their instability in certain foods or environments need to be addressed (Abee et al., 1995). Recombinant DNA technology has been employed to improve production levels (Abee et al., 1995; Rodríguez et al., 2003; Zhou et al., 2006; Gálvez et al., 2007), transfer bacteriocin genes to other species (Abee et al., 1995; Du Toit et al., 2002; Van Reenen et al., 2003), and to create mutations and selection of bacteriocin variants with enhanced and/or broader spectra of activity (Abee et al., 1995). The precise point of bacteriocin production could be achieved with the aid of inducible production systems (Zhou et al., 2006).

Transposon-encoding nisin production and immunity was transformed into a commercial *Lact. lactis* starter culture for Gouda cheese (Abee et al., 1995). Since *Pediococcus* spp. do not have any application as cheese starter cultures, the pediocin-encoding plasmid was expressed in *Lact. lactis* to enhance the preservation of cheddar cheese and to improve the microbial quality of the fermentation process (Buyong et al., 1998). In this study, control cheese made from milk spiked with 10^6 cfu/ml *List. monocytogenes* had 10^7 cfu/g after 2 weeks of ripening, while cheese made with the pediocin-producing strain had only 10^2 cfu/g after 1 week. Thus, it was concluded that the pediocin producing strain was much better in controlling bacterial growth as opposed to the control strain. Pediocin PA-1 has also been expressed in *Streptococcus thermophilus*, an important bacterium in dairy fermentations (Coderre and Somkuti, 1999). Horn et al. (1999) co-expressed pediocin PA-1 and nisin in *Lact. lactis*. The production level of the transformed cells was only 11.8% compared to the control pediocin producer, however Cleveland et al. (2001) argued that the co-production of bacteriocins may have major applications in improving food safety and minimizing the likelihood of resistant organisms. Schoeman et al. (1999) also expressed Pediocin PA-1 in the yeast *S. cerevisiae* for the potential of improving the preservation of wine, bread and other food products where yeast is used. However, the production levels need to be optimised. Cleveland et al. (2001) also suggested that the term "natural" is compromised when the bacteriocin is produced by genetically modified systems like bacteria.

2.12.2 Bacteriocins as part of Hurdle Technology

The concept of hurdle technology started application in the food industry after the observation that survival of microorganisms greatly decrease when they encountered multiple antimicrobial factors (Leistner and Gorris, 1995; Leistner, 1978, 2000). Treating a bacterial population with a single antimicrobial factor results in a heterogeneous response, which amongst many other factors depend on the intensity of the treatment. A portion of the population may receive a lethal dose of the treatment, resulting in cell death, but

the remaining part may survive for various reasons: (i) receiving a sub-lethal dose; (ii) showing an increased resistance because of its physiological state (e.g. stationary phase cells, or cells already stressed in response to other unfavourable environmental conditions), and (iii) cells naturally resistant to the antimicrobial agent. Cells that are sub-lethally injured and cells with increased resistance may recover from the damage inflicted by an antimicrobial agent and survive. Such cells stand a good chance to develop mechanisms of resistance or adaptation that would enable immunity to future exposure. In contrast, when cells are treated with a combination of antimicrobial factors, the damage intensity may be higher as some of the antimicrobial factors may have the same cellular target. In order to recover from multiple damages may also be energy costly, resulting in energy depletion and subsequent cell death. Therefore, cells that are exposed to multiple hurdles have low potential of survival and proliferation. Furthermore, antimicrobial factor combinations will enable the use of lower doses compared to those used individually (Gálvez et al., 2007).

Over 60 potential hurdles have been pronounced to improve food stability and/or quality (Leistner, 1999). The use of bacteriocins as part of hurdle technology has attracted much attention in recent years (Chen and Hoover, 2003; Ross et al., 2003; Deegan et al., 2006) as bacteriocins can be used in synergy with selected hurdles to increase microbial inactivation (Figure 2.6) (Gálvez et al., 2007). The combination of hurdles to be used depends much on the food type and its microbial composition. This is very important as different hurdles have varying effects on different members of a microbial community. For instance, acidification of food may favour aciduric bacteria, while heat treatment favours endospore forming bacteria. The elimination of some members of the population may create a more conducive environment for others due to the lack of competition. The application of different antimicrobial factors may also modify their individual antimicrobial spectra. For example, Gram-negative bacteria may be sensitized to bacteriocins and other molecules when exposed to treatments that disrupt the bacterial outer membrane (Gálvez et al., 2007).

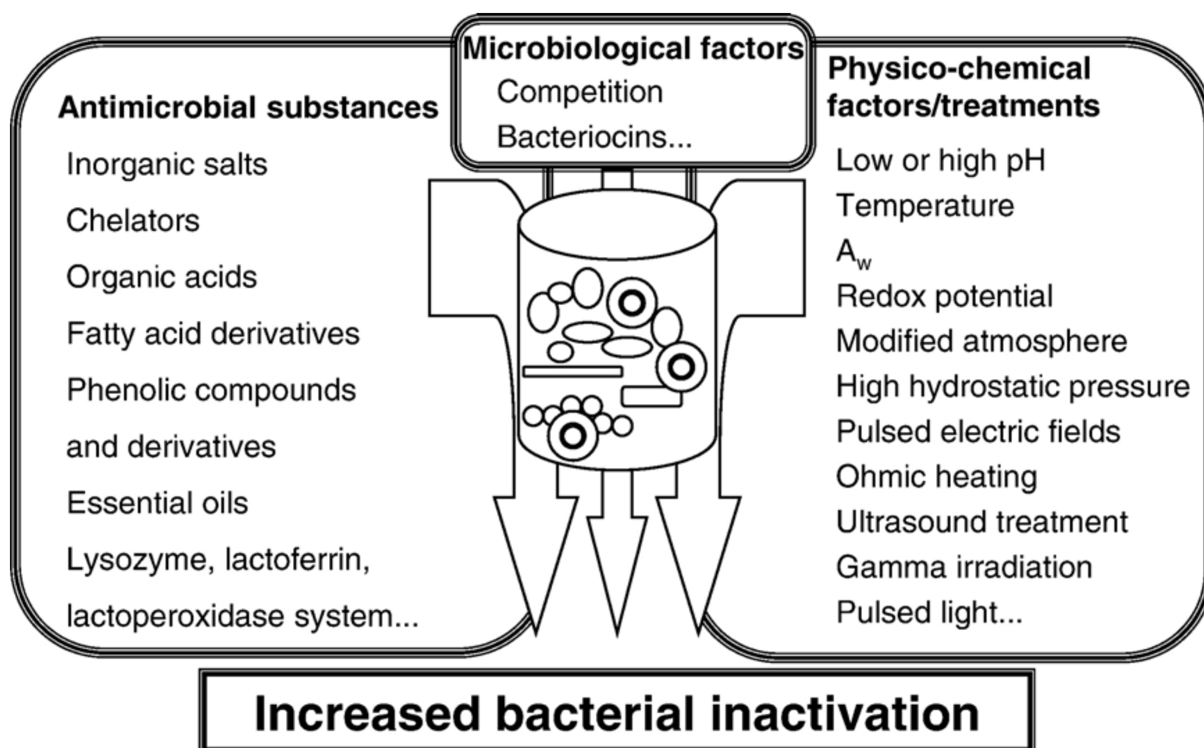

Figure 2.6: Application of bacteriocins as part of hurdle technology (Gálvez et al., 2007).

2.12.2.1 Bacteriocins and Pulsed Electric Field Technology

Pulsed electric field (PEF) technology is a non-thermal process where microbial inactivation is achieved by application of high-voltage pulses between a set of electrodes (Vega-Mercado et al., 1997; Gálvez et al., 2007). PEF has similar effects to bacterial electroporation, but the higher intensity of PEF causes severe damage to the bacterial cell membrane. In recent years, PEF has gained much attraction individually or in synergy with other hurdles such as bacteriocins. Since most bacteriocins act on the bacterial cytoplasmic membrane, it is thus expected that a combination of bacteriocins and PEF will induce an increased bactericidal effect. Bacteriocins could show bactericidal activity against bacteria that survive PEF treatment, such as sub-lethally injured cells or bacterial endospores (Figure 2.7). It can also serve to extend the antimicrobial spectrum of bacteriocins by disrupting the bacterial outer membrane and thus enabling bacteriocin molecules to reach their target (Figure 2.7). The efficacy of PEF and bacteriocin synergy is dependent on several factors related to the PEF treatment, such as field strength, number of pulses, wave form or pulse duration. Other factors include the food microbial load, composition and physiological stage, the added bacteriocin, and other environmental factors (Wouters et al., 2001; Bendicho et al., 2002; Heinz et al., 2002). These factors may have an influence on the numbers and types of bacteria withstanding the combined treatment and their proliferation during the shelf life of the processed food. Thus, applications for PEF and bacteriocin combinations must be thoroughly investigated for each food type and target bacteria (Gálvez et al., 2007).

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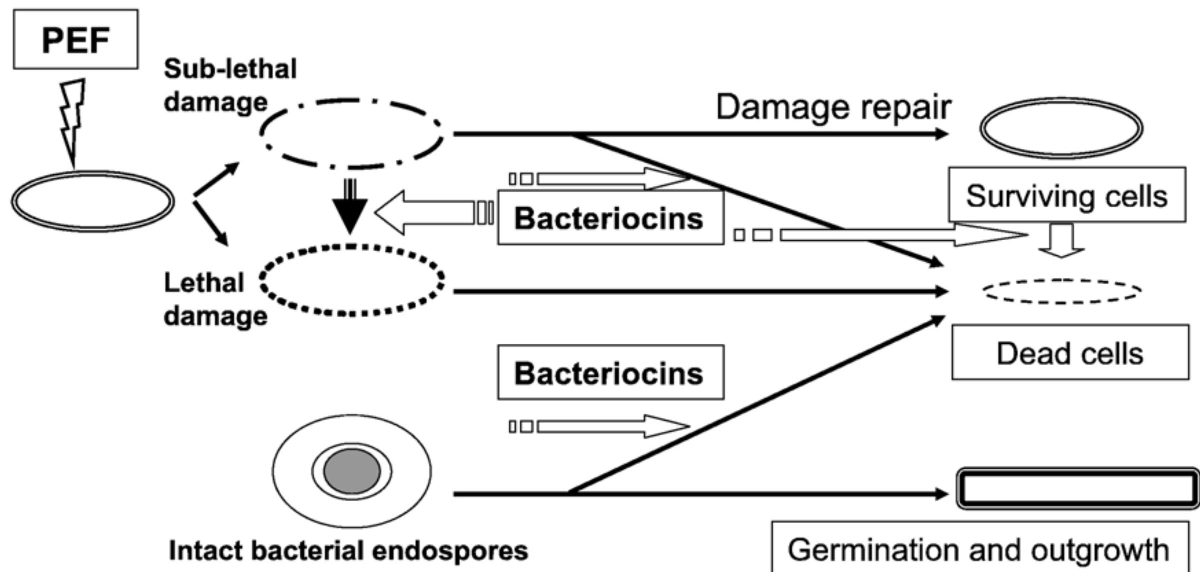

Figure 2.7: Effects of pulsed electric fields in combination with bacteriocins on microbial populations (Gálvez et al., 2007).

A combination of PEF with bacteriocins and lysozyme has been used to improve the protection of fruit juices from spoilage microorganisms (Liang et al. 2006; Mosqueda-Melgar et al. 2008; Bartowsky, 2009). Some recent trials have also demonstrated that a combination of PEF with low concentrations of SO_2 have no negative effect on the formation of volatile compounds in grape must (Garde-Cerdan et al. 2008; Bartowsky, 2009). Therefore, PEF technology remains to be further investigated for its potential of controling wine spoilage bacteria during wine storage prior to bottling (Bartowsky, 2009). There are several other studies that have been done on the efficacy of combining PEF and bacteriocin treatments (Table 2.2) (Gálvez et al., 2007).

Table 2.2. Application of nisin and pulsed-electic fields (PEF) for bacterial inactivation (adapted from Gálvez et al., 2007).

Observed effects	**References**
–Increased inactivation of *Micrococcus luteus* in phosphate buffer	Dutreux et al. (2000)
–Increased inactivation of *Pseudomonas aeruginosa*	Santi et al. (2003)
–Increased inactivation of *S. aureus* in skim milk	Sobrino-Lopez and Martin Belloso (2006)
–Increased inactivation of *List. innocua* in liquid whole egg, skim milk, and liquid whey protein concentrate	Calderon-Miranda et al. (1999a,b&c); Gallo et al. (2007).
–Increased inactivation of *Eschericia coli* in simulated milk ultrafiltrate media. Observed synergism with reduced water activity	Terebiznik et al. (2000, 2002)
–Increased inactivation of *B. cereus* vegetative cells (more efficient in buffer than in skim milk) Observed synergism with carvacrol	Pol et al. (2000, 2001a,b)
–Inactivation of *Salmonella* in orange juice. Observed synergism with lysozyme	Liang et al. (2002)
–Inactivation of *E. coli* O157:H7 in fresh apple cider. Observed synergism with cinnamon	Iu et al. (2001)
–Inactivation of *Lb. plantarum* in model beer	Ulmer et al. (2002)
–Inactivation of *List. innocua* in liquid whey protein concentrate	Gallo et al. (2007)
–Disinfection of collagen gels to be used for tissue engineering applications	Griffiths et al. (2009)
–Inactivation of *S. epidermidis* in collagen gels	Griffiths et al. (2011)

2.12.2.2 Bacteriocins and High Hydrostatic Pressure Technology

High hydrostatic pressure (HHP) is an innovative food processing and preservation method that causes injury and killing of microbial cells (Kalchayanand et al., 1994; Farkas and Hoover, 2000; Patterson, 2000; Ray, 2002). HHP alters the structure and function of microbial cells by inducing the disruption of H-bonds, ionic bonds and hydrophobic interactions of the macromolecules. The sub-lethal damage is provoked by membrane phase transitions (Kato and Hayashi, 1999), which mainly affect proteins generating ATP and transport proteins (Gálvez et al., 2007). Cell death caused by HHP individually and by synergism with bacteriocins increases with pressure. Because most bacteriocins act on the bacterial cytoplasmic membrane, it can then be deduced that the combination effect of bacteriocins and HHP is due to the gradual damage to this structure. However, the interaction between bacteriocin and membrane during phase transition at high pressure has never been investigated. After HHP treatments, a tailing effect is frequently observed, which indicates that cell death results from either multiple events or cumulative cell damage. Sub-lethally injured vegetative cells which survive HHP treatment may develop pressure resistance, a phenomenon observed in *E. coli* (García- Graells et al., 1998) and *List. monocytogenes* (Karatzas and Bennik, 2002). However, the increased cell damage resulting from the combined effect of HHP treatment and bacteriocins could prevent the tailing effect, and thus provide an additional hurdle against the selection of pressure-resistant vegetative cells (Gálvez et al., 2007).

The effect of HHP treatment on bacteria increases with temperature. Temperature determines different forms of HHP treatments such as cold HHP pasteurisation (ca. 5 °C), HPP-assisted pasteurisation (ca. 40 °C), or HHP-assisted sterilisation (ca. 90 °C). The pH is another influencing factor as bacteria are usually more resistant to HHP treatment in low acid foods. Thus, the addition of bacteriocins could enhance the effectiveness of HHP treatments in foods and compensate for the need for higher pressure or temperature (Gálvez et al., 2007).

The type of bacteria and the physiological stage (e.g. vegetative cells or endospores) may greatly affect HHP efficacy (Chen et al., 2006). Bacteriocins are generally inactive on Gram-negative bacteria, however HHP treatment sensitizes Gram-negative bacteria by damaging the outer membrane (Kalchayanand et al., 1994; Hauben et al., 1996; Masschalck et al., 2001; Black et al., 2005). HHP treatment also causes long lasting sensitisation of Gram-negative bacteria to small diffusible antimicrobial molecules (García-Graells et al., 1998, 2000), which may be used in combination with other hurdles (Gálvez et al., 2007). Bacterial endospores are resistant to HHP treatments that are employed in foods (Smelt, 1998). This treatment can even induce endospore germination (Gálvez et al., 2007). Thus the addition of bacteriocins as a second hurdle against surviving endospores could also improve the safety and shelf life of HHP-processed foods (Shearer et al., 2000). Several studies on the combined effects of bacteriocins and HHP on bacteria have been conducted (Table 2.3) (Gálvez et al., 2007).

Table 2.3. Application of bacteriocins and high hydrostatic pressure (HHP) for bacterial inactivation (adapted from Gálvez et al., 2007).

Bacteriocin	Observed Effects	References
Nisin	Increased inactivation of *Bacillus coagulans*, *Bacillus subtilis* and *Clostridium* spores	Roberts and Hoover (1996); Stewart et al. (2000)
	Increased bactericidal activity and spectrum (*E. coli*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, Shigella flexneri, Pseudomonas fluorescens and S. aureus)	Masschalck et al. (2001)
	Increased inactivation of bacteria associated with milk (*E. coli*, *P. fluorescens*, *List. innocua*, and Lactobacillus viridescens)	Garcia-Graells et al. (1999); Black et al. (2005)
	Improved bactericidal effect on spores and aerobic mesophilic bacteria in cheese	Capellas et al. (2000)
	Increased inactivation of *B. cereus* spores and inhibition of the surviving fraction in cheese	López-Pedemonte et al. (2003)
	Increased inactivation of *List. monocytogenes* Scott A in cheese inoculated with a nisin-producing strain	Arqués et al. (2005)
	Increased inactivation of *E. coli* and *List. innocua* in liquid whole egg	Ponce et al. (1998)
	In a meat model system, nisin reduced viable counts of *E. coli*, reduced growth of *S. aureus*, and suppressed slime-producing bacteria	Garriga et al. (2002)
Pediocin AcH	Increased inactivation of food spoilage and pathogenic bacteria (*S. aureus*, *List. monocytogenes*, *S. typhimurium*, *E. coli* O157:H7, *Lb. sakei*, *Leuc. mesenteroides*, *Serratia liquefaciens*, and *P. fluorescens*) suspended in peptone water	Kalchayanand et al. (1998b)
	Increased cell lysis through cell wall degradation in *Leuc. mesenteroides*	Kalchayanand et al. (2002)
	Reduction of *List. monocytogenes* viable counts and inhibition of proliferation during storage in meat model system	Garriga et al. (2002)
Pediocin AcH+nisin	Increased inactivation of *S. aureus*, *List. monocytogenes* Scott A, *S. thyphimurium* and *E. coli* O157:H7	Kalchayanand et al. (1998a,b, 2004a)
	Killing of *Clostridium* spores induced to germinate	Kalchayanand et al. (2004b)
Sakacin K, enterocins A and B	Reduction of *List. monocytogenes* viable counts and inhibition of proliferation during storage in meat model system	Garriga et al. (2002)
Lacticin 3147	Increased inactivation of *List. monocytogenes* and *S. aureus* in milk and in whey	Morgan et al. (2000)
Enterocin A & B	Reduction and inhibition of *List. monocytogenes* in cooked ham	Marcos et al. (2008)
	Reduction of *List. monocytogenes* and *S. enterica (should be written out since you haven't used it before in* low acid dry fermented sausages	Jofré et al. (2009)
	Reduction of *List. monocytogenes* and *S. enterica* in dry cured ham and cooked ham	Jofré et al. (2008)
Enterocin LM-2	Suppressed the growth of microflora, inhibited the accumulation of TVB-N and decelerated lipid oxidation and kept a better sensory profile during the storage period of refrigerated sliced cooked ham	Liu et al. (2012)
Enterocin AS-48	Control *S. enterica* in a low acid fermented sausage	Ananou et al. (2010)

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2.13. Bacteriocin Safety Considerations

LAB-produced bacteriocins have received much attention because LAB have a qualified presumption of safety status. LAB and their metabolites are regarded as safe for human consumption because they have been consumed in fermented foods for many generations without causing any health problems (Cleveland et al., 2001; Ruiz-Larrea et al. 2007). In addition, LAB form part of the natural microbiota in as early as a few days after birth. Thus bacteriocins have found important applications as natural preservatives (Ruiz-Larrea et al. 2007). They are believed to be safe for consumption in food because of their proteinaceous nature which allows inactivation by the enzymes of the gastro-intestinal tract (Lloyd and Drake, 1975). When purified, bacteriocins are colourless, tasteless and odourless. A number of bacteriocins have been tested extensively and proven to be non-toxic and safe for human consumption (Abee et al., 1995; Bauer et al., 2005; Dicks et al., 2009). Moreover, because of the narrow spectrum of antimicrobial activity, bacteriocins would not have a remarkable effect on the normal population of intestinal bio-microflora (De Vuyst and Vandamme 1994, Verellen et al. 1998; Dicks et al., 2009).

The use of nisin was approved on the basis of both published and unpublished data, not on the history of common use (U.S. Food and Drug Administration, 1988; Cleveland et al., 2001). Many studies on acute chronic and subchronic, reproduction, sensitization, *in vitro* and cross-resistance proved that nisin is safe for human consumption at an Acceptable Daily Intake (ADI) of 2.9 mg/person/day (U.S. Food and Drug Administration, 1988).

The safety of other emerging bacteriocins like pediocin PA-1 has also been evaluated. When pediocin PA-1 was injected into mice and rabbits, the immunoblotting data revealed that it was non-immunogenic in both animals. Pediocin was also inactivated by trypsin and chymotrypsin (Bhunia et al., 1990).

2.14. Bacteriocin Regulatory Considerations

For a bacteriocin to be approved it must have been chemically identified and characterized, and its use and efficacy clearly shown. In addition to that, toxicological data as well as detailed information on what happens after ingestion must be provided (Cleveland et al., 2001). In some countries it is important to clearly distinguish bacteriocins from antibiotics, since the presence of antibiotics is usually not allowed in food. In Denmark, bacteria producing food additives must not co-produce toxins or antibiotics (Wessels et al., 1998). The use of starter cultures that produce bacteriocins as food ingredients are exempted from special consideration in the United States if the microorganism has a generally recognized as safe (GRAS) status. Such microorganisms have a history of safe use by food industries before the 1958 Food Additives Ammendment (Muriana, 1996). According to the Code of Federal Regulations, a purified bacteriocin used as a food preservative may be assumed to have GRAS status by a company that produces it, however, the Food and Drug Administration (FDA) may require justification for approval (US Government Printing Office, 1990). The European Union (EU) gives food additives “E” numbers. Nisin has been listed as E234 and may

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also be labelled as “nisin preservative” or “natural preservative” (Cleveland et al., 2001). It has been selling under the trade name of *Nisaplin*® since 1953. The approval of nisin as a food preservative is based on characteristics such as non-toxicity, its production by a *L. lactis* strains which has GRAS status and it has no clinical use. In addition to that, nisin has no record of cross-resistance with health related bacteria and hence poses no risk on antibiotic therapeutics, and it is digested quickly (Delves-Broughton, 2005). Table 2.4 shows concentrations of nisin used in various foods and the typical target organisms.

In the United States where antibiotics are not allowed in foods, nisin received GRAS approval by the FDA in 1988 (Cleveland et al., 2001). Many authors have discussed the issues involved in the approval of new bacteriocins as safe for consumption in foods (Harlander, 1993; Fields, 1996; Post, 1996). The guidelines for safety assessment of a new preservative are published by the United States Department of Agriculture (U.S. Food and Drug Administration, 1993).

Table 2.4. Typical addition levels of nisin and *Nisaplin*® in food applications (Delves-Broughton, 2005).

Food application	**Typical target organisms**	**Level of nisin (mg/kg or mg/L)**	**Level of Nisaplin® (mg/L)**
Processed cheese	*Clostridium* spp. *Bacillus* spp.	5–15	200–600
Pasteurised milk and milk products	*Clostridium* spp. *Bacillus* spp.	0.25–10.0	10–400
Pasteurised chilled soups	*B. cereus*, *Clostridium pasteurianum*	2.5–6.25	100–200
Crumpets	*B. cereus*	4–6.25	150–250
Canned foods (high acid)	*Clostridium botulinum* and *thermosaccharolyticum*	2.5–5.0	100–200
Ricotta cheese	*List. monocytogenes*	2.5–5.0	100–200
Continental type cooked sausage	Lactic acid bacteria, *Brochothrix thermosphacta*, *List. monocytogenes*	5–25	200–1000
Dipping sauces	Lactic acid bacteria	1.25–6.25	50–250
Salad dressings	Lactic acid bacteria	1.25–5	50–200
post fermentation	Lactic acid bacteria, eg. *Lactobacillus,*	25.0–37.5	1000–1500
	Pediocococcus	0.25–1.25	10–50

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2.15 Concluding Remarks

Although bacteriocins have been researched extensively, only a few have been approved for use in food systems and are commercially available. Cost implications may be one of the contributing factors why bacteriocins use is not yet approved in the wine industry. Therefore, identifying more suitable bacteriocinogenic strains could help in reducing the cost involved in the large scale bacteriocin production and purification for commercial use. Researchers also have an important role in educating the wine industry (both winemakers and consumers) about the benefits of using natural preservatives in wine, and as well as addressing the misconceptions they may have.

It is also evident that the use of novel preservation technologies presents new opportunities to employ bacteriocins as part of hurdle technology. More studies on the synergistic reactions between bacteriocins and other natural preservatives, and in combination with other hurdles such as PEF and HHP could lead to the replacement of chemical preservatives or enable minimal food processing treatments, while maintaining the adequate microbial food safety and quality (Abee et al., 1995). However, the combined effect of bacteriocins with many other technologies, such as ultrasonication, irradiation, microwave and ohmic heating, or pulsed light still remains to be investigated (Gálvez et al., 2007).

While it is important to identify more bacteriocins for application in foods, it is equally important to investigate and develop alternative systems (Bauer et al., 2003) that could prevent the development of bacteriocin resistance by target microorganisms. This could prolong the lifespan of bacteriocins and their application in foods (Cleveland et al., 2001).

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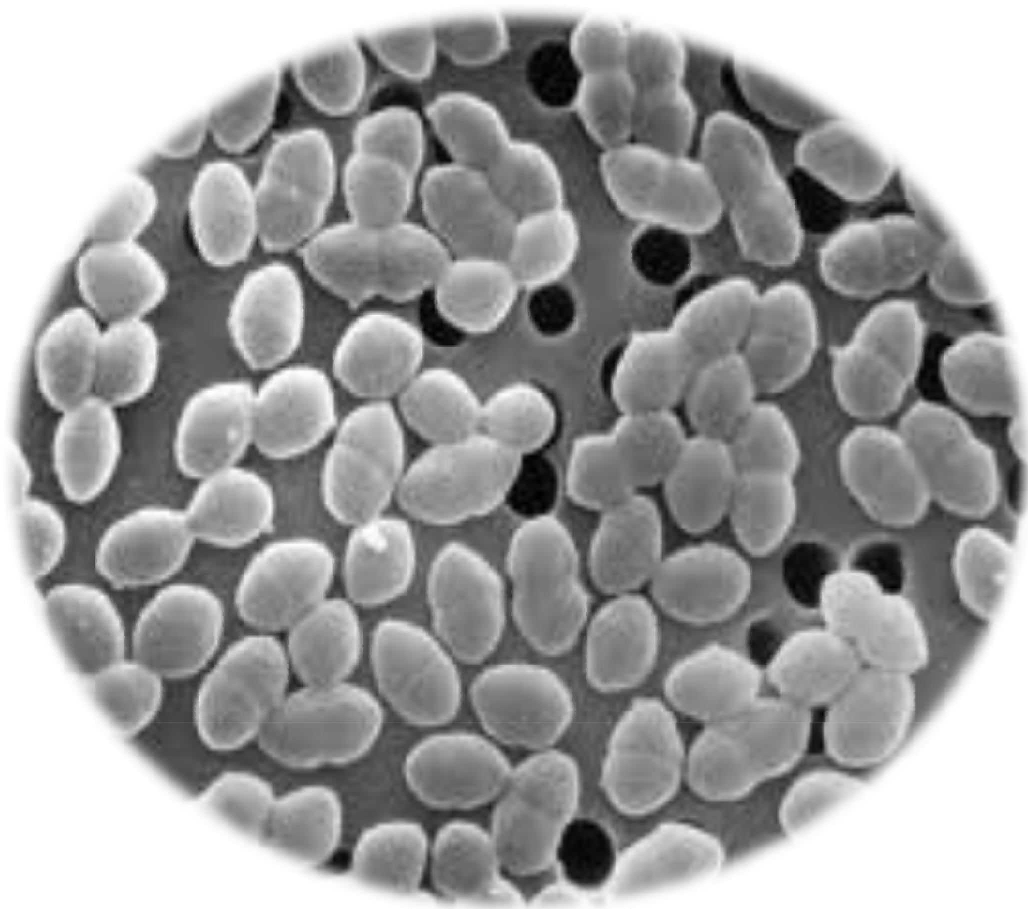
CHAPTER 3

Research Results



Screening, identification and characterization of bacteriocins produced by wine-isolated LAB strains

CHAPTER 3

3.1 Abstract

One hundred and fifty five (155) LAB strains isolated from South African red wines undergoing spontaneous malolactic fermentation were screened for bacteriocin production. Eight isolates (5%) were identified to be bacteriocin producers and were identified to a species level as belonging to *Enterococcus faecium*. The strains were all identical as they shared the same phenotypic and genotypic profiles. Peptide size was determined by tricine-SDS-PAGE to be below 4.6 kDa. The peptides were identified as enterocin P using mass spectrometry. PCR amplification with enterocin P primers and sequencing confirmed the mass spectrometry result that the *Ent. faecium* strains produced enterocin P. The peptide activity was inhibited by proteolytic enzyme treatments with α-Chymotrypsin, papain and proteinase K, but not inhibited by treatments with lysozyme, lyticase, catalase and lipase. Heat treatments showed *Ent. faecium* produced bacteriocins to be heat stable at 37°C, 60°C, 80°C and 100°C but showed 50% decrease in activity at 121 °C. The peptides were active over a broad pH range of 2–10. Growth and production kinetics revealed that bacteriocin production followed primary metabolite kinetics. Mode of action assays showed that the peptides employed a bactericidal mode of action against the sensitive organism, *Lactobacillus pentosus* DSM 20314.

Keywords: *Enterococcus faecium*, enterocin P, bacteriocin-screening, wine

3.2. Introduction

Wine grapes provide a habitat for a complex microbiota that includes filamentous fungi, yeasts and bacteria. These micro-organisms have unique physiological characteristics and various impacts on winemaking. Some species only occurs on grape surfaces and freshly extracted juice, while others can survive different stages of the winemaking process up to the final product (Barata et al., 2012).

Even though occurring in relatively smaller concentrations (Barata et al., 2012), lactic acid bacteria (LAB) form part of the wine grape microbiota. Species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Weissella* are commonly found on grapes and freshly extracted grape must (Marcobal et al., 2004; Renouf et al., 2005; Dicks et al., 2009; Capozzi et al., 2011; Kačániová et al., 2012). *Oenococcus* is rarely isolated from grapes (Bae et al., 2006; Barata et al., 2012). Malolactic fermentation is characterized by *Oenococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, with *O. oeni* occurring predominantly (Bartowsky, 2005; Ruiz-Larrea, 2010). *Enterococcus* is rarely found in wine (Capozzi et al., 2011; Kačániová et al., 2012) but occurs predominantly in the intestines of humans and other animals (Khan et al., 2010; Javed et al., 2011).

LAB play a vital role in reducing wine acidity and also contributing to its aroma and flavour. However, they can also be responsible for many wine spoilage problems that compromise the quality and value of wine. While *O. oeni* contributes positive characteristics to the sensory quality of the wine, species of

the genera, *Lactobacillus* and *Pediococcus* can affect the wholesomeness of wine by producing undesirable volatile compounds (Bartowsky, 2009), such as biogenic amines and ethyl carbamate (Du Toit et al., 2002).

Chemical preservatives like sulphur dioxide (SO_2) are used to prevent the growth of spoilage micro-organisms during the wine making process (Bartowsky, 2009). SO_2 also acts as a reducing agent and maintains the benefits of antioxidant properties of the polyphenols of wine (Oliveira et al., 2002; Rojo-Bezares et al., 2007b; Bartowsky, 2009). However, there is a worldwide demand to reduce SO_2 levels due to the increasing health risks for sulphite-sensitive individuals (Enrique et al., 2007; Rojo-Bezares et al., 2007b), consumer preference for more natural products, and the strict legislation regarding preservatives (Du Toit and Pretorius, 2000; Bauer et al., 2003; Enrique et al., 2007; Ruiz-Larrea, 2010). Additional or novel antimicrobial agents are therefore required in wines. Molecular SO_2 is the only form of SO_2 that demonstrates antimicrobial activity and only 5 -10% of it is present in wines at pH 3 and close to zero in wines at pH 4. However, several bacterial species (Bartowky, 2009) and some wine spoilage yeasts (Du Toit and Pretorius, 2000; Enrique et al., 2007) may also be resistant to the maximum permitted levels of SO_2.

All these considerations have increased the interest in research to look for new preservation strategies. For the past decades, the research focus has been on the naturally occurring antimicrobials, like bacteriocins as an additional hurdle to fight unwanted bacterial growth and prevent food spoilage (Cleveland et al., 2001; Du Toit et al., 2002, Bauer et al., 2003; Martínez and Rodríguez, 2005). Bacteriocins are antimicrobial proteinaceous substances that are produced by some bacteria against other bacteria that are closely related to the producing organism growing in the same medium (Klaenhammer, 1988; Navarro et al., 2000; Cleveland et al., 2001; Hernandez et al., 2005; Ruiz-Larrea et al., 2007).

LAB-produced bacteriocins play a very important role in the food fermentation industry as natural preservatives, since they are capable of inhibiting the growth of many food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (Hernandez et al., 2005; Ruiz-Larrea et al., 2007). Various types of LAB-produced bacteriocins have been identified and characterised (Navarro et al., 2000; Hernández et al., 2005; Badarinath and Halami, 2011). However, there are few reports on bacteriocins produced by LAB of oenological origin or on bacteriocins present in the finished wine (Rojo-Bezares et al., 2007a; Saenz et al., 2009). For example, no *O. oeni* strains have been reported to produce bacteriocins and these species have demonstrated sensitivity towards bacteriocins such as nisin and pediocin (Knoll et al., 2008).

Nisin is a bacteriocin produced by some strains of *Lactococcus lactis* of non-oenological origin (Navarro et al., 2000; Hernandez et al., 2005) and active against a wide range of Gram-positive bacteria (Hernandez et al., 2005). It could be of winemaking interest since it has been used to control spoilage by undesired LAB in wine and beer (Delves-Broughton et al., 1996; Navarro et al., 2000; Bauer et al., 2003).

It has been proposed that a combination of nisin and SO_2 could be a means to reduce the use of SO_2 in winemaking (Rojo-Bezares et al., 2007; Knoll et al., 2008; Bartowsky, 2009). Fairly recently, a bacteriocin-like inhibitory substance has been shown to be active against wine *Lactobacillus* species (Yurdugül and Bozoglu, 2002; 2008). There is very strong evidence that these antimicrobials may have an

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essential role in wine fermentation (Navarro et al., 2000; Du Toit et al., 2002; Rojo-Bezares et al., 2007a&b; Ruiz-Larrea et al., 2007; Saenz et al., 2009). However, even though their use to control wine LAB is of great potential, they have not yet been approved for use in winemaking (Bartowsky, 2009).

According to Ruiz-Larrea et al. (2007), bacteriocins used in the food industry should meet some of the following requisites: the producing strain should preferably have QPS (qualified presumption of safety) status, preferably be heat stable, be active against pathogenic or spoilage bacteria, should have no health risks, if added in food products it should demonstrate beneficial impacts such as improved safety, quality or flavour, should not be added in higher concentrations than those found in its natural source, and lastly, should have high specific activity and should only inhibit pathogenic and spoilage organisms. Nisin and pediocin, the current biopreservatives used in the food industry, have demonstrated all of the above-mentioned characteristics. For experimental purposes, these biopreservatives are either used individually or in combination with other preservation techniques like: heat treatment, high pressure or modified atmosphere packaging (Allende et al., 2006; Ruiz-Larrea et al., 2007). Additionally, they have been demonstrated to inhibit LAB found in wine (e.g. *Lb. plantarum, Lb. paracasei*, *Lb. brevis*, *Lb. hilgardii*, *Lb. pentosus*, *Leuc. mesenteroides*, *Ped. pentosaceus* and *O. oeni*), to be stable under winemaking conditions, and also show no negative effect on yeast growth or the sensorial wine profile (Radler, 1990a, b; Bauer et al., 2003; Rojo-Bezares et al., 2007b; Knoll et al., 2008a; Knoll et al., 2008b). Some studies have also demonstrated that bacteriocins may be used to control biofilm formation in steel tanks, suggesting that they are natural and bio-safe sanitizers (Bauer et al., 2002, 2003; Nel et al., 2002; Dicks et al., 2009).

The aim of the present study was to screen and identify wine LAB strains other than *Lb. plantarum* for bacteriocin production and also to identify and characterise the bateriocins they produce.

3.3. Materials and Methods

3.3.1. Strains and Culture Conditions

One hundred and fifty-five (155) LAB isolates from South African red wines undergoing spontaneous malolactic fermentations (IWBT collection), and sixteen indicator organisms (13 LAB and 3 non-LAB strains) (Table 3.1) were used in this study. All bacterial cultures were kept at -80°C in 40% glycerol until needed. Lactic acid bacteria strains were grown in De Man, Rogosa and Sharpe (MRS) (Biolab Diagnostics (PTY) Ltd, Wadeville, Gauteng, South Africa) or MRS supplemented with tomato juice (MRST) broth and agar plates; and non-LAB were grown in Brain Heart Infusion (BHI) (Biolab Diagnostics) broth medium and agar plates. All the LAB strains were sub-cultured twice in MRS broth at 30°C in semi-anaerobic conditions and non-LAB strains were grown in BHI broth at 37°C in aerobic conditions (in a 50 rpm rotary shaker).

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Table 3.1. Bacteria species that were used as sensitive/indicator organisms in the present study.

Indicator organism	Strain	Source
Lactobacillus casei	LMG 13552	LMG collection
Lactobacillus fermentum	ATCC 9328	ATCC collection
Lactobacillus hilgardii	B 203	IWBT collection
Lactobacillus paracasei	B 164	IWBT collection
Lactobacillus pentosus	B 205	IWBT collection
Lactobacillus pentosus	DSM 20314	DSM collection
Lactobacillus plantarum	LMG 13556	LMG collection
Lactobacillus plantarum	B 213	IWBT collection
Lactobacillus plantarum	B 130	IWBT collection
Leuconostoc mesenteroides	B 301	IWBT collection
Leuconostoc mesenteroides	B 296	IWBT collection
Leuconostoc mesenteroides	DIIIM: I	IWBT collection
Listeria innocua	LMG 13568	LMG collection
Listeria monocytogenes	127	BFE collection
Listeria monocytogenes	WS 2250	WS collection
Pediococcus pentosaceus	NCDO 813	NCDO collection

LMG: Culture Collection of the Laboratory of Microbiology
IWBT: Institute for Wine Biotechnology (Stellenbosch, South Africa)
ATCC: American Type Culture Collection (Rockville, MD., USA)
DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen
NCDO: National Collection of Dairy Organisms (Reading, UK)
BFE: Bundesforschungsanstalt für Ernährung, now Max Rubner-Institute (Karlsruhe, Germany)
WS: Institut fur Mikrobiologie, Forschungszentrum fur Milch und Lebensmittel, TU Munchen, Freising/ Weihenstephan.

3.3.2. Initial screening for antimicrobial activity of bacterial isolates

Antimicrobial activity was detected by the agar spot test method (Schillinger and Lücke, 1989), using buffered bacteriocin screening medium (BSM) (Tichaczek et al., 1992). Five microlitres (5 µl) of overnight cultures of the LAB isolates were spotted onto BSM plates and incubated further for 24 hours at 30°C. Ten millilitres of MRS soft agar (0.7% w/v agar) were seeded with 100 µl of each of the overnight grown indicator strains and was used to overlay LAB cultures and incubated for 24 h at 30 °C. In case of non-LAB indicator strains, BHI soft agar (0.7% (w/v) agar) was used instead of MRS, and the incubation temperature was 37°C. After 24 h incubation, the plates were examined for clear inhibition zones which form if a particular strain is a producer of bacteriocins. LAB isolates that showed antimicrobial activity against one or more indicator strains were selected for identification and further experiments.

3.3.3. Phenotypic identification of bacteriocin-producing strains

Physiological and biochemical characteristics were used to identify the isolates. For physiological tests, bacteriocin-producing isolates were evaluated for the production of gas (CO_2) from glucose,, growth in

MRS broth (Merck, Darmstadt, Germany) at 10°C, 37°C and 45°C, growth in MRS broth at pH 9.6, and in MRS broth with 6.5% NaCl using the methods of Schillinger and Lücke (1987) and Andrighetto et al. (2001). Biochemical characteristics were established by perfoming the enzymatic tests and the sugar fermentation tests using the API 20 Strep system (bioMérieux Germany, Nürtingen, Germany) according to the manufacturer's instructions.

3.3.4. Genotypic characterization of bacteriocin-producing strains

3.3.4.1. 16S rRNA gene sequencing

The partial 16S rDNA of selected strains was amplified by PCR using the primers 16Sseqfw (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16Sseqrev (5'-GGN TAC CTT GTT ACG ACT TC-3') corresponding to positions 8 to 27 and 1511 to 1491 of the corresponding 16S rDNA gene of *E. coli*, respectively. DNA was amplified in 32 cycles (94°C, 1 min; 56°C, 1 min, 72°C, 2 min) in a 50 µl reaction volume containing 1.5 U *Taq* DNA polymerase (Amersham Pharmacia), 1 x polymerase buffer (Amersham Pharmacia), 25 pM of each primer and 20 µM dNTP's. The PCR products were cleaned using the PeqGOLD MicroSpin Cycle-pure kit (Peqlab, Erlangen, Germany) and sequenced bi-directionally at GATC Biotech (GATC, Konstanz, Germany). The 16S rRNA gene sequences obtained were compared to the GenBank database using the pairwise clustering comparison option of the Bionumerics sequence types module.

3.3.4.2. RAPD-PCR fingerprinting

For RAPD-PCR fingerprinting, total genomic DNA from enterococci isolates was isolated according to the methods of Pitcher et al. (1989) as modified for Gram-positive bacteria by Björkroth and Korkeala (1996). Two RAPD-PCR reactions were done for each isolate, each employing a different primer, M13 (5'-GAG GGT GGC GGT TCT-3') (Huey and Hall, 1989) and LB2 (5'-GGT GAC GC-3') (Ben Omar et al., 2000). DNA was amplified using methods and amplification conditions described by Andrighetto et al. (2001). PCR products were separated by electrophoresis on a 1.8% (w/v) agarose gel using 1 x TBE buffer (Sambrook et al. 1989). The gels were stained in ethidium bromide and photographed on an UV transilluminator. Photo-positives were digitalised by scanning and scanned images were normalised and subsequently analysed using the Bionumerics (version 2.5) software package (Applied Maths, Sint-Martens-Latem, Belgium).

3.3.5. Obtaining crude and partially purified bacteriocin extracts

The method followed for obtaining crude bacteriocin extracts was adapted from Schillinger and Lücke (1989) and Chaimanee et al. (2009). Bacteriocin-producing isolates were grown in 100 ml MRS broth at 30°C for 24 hours. Cells were harvested by centrifugation at 14 000 rpm for 10 minutes at 4°C. Cell-free supernatants were heated at 100°C for 5 minutes in order to inactivate remaining cells (Franz et al., 1996). Following that, the supernatants were filtered through a 0.2 µm pore-size nylon syringe filter and were kept

at 4°C for further purification and bacteriocin characterisation experiments. These were referred to as crude extracts (Callewaert et al., 1999).

To partially purify bacteriocins, the crude extracts (cell free culture supernatants, CFS) were concentrated with ammonium sulphate precipitation at 85% (w/w) saturation, followed by sample dialysis and freeze-drying (Bollag and Edelstein, 1991). Partially purified bacteriocins were stored at -20°C and used for identification and characterisation experiments. Antimicrobial activity of partially purified bacteriocins was carried out by following the agar spot test method by Schillinger and Lücke (1989), using buffered BSM as previously described by Tichaczek et al. (1992). Alternatively, crude CFS samples were concentrated through an Amicon Ultra-15 10 K device (Amicon® Ultra-15, Merck Millipore, South Africa) and centrifuged at 5 000 rpm for 10 minutes.

3.3.6. Activity units assay

The producer strains were inoculated in MRS broth and grown for 24 hours at 30°C and 37°C[1]. The stationery phase grown cultures were centrifuged at 14 000 rpm for 10 minutes at 4°C and the resulting extracts were partially purified as described in 3.2.5. Activity units (AU/ml) of partially purified enterocins were determined by preparing appropriate two-fold serial dilutions. The dilutions were tested against *Lb. pentosus* DSM 20314 as a sensitive strain, using the agar spot test method (Schillinger and Lücke, 1989). The antimicrobial activity, expressed in activity units per milliliter (AU/ml) was defined as a reciprocal of the highest dilution showing inhibition of an indicator organism (Chaimanee et al., 2009).

3.3.7. Protein identification using mass spectrometry

The protein identification was done at the Central Analytical Facility (MS Unit, Proteomics Laboratory), Stellenbosch University. A gel trypsin digestion method was used to purify and prepare the gel pieces (cut from a tricine SDS-PAGE gel) before the injections for nano-LC chromatography were made. For mass spectrometry analysis, all the experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. For liquid chromatography analysis, separation was performed on an EASY-column (2 cm, ID 100 µm, 5 µm, C18) pre-column followed by an EASY- column (10 cm, ID 75 µm, 3 µm, C18) column with a flow rate of 300 nl/min. Data analysis to identify proteins were performed using a Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany). This was done via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against the Swissprot 57.15 database. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per protein, a Mascot or Sequest score of more than $p<0.05$ as determined by Proteome

[1] Producer strains in which the crude extracts were obtained were initially grown at 30°C. After identification of the enterococci, the following samples were prepared from strains grown at 37°C (an optimal growth temperature for enterococci). Later it was discovered that 30°C and not 37°C is the optimal temperature for bacteriocins production.

Discoverer 1.3. Percolator was also used for validation of search results. In Percolator a decoy database was searched with a FDR (strict) of 0.02 and FDR (relaxed) of 0.05 with validation based on the q-value.

3.3.8. Molecular weight of bacteriocins

The molecular size of bacteriocins was determined by tricine SDS-PAGE described by Schägger (2006). Two polyacrylamide gels were prepared and loaded with aliquots of each partially purified CFS. After electrophoresis, gels were subjected to different treatments: one gel was fixed and stained with Coomassie blue solution to determine the peptide sizes using a Spectra Multicolor Low Range Protein Ladder (Thermo Scientific). The other gel was fixed in 20% (v/v) isopropanol and 10% (v/v) acetic acid solution for 30 min at room temperature, followed by rinsing for 2 h in MilliQ water and then left in MilliQ water overnight while shaking. The following day, the gel was rinsed with MilliQ water for 30 min, placed onto a thin layer of BSM agar and overlaid with 0.7% (w/v) MRS soft agar containing *Lb. pentosus* DSM 20314 as a sensitive indicator organism. The gel was then incubated for 24 h at 30°C followed by inspection for inhibition zones.

3.3.9. Identification of the enterocin genes and the encoded proteins

In order to identify the genes encoding the bacteriocins, DNA isolations were performed by extracting the genomic DNA using a phenol/chloroform protocol as described by Vaquero et al. (2004) and modified by Mtshali et al. (2010). PCR amplifications of the genomic DNA were performed using the enterocin P[2] (EntP) primers (EntPF: 5’ ATG AGA AAA AAA TTA TTT AG 3’ and EntPR: 5’ TTA ATG TCC CAT ACC TGC CA 3’) (Integrated DNA Technologies). The final concentrations of the reagents used in the PCR reactions were, Flexi buffer (1×), forward and reverse primer (0.125 µM each), dNTPs (0.25 mM), $MgCl_2$ (2.08 mM), GoTaq DNA polymerase (2.5 U/µl), and sterile MilliQ H_2O to a total volume of 30 µl per reaction. The reagents used were either purchased from Promega Madison WI (USA) or from Takara Biotechnology (Dalian). The PCR experiment was run in Gene Amp® PCR System 9700 (Applied Biosystems) for the duration of 35 cycles, under the following conditions: Initial denaturation (95°C for 2 min), denaturation (95°C for 1 min), primer annealing (45°C for 1 min), extension (72°C for 1 min), final extension (72°C for 10 min) and cooling (4°C for infinity). PCR products were analysed by electrophoresis onto a 3% gel (White *Sci*), with a Generuler™ 50 bp DNA Ladder, as a molecular weight marker (ThermoScientific). The fragments were visualised with ethidium bromide under ultraviolet light. After electrophoresis analyses, the PCR products were purified and sequenced (CAF, Stellenbosch University). The sequencing data were analysed and the resulting DNA sequences searched against existing data bases, using the BLAST tool (National Centre for Biotechnology Information; NCBI).

The same sequences were translated into proteins using the online DNA to protein translation tool ExPASy (SIB Swiss Institute of Bioinformatics). This was followed by alignment with the enterocin

[2] It was decided to amplify the enterocin P gene because the mass spectrometric result for peptide identification gave an indication that the peptide could be enterocin P.

sequences from NCBI and BACTIBASE (database dedicated to bacteriocins) showing the highest percentage identity, using the online alignment tool: CLUSTALW.

3.3.10. Bacteriocin characterisation

3.3.10.1. Enzyme treatment, heat resistance and pH sensitivity

Bacteriocin sensitivity to enzyme treatments were determined by treating partially purified bacteriocin samples (800 AU/ml) with the following enzymes at a final concentration of 1 mg/ml: lysozyme (pH 7, Sigma), lyticase (pH 7, Sigma), α-chymotrypsin (pH 7, Sigma), catalase (pH 7, Sigma), papain (pH 7, Sigma), lipase (pH 7, Sigma), and proteinase K (pH 7, Sigma). The samples were incubated at 37°C for 2 h, followed by heating for 5 minutes at 100°C to inactivate enzyme activity. Untreated samples were used as controls (Franz et al., 1996). After all treatments, the samples were assayed for bacteriocin activity using the agar spot test method (Schillinger and Lücke, 1989). The effect of heat treatment on bacteriocin activity were determined by subjecting partially purified bacteriocin samples (6400AU/ml) to 37°C for 3 hours, 60°C for 30 min, 80°C for 30 min, 100°C for 30 min, and 121°C for 15 min, respectively, followed by activity analysis. To investigate pH effect on antimicrobial activity, bacteriocin samples were adjusted to a pH range of 2 to 10 with either 1M HCL, 5M or 1M NaOH. Serial dilutions of heat treated samples and pH adjusted samples were prepared. The activity units (AU/ml) of the samples was evaluated by spotting against a sensitive organism and incubated overnight at 30°C and compared to untreated samples, also serially diluted. Three independent experiments were done (on different days) for each treatment.

3.3.10.2. Production kinetics and the mode of action of enterocins

Bacteriocin production kinetics was determined as described by Ferreira et al. (2007), with minor modifications. Three independent experiments were performed on different days. Producer strains, 16.3 and 128.1 were grown in MRS medium for 24 h at 30°C. Aliquot samples were taken at 2 h intervals to determine bacteriocin activity (expressed in AU/ml) against *Lb. pentosus* DSM 20314 as a sensitive indicator organism. The samples were centrifuged at 10 000 rpm for 15 minutes and the crude supernatants were serially diluted and spotted using the agar spot test method on agar plates lawned with the indicator organism (Schillinger and Lücke, 1989). At each sampling point, bacterial growth analyses were determined by plating onto MRS agar plates, incubated at 30°C and determining cell counts (CFU/ml).

In order to investigate the mode of action of bacteriocins, a method described by Franz et al. (1996) and Du Toit et al. (2000) was followed with few a modifications. Three flasks with 50 ml MRS broth were inoculated with an overnight culture of the sensitive indicator organism (*Lb. pentosus* DSM 20314) to a final concentration of 10^6 CFU/ml. This was followed by addition of 1 ml 16.3 partially pure enterocin sample (25600 AU/ml) in one flask and 1 ml of 128.1 sample (25600 AU/ml) in the second flask while the third flask was used as a negative control. The cultures were incubated at 30°C and viable cells were determined for a period of 13 hours (0, 1, 2, 3, 5, 7, 9, 11 and 13 h time intervals) by plating onto MRS agar plates,

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incubated at 30°C and determining cell counts (CFU/ml). Three independent experiments were done (on different days) for the mode of action investigation.

3.4. Results

3.4.1. Screening of antimicrobial activity

Of the one hundred and fifty-five LAB isolates that were screened for bacteriocin production, only eight were identified as producers (Table 3.2.). Of the 16 indicator organisms used, ten showed detectable sensitivity towards the producing isolates, namely: *Lb. paracasei* B 164, *Lb. pentosus* B 205, *Lb. pentosus* DSM 20314, *Lb. plantarum* B 130, *Lb. plantarum* B 213, *Leuc. mesenteroides* B 296, *List. innocua* LMG 13568, *List. monocytogenes* WS 2250, *List. monocytogenes* 127 and *Ped. pentosaceus* NCDO 813. *Lb. casei* LMG 13552 showed a very slight to non-detectable sensitivity, whereas *Lb. fermentum* ATCC 9328, *Lb. hilgardii* B 203, *Lb. plantarum* LMG 13556, *Leuc. mesenteroides* DIIIM: I and *Leuc. mesenteroides* B 301 showed no sensitivity at all. Three of these indicator organisms were identified as most sensitive to the identified producers and for further analysis they were used, namely, *Lb. paracasei* B 164; *Lb. pentosus* DSM 20314; *Lb. plantarum* B 130 and eight producer isolates (#3; 6; 16.3; 33.5; 35.3; 60.3; 112.4 and 128.1) were used for further study. Figure 3.1 shows the inhibition zones formed by eight producers against three sensitive strains. The three sensitive bacteria demonstrated the same degree of sensitivity towards the producers as evidenced by the same-sized inhibition zones formed against each of them.

Table 3.2. LAB isolates showing antimicrobial activity against indicator strains.

	LAB Strains							
Indicator organism	**3**	**6**	**16.3**	**33.5**	**35.3**	**60.3**	**112.4**	**128.1**
Lactobacillus casei LMG 13552	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Lactobacillus fermentum ATCC 9328	-	-	-	-	-	-	-	-
Lactobacillus hilgardii B 203	-	-	-	-	-	-	-	-
Lactobacillus paracasei B 164	++	++	++	++	++	++	++	++
**Lactobacillus pentosus* B 205	++	++	++	++	++	++	++	++
Lactobacillus pentosus DSM 20314	++	++	++	++	++	++	++	++
Lactobacillus plantarum B 130	++	++	++	++	++	++	++	++
Lactobacillus plantarum LMG 13556	-	-	-	-	-	-	-	-
Lactobacillus plantarum B 213	+	+	+	+	+	+	+	+
Leuconostoc mesenteroides DIIIM:I	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides B 301	-	-	-	-	-	-	-	-
Leuconostoc mesenteroides B 296	+	+	+	+	+	+	+	+
Listeria innocua LMG 13568	+	+	+	+	+	+	+	+
Listeria monocytogenes WS 2250	+	+	+	+	+	+	+	+
**Listeria monocytogenes* 127	++	++	++	++	++	++	++	++
**Pediococcus pentosaceus* NCDO 813	++	++	++	++	++	++	++	++

+/- = very slight to non detectable inhibition (+/- 1 mm)

+ = detectable inhibition zone (> 2 mm)

++ = very strong inhibition (> 8 mm)

***sensitive towards culture on an agar plate, but partially sensitive to supernatants with a spot on lawn assay**

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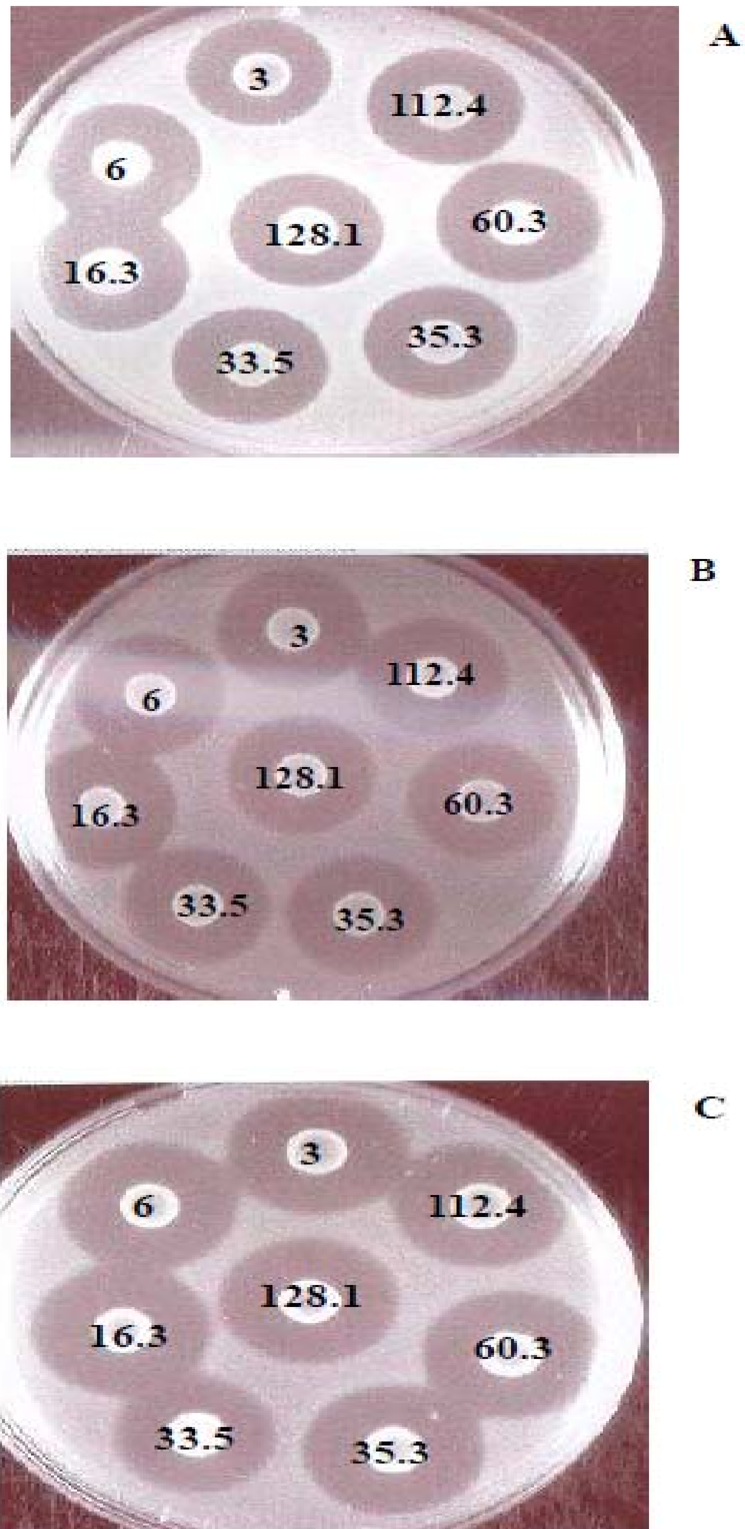

Figure 3.1. Antimicrobial activity of the bacteriocin-producing cultures against the selected sensitive organisms, **(A)** *Lb. pentosus* DSM 20314, **(B)** *Lb. paracasei* B 164 and **(C)** *Lb. plantarum* B 130, respectively.

3.4.2. Phenotypic and genotypic identification of bacteriocin-producing strains

For physiological characteristics, all isolates produced gas from glucose; they grew in MRS broth at 10°C, 37°C and 45°C. They also grew at pH 9.6 and MRS containing 6.5% NaCl, which is typical of

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Enterococcus (Table3.3). Biochemical and sugar fermentation profiles tested using the API 20 Strep systems (bioMérieux Germany, Nürtingen, Germany) were identical among all tested isolates (Table 3.4). They were all Voges-Proskauer (VP) positive, hippuric acid (HIP) negative, esculin (ESC) negative, pyrrolidonyl arylamidase (PYRA) negative, α-galactosidase (α GAL) negative, β-glucuronidase (β GUR) negative, β-galactosidase (β GAL) negative and alkaline phosphatase (PAL) negative, and leucine amino peptidase (LAP) and arginine dihydrolase (ADH) positive. Sugar fermentation profiles were: ribose (RIB) positive, arabinose (ARA) positive, mantiol (MAN) positive, sorbitol (SOR) negative, lactose (LAC) positive, trehalose (TRE) positive, inulin (INU) negative, raffinose (RAF) positive, amidon acidification (AMD) negative and glycogen (GLY) negative.

Table 3.3. Physiological profiles of the enterococci isolates tested under different growth conditions.

Strain #	Prod. of Gas (Glucose)	MRS (10°C)	MRS (37°C)	MRS (45°C)	MRS pH 9.6	MRS 6.5% NaCl
#3	-	+	+	+	+	+
#6	-	+	+	+	+	+
#16.3	-	+	+	+	+	+
#35.3	-	+	+	+	+	+
#60.3	-	+	+	+	+	+
#112.4	-	+	+	+	+	+
#128.1	-	+	+	+	+	+

Table 3.4. Biochemical and sugar fermentation profiles of putative enterococci isolates tested using the API 20 Strep system.

Strain #	VP	HIP	ESC	PYRA	α-GAL	β-GUR	β-GAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG
#3	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#6	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#16.3	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#35.3	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#60.3	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#112.4	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-
#128.1	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	-

VP= Voges-Proskauer; **HIP**= hippuric acid; **ESC**= esculin; **PYRA**= pyrrolidonyl arylamidase; **α-GAL** = α-galactosidase; **β-GUR** = β-glucuronidase; **PAL**= alkaline phosphatase; **LAP**= leucine amino peptidase; **ADH**= arginine dihydrolase; **RIB**=: ribose; **ARA**= arabinose; **MAN**= mantiol; **SOR**= sorbitol; **LAC**= lactose; **TRE**= trehalose; **INU**= inulin; **RAF**=, raffinose; **AMD**= amidon acidification; **GLYG**= glycogen

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The seven 16S rRNA gene sequences obtained for the seven[3] tested isolates: #3; 6; 16.3; 35.3; 60.3; 112.4; 128.1 showed 99.9% similarity to the 16S rRNA gene sequences of *Ent. faecium* type strains obtained from the GenBank database. The pairwise clustering comparison (Figure 3.2) shows that all seven isolates group with the *Ent. faecium* type strains. In two RAPD-PCR finger printing reactions performed with primers, M13 and LB2, all seven isolates gave the same genetic profile with both of the primers used, showing that they are genotypically identical (Figure 3.3).

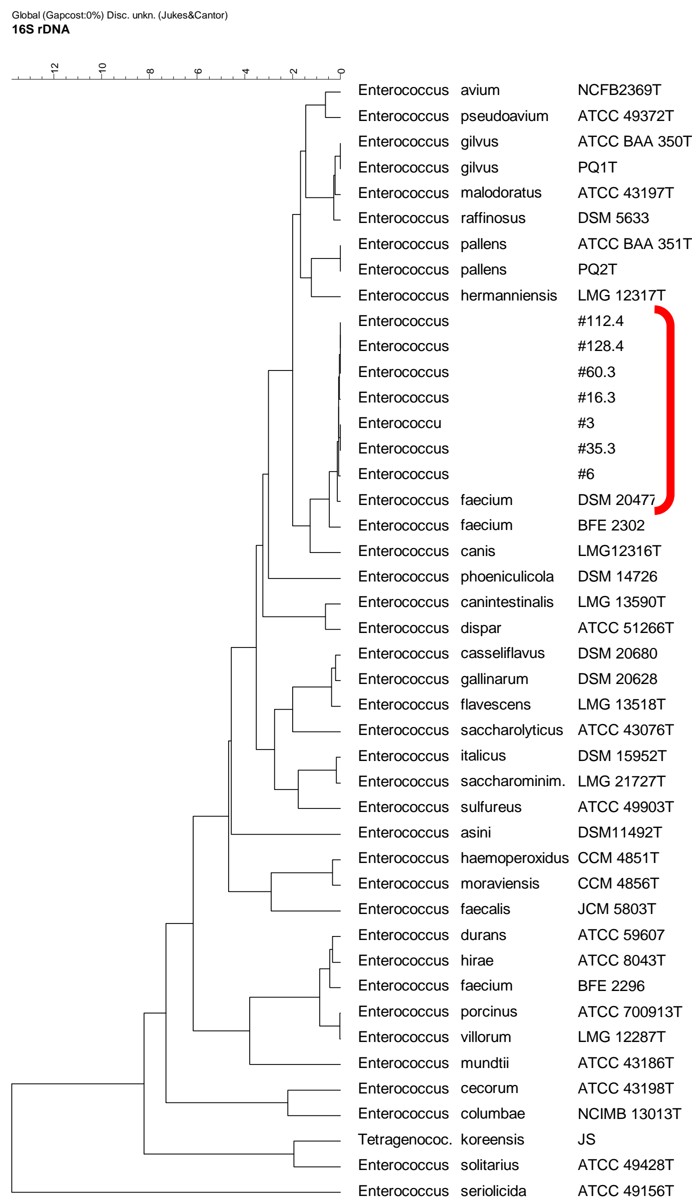

Figure 3.2. Dendogram showing the relationship between the 16S rRNA gene sequences of the seven strains (#112.4, 128.1, 60.3, 16.3, 3, 35.3 and 6) tested in this study and the 16S rRNA gene sequences of *Enterococcus* spp. type strains obtained from the GenBank database using the pairwise clustering comparison option of the Bionumerics sequence types module.

[3] #33.5 was not further identified because it lost its antimicrobial activity. However, it proved to be similar to the other isolates in all other tests performed.

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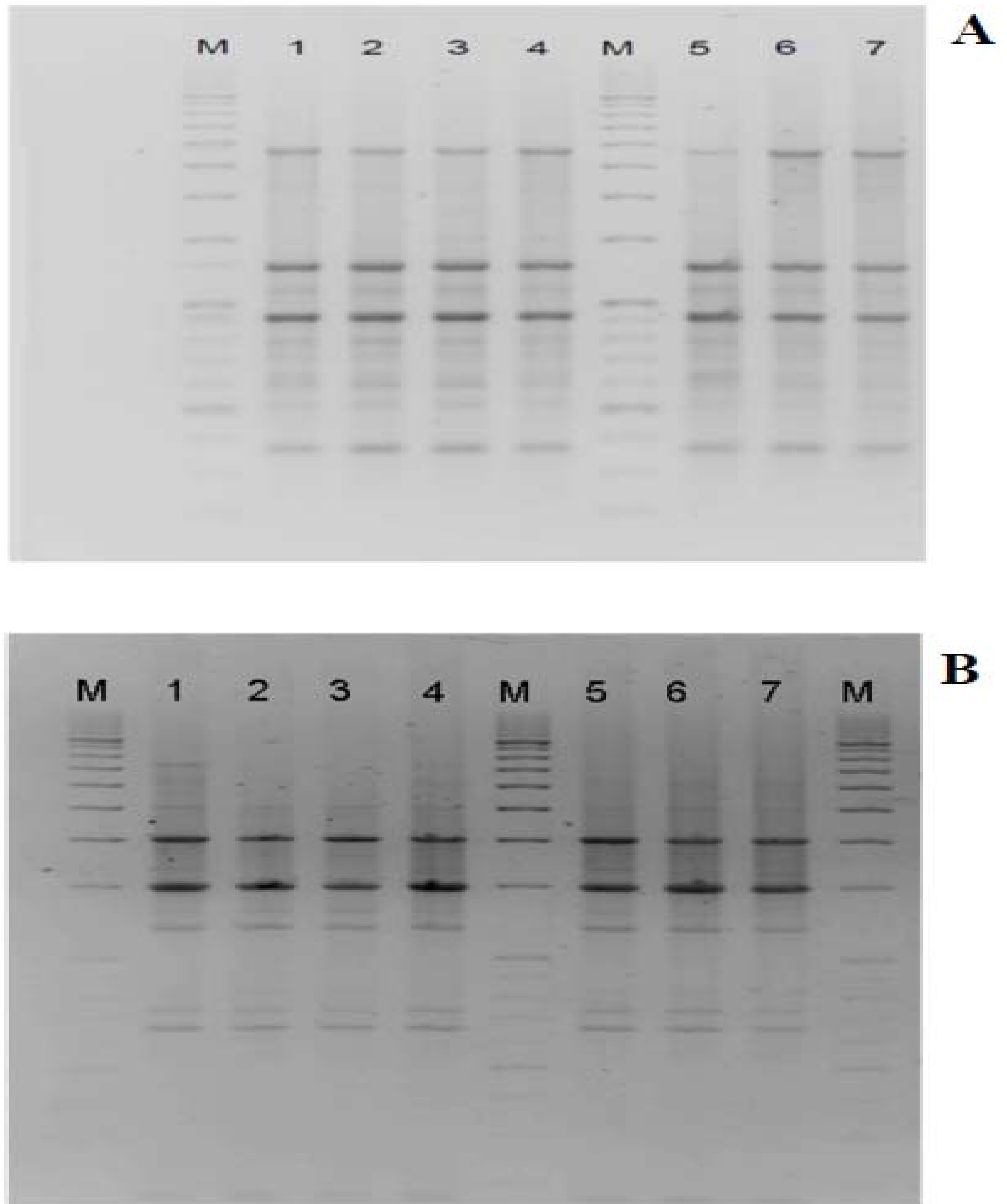

Figure 3.3. RAPD-PCR fingerprinting profiles of *Enterococcus* strains using primer M13 (A) and primer LB2 (B). Lanes 1-7: #3, 128.1, 35.3, 60.3, 6, 16.3, 112.4, respectively. Lanes M: Molecular weight marker.

3.4.3. Partial purification of enterocins

The testing of protein precipitated with 85% (w/v) ammonium sulphate, followed by dialysis and freeze drying gave clear zones of antimicrobial activity against indicator organisms (Figure 3.4.). For each strain, 5 µl of a partially purified fraction was spotted onto a lawn seeded with sensitive organism. In each strain the zones were ± 10 mm in diameter. Samples that were concentrated through a 10 kDa column showed more or less the same diameter inhibition zones as the ones formed by ammonium sulphate treated samples (results not shown). Inhibition zones formed by crude extracts against the same three sensitive organisms were smaller in size (results not shown).

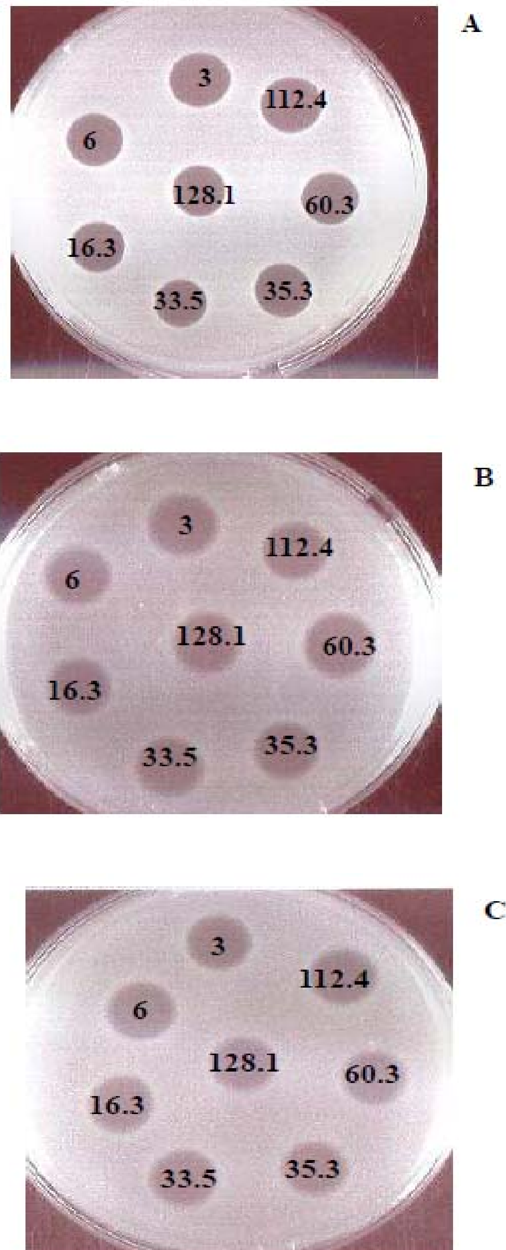

Figure 3.4. Antimicrobial activity of ammonium sulphate precipitated peptides (Bollag and Edelstein, 1991) from *Enterococcus* strains against **(A)** *Lb. pentosus* DSM 20314, **(B)** *Lb. paracasei* B 164 and **(C)** *Lb. plantarum* B 130, respectively.

3.4.4. Activity units of partially purified enterocins

The activity units per millilitre (AU/ml) of partially purified enterocins were determined to be the same for all the *Ent. faecium* strains tested under the same conditions (Table 3.5.).

Table 3.5. Bacteriocin activity of partially purified enterocin extracts against *Lb. pentosus* DSM 20314.

Bacteriocin activity (AU/ml)			
Strain	**Partially purified supernatants**		
	Sample 1 (37°C)	Sample 2 (37°C)	Sample 3* (30°C)
# 3	6400	3200	
#6	6400	3200	
#16.3	6400	3200	25 600
#33.5	6400	3200	
#35.3	6400	3200	
#60.3	6400	3200	
#112.4	6400	3200	
#128.1	6400	3200	25 600

***Only 16.3 and 128.1 partially purified CFS were tested.**

-Sample 1 and 2 were prepared from cultures grown at 37°C, while #16.3 and 128.1 were grown at 30°C (optimal temperature for bacteriocin production) to form sample 3.

3.4.5. Protein identification using mass spectrometry

Mass spectrometric results shed some light on the possible proteins contained by the partially purified extracts from the *Ent. faecium* strains. This result showed isolates #3, 6, and 35.3 to possibly contain protein P54 from *Ent. faecium*; isolates #60.3 and 128.1, enterocin P from *Ent. faecium* and isolates #16.3 and 112.4 to be likely to contain both protein P54 and enterocin P (Table 3.6). The proteins identified from each sample had a Mascot score above 24, which is considered to be significant. They also had sequence coverage above 5%, which represents a good coverage. However, the mass spectrometry result was not absolute. This was because the method used to prepare the samples, gel trypsin digestion, does not employ the pure fraction of the mature peptide. For this reason, the putatively identified proteins needed to be confirmed further.

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3.4.6. Identification of the enterocin genes and the encoded proteins

The presence of enterocin P in all the isolates was confirmed by performing PCR amplification of the enterocin gene. The PCR amplifications of the genomic DNA from eight *Ent*. *faecium* isolates revealed that all eight strains possessed the enterocin P gene of ~ 220 bp in size (Figure 3.5). The database search for sequence similarity revealed a 100% identity to the enterocin P gene from *Ent*. *faecium* (ascession no. AF005726.1). The *in silico* translated gene sequences, using the ExPASy tool and alignment with the enterocin P peptide from NCBI and BACTIBASE showed a 100% identity (Figure 3.6). Protein P54 was not investigated further.

Table 3.6. Mass spectrometric identification of proteins identified from the *Ent. faecium* isolates.

Isolate No.	Accession	Description	Score	Coverage	Unique peptides	MW (kDa)	calc. pI
#3	P13692	*Ent. faecium* (Protein P54)	126.57	9.11	3	54.6	4.88
#6	P13692	*Ent. faecium* (Protein P54)	99.59	8.91	2	54.6	4.88
#16.3	P13692	*Ent. faecium* (Protein P54)	93.86	6.98	3	54.6	4.88
	O30434	*Ent. faecium* (Enterocin-P)	47.97	28.17	1	7.5	9.35
#35.3	P13692	*Ent. faecium* (Protein P54)	119.64	6.98	3	54.6	4.88
#60.3	O30434	*Ent. faecium* (Enterocin-P)	82.32	28.17	1	7.5	9.35
#112.4	O30434	*Ent. faecium* (Enterocin-P)	68.21	28.17	1	7.5	9.35
	P13692	*Ent. faecium* (Protein P54)	53.71	6.2	2	54.6	4.88
#128.1	O30434	*Ent. faecium* (Enterocin-P)	92.13	45.07	2	7.5	9.35

Ascession: The protein nr as found for the specific database; **Name**: Protein name as assigned by database; **Score**: Mascot score, anything above 24 is usually considered significant; **Coverage**: The % sequence coverage of the protein as detected by the MS. A sequence coverage of 5% is considered good; **Unique peptides**: Nr of unique peptides detected for the specific protein; **MW**: Molecular weight of the protein as given in database; **pI**: pI of protein as given in database.

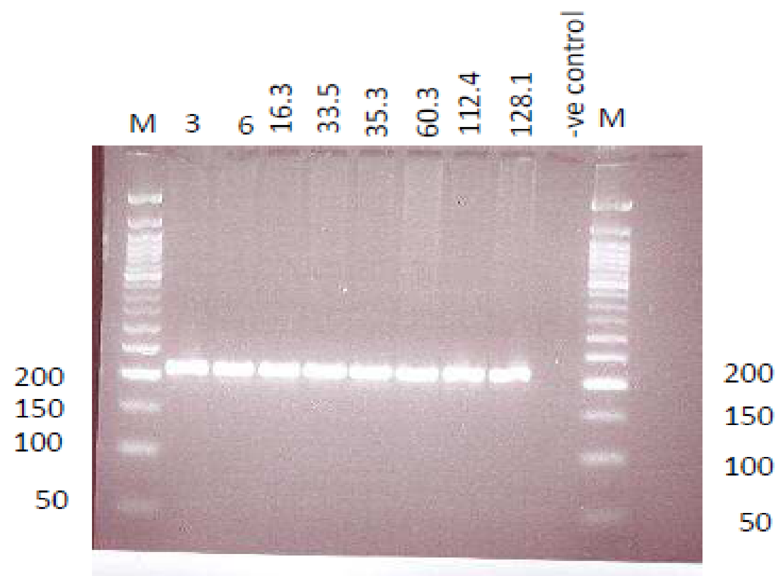

Figure 3.5. PCR amplification of the enterocin P gene in *Ent. faecium* isolates (#3 – 128.1). M: is a 50 base pair molecular marker; -ve control: is a negative PCR control.

1. ENTP	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
2. 3	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
3. 6	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
4. 16.3	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
5. 33.5	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
6. 35.3	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
7. 60.3	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
8. 128.1	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH
9. 112.4	100.0%	MRKKLFSLALIGIFGLVVTNFGTKVDAATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH

Figure 3.6. Multiple sequence alignments (BACTIBASE: ClustalW) of the EntP peptide from the online database and the *Ent. faecium* isolates from this study. 1: EntP from online database, 2 – 9: EntP from isolates in numerical order.

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3.4.7. Molecular weight of peptides

The peptide size of (#3, 6, 16.3, 33.5, 35.3, 60.3, 112.4 and 128.1) was determined by Tricine SDS-PAGE to be below 4.6 kDa (Figure 3.7. A). The overlaid gel confirmed the peptide size as evidenced by the inhibition zones that formed against the sensitive organism, *Lb. pentosus* DSM 20314 (Figure 3.7. B).

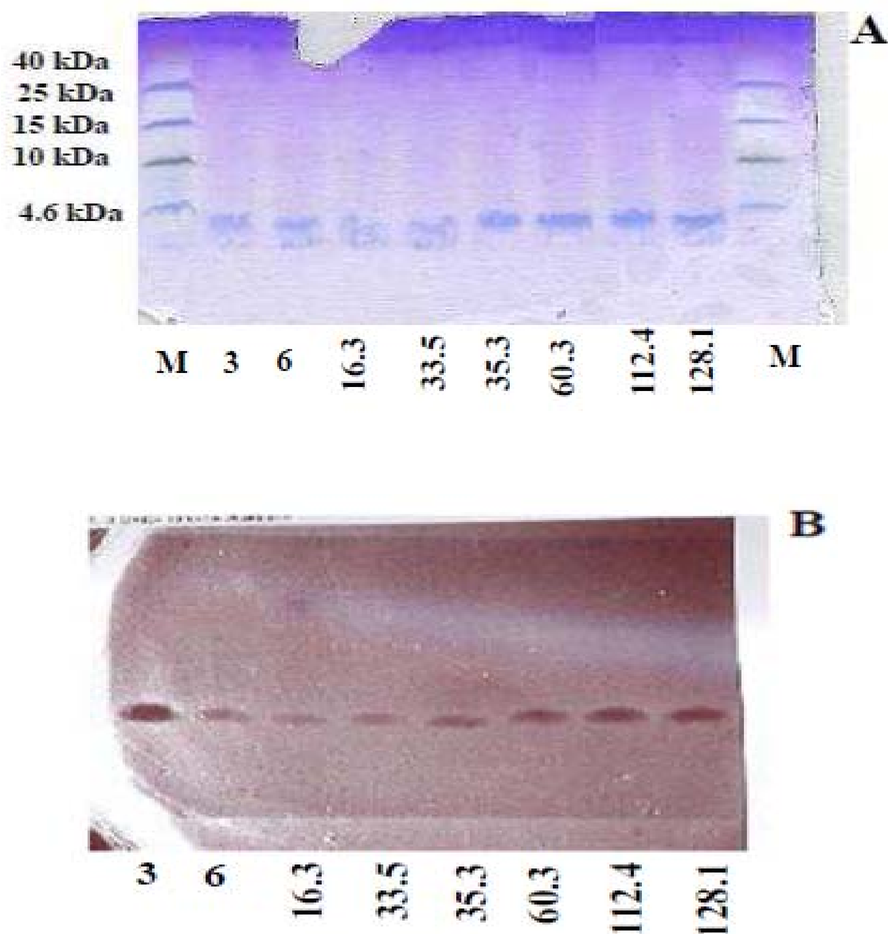

Figure 3.7. Tricine SDS-PAGE gel of the partially purified fractions of all eight *Ent. faecium* strains (A) and a gel overlaid with an MRS soft agar seeded with *Lb. pentosus* DSM 20314 (B). M: Spectra Multicolor Low Range Protein Ladder.

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3.4.8. Bacteriocin characterization

3.4.8.1 Effect of proteolytic enzymes on enterocin activity

The partially purified enterocin fractions from all eight samples were treated with several enzymes and their activity tested against *Lb. pentosus* DSM 20314. Lysozyme, lyticase, catalase and lipase could not inactivate enterocin samples while α-chymotrypsin, papain and proteinase K completely inhibited activity of all samples (Table 3.7). All untreated (control) enterocin samples demonstrated activity against *Lb. pentosus* DSM 20314.

Table 3.7. Effect of enzyme treatments on enterocin activity against *Lb. pentosus* DSM 20314.

	# 3	**6**	**16.3**	**33.5**	**35.3**	**60.3**	**112.4**	**128.1**
Control	+	+	+	+	+	+	+	+
Lysozyme	+	+	+	+	+	+	+	+
Lyticase	+	+	+	+	+	+	+	+
α-Chymotrypsin	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Papain	-	-	-	-	-	-	-	-
Lipase	+	+	+	+	+	+	+	+
Proteinase K	-	-	-	-	-	-	-	-

+, no inhibition; - , inhibition

3.4.8.2 Effect of heat treatments and pH

The partially purified enterocins demonstrated a fair resistance towards heat (Table 3.8.). At a treatment of 95°C for 30 minutes, all bacteriocin samples were still showing the same activity (6400 AU/ml) as in the control (untreated). At 121°C (15 min), the activity dropped to 800 AU/ml. However, at a treatment of 37°C for 3 hours, samples from #16.3, 60.3, 112.4 and 128.1 dropped to 3200 AU/ml. At a 60°C treatment for 30 minutes, #60.3, 112.4 and 128.1 samples dropped to 3200 AU/ml, 1600 AU/ml and 3200 AU/ml, respectively, and at a treatment of 80 for 30 minutes, samples #112.4 and 128.1 dropped to 3200 AU/ml.

Table 3.8. Effect of heat treatments on enterocin activity against *Lb. pentosus* DSM 20314

	Activity Units (AU/ml)*						
Heat Treatment	**3**	**6**	**16.3**	**35.3**	**60.3**	**112.4**	**128.1**
Control	6400	6400	6400	6400	6400	6400	6400
37 °C (3 hrs)	6400	6400	3200	6400	3200	3200	3200
60 °C (30 min)	6400	6400	6400	6400	3200	1600	3200
80 °C (30 min)	6400	6400	6400	6400	6400	3200	3200
95 °C (30 min)	6400	6400	6400	6400	6400	6400	6400
121 °C (15 min)	800	800	800	800	800	800	800

***Sample 1 (37 °C) partially purified enterocin extracts with 6400AU/ml were used for heat treatments.**

#33.5 was not included due to loss of activity.

Enterocin samples from all eight strains showed high tolerance to pH treatments (pH 2-9), with the AU/ml of 6400 and 3200 throughout the treatments (Table 3.9). The activity of the enterocins was significantly reduced at pH 10 except for # 33.5 which maintained the original AU/ml through out the treatments. #3 and #6 were reduced to 800 AU/ml, #16.3 and #128.1, reduced to 1600 AU/ml and #35.3, 60.3 and #112.4 only reduced to 3200 AU/ml.

Table 3.9. Effect of pH on enterocins activity against *Lb. pentosus* DSM 20314

	AU/ml*							
pH	**3**	**6**	**16.3**	**33.5**	**35.3**	**60.3**	**112.4**	**128.1**
2	3200	3200	6400	6400	6400	6400	6400	6400
3	6400	3200	6400	6400	6400	6400	6400	6400
4	6400	3200	6400	6400	6400	6400	6400	6400
5	6400	6400	6400	6400	6400	6400	6400	6400
6	3200	6400	6400	6400	6400	6400	6400	6400
7	3200	6400	6400	6400	6400	6400	3200	6400
8	3200	3200	6400	6400	6400	6400	3200	6400
9	3200	3200	6400	6400	3200	6400	3200	3200
10	800	800	1600	6400	3200	3200	3200	3200

*** Partially purified enterocin extracts (6400 AU/ml) were used for pH treatments.**

3.4.9. Enterocin production kinetics and mode of action

For production kinetics and mode of action assays, only #16.3 and 128.1 *Ent. faecium* strains were used. This was because all strains were identical and they were characterized to be the same according to enzymatic, heat and pH treatments.

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3.4.9.1. Production kinetics

Growth of *Ent. faecium* strains, #16.3 and 128.1 reached the mid exponential phase after six hours of incubation with the cell counts of log 8.55/ml and log 8.43/ml, respectively (Figure 3.8). After incubating for 12 hours, the cell growth was at the late exponential phase with the cell counts of log 8.9/ml and 9.63/ml, respectively. This was followed by a long stationery phase which lasted to 72 hours. Bacteriocin production started after 6 hours of incubation at 200 AU/ml. From 8 to 54 hours, the production exponentially increased to 400 AU/ml. After that it diminished to 200 AU/ml at 60, 64 and 72 hours. This was observed for both #16.3 and 128.1.

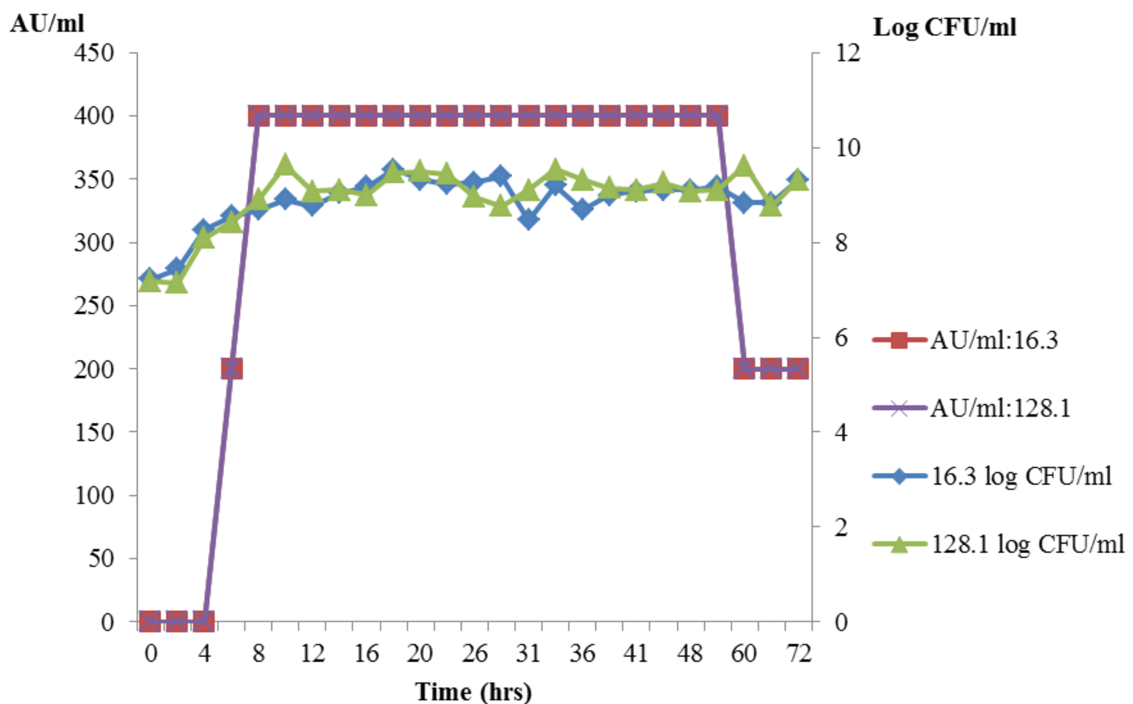

Figure 3.8. Growth of *Ent. faecium* strains #16.3 and 128.1 over time together with the production of enterocins and their antimicrobial activity in AU/ml against *Lb. pentosus* DSM 20314.

3.4.9.2. Mode of action

Figure 3.9 shows the results of the *Lb. pentosus* DSM 20314 cell counts (in log CFU/ml) on MRS plates over a period of 13 hours after the addition of partially purified enterocin extracts. Both the control and the flasks co-inoculated with partially purified enterocin from #16.3 and 128.1 had the same cell numbers at time zero (log 6.2, 6.35 and 6.36 CFU/ml, respectively). The control flask cell counts increased exponentially over the period of 13 hours to reach log 9.39 CFU/ml; whereas cell counts from the other two flasks decreased gradually and reached zero after 3 hours for 16.3 and 5 hours for 128.1.

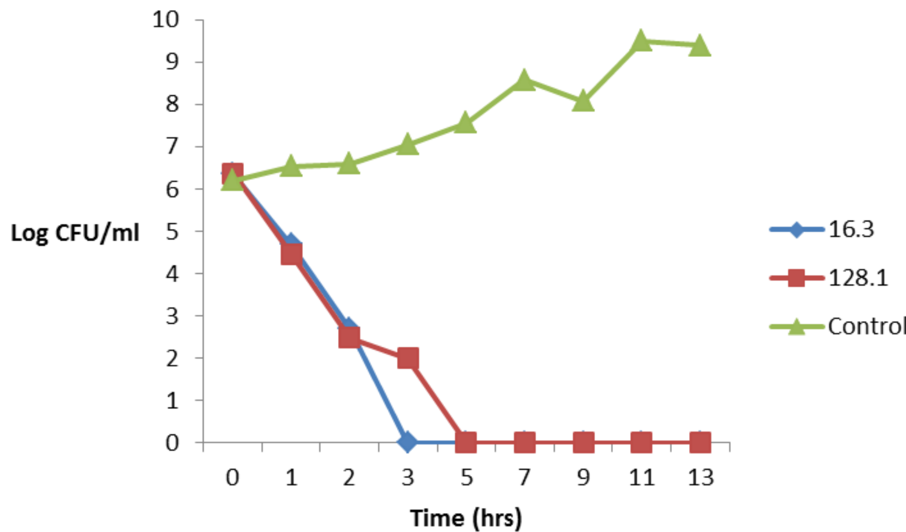

Figure 3.9. Bactericidal effect of enterocin produced by #16.3 and 128.1 on *Lb. pentosus* DSM 20314 over time.

3.5. Discussion

Enterococcus occurs in various environments but is most common in the intestines of humans and other animals (Khan et al., 2010; Javed et al., 2011). Like other LAB that are natural microbiota of the human digestive system (Ogier and Serror, 2008), *Enterococcus* have many benefits (Khan et al., 2010). This genus does however not have GRAS status (Ogier and Serror, 2008) and is commonly associated with pathogenicity to humans and other animals (Franz et al., 1999a; Eaton & Gasson, 2001; Franz et al., 2001; Khan et al., 2010). Some enterococci are significant to public health, medical microbiology and various foods (Franz et al., 2007) such as milk (Giraffa et al., 1995; Achemchem et al., 2006; Muñoz et al., 2007), cheese (Giraffa and Carminati, 1997; Muñoz et al., 2004; Liu et al., 2008; Izquierdo et al., 2009), sausages (Callewaert et al., 2000; Sabia et al., 2003) and meat (Aymerich et al., 2002; Khan et al., 2010). Some have suggested that strains isolated from traditional fermented products could be regarded as safe for use as starter cultures for fermented products, provided they are non-hemolytic and sensitive to antibiotics (Fields, 1996; Holzapfel et al., 1995; De Vuyst et al., 2003; Khan et al., 2010). *Enterococcus* also produce enterocins that belong to classes I, II, III and IV of bacteriocins (Franz et al., 2007). Enterocins have a broad spectrum of antimicrobial activity against food-borne pathogens such as other enterococci, *List. monocytogenes*, *S. aureus* and *Clostridium* spp. (Cintas et al., 1997; Franz et al., 1999; Wijaya, 2003; Gutie´rrez et al., 2005; Franz et al., 2007; Aguilar-Galvez et al., 2011).

The present study screened 155 LAB strains from IWBT collection for bacteriocin production. Eight strains (5%) were identified to be producers, as evidenced by strong inhibition zones formed against a few sensitive organisms on agar plates. The producers demonstrated a broad spectrum of antimicrobial activity by inhibiting different *Lactobacillus* spp., *Leuc. mesenteroides*, *List. monocytogenes* and *Ped. pentosaceus*. These bacteriocin-producing strains may be of oenological importance since *Lactobacillus*, *Leuconostoc* and *Pediococcus* genera are found in the winemaking environment such as winery equipment, grape juice and

also in the finished wine (Ruiz-Larrea, 2010). These three bacterial genera are important since they are known to be potential wine spoilage bacteria. Hence these isolated strains and/or the bacteriocins they produce could find application in the food fermentation industry.

The physiological results, biochemical tests and sugar fermentation profiles all gave the same results for the strains, which were indicative of enterococci. The isolates identification was confirmed by 16S rRNA gene sequencing showing that all isolates were *Ent. faecium*. RAPD-PCR fingerprinting gave the same profiles for all strains and this confirmed that the strains are all identical on genetic level, which could be that the same strain was isolated at different occasions from the same farm and tank monitored. It is difficult to distinguish enterococci from other Gram-positive, catalase-negative, ovoid coccus-shaped bacteria using phenotypic characteristics (Devriese et al., 1993; Wijaya, 2003). Characteristics like group D antigen, capability to grow in 10°C, 45°C, in 6.5% NaCl at pH 9.6 and heat stability at 60°C for 30 minutes can distinguish most *Enterococcus* species from other coccus-shaped bacteria (Hardie and Whiley, 1997). But it is necessary to complement phenotypic characteristics with genotypic methods like DNA:DNA hybridisation or 16S rDNA sequencing in order to distinguish *Enterococcus* species from other coccus-shaped bacteria (Wijaya, 2003).

The AU/ml of the partially purified enterocins was the same among all *Ent. faecium* isolates. This further confirmed that the isolates were all the same as semi-purified samples prepared on different dates consistently gave similar AU/ml when tested. Optimal growth temperature of the producer strain may not be the optimal temperature for bacteriocin production. This is because bacteriocin production normally occurs under strictly controlled temperature and pH conditions. The optimal temperature for their production is normally lower than the optimal temperature of the producer strain (Herranz et al., 2001; Todorov et al., 2004; Bauer et al., 2001). In this study, enterocin production was lower at 37°C compared to 30°C and correlates with the production of *Ent. faecium* P13 which produced the highest enterocin P levels at pH 6.2 and 6 at 32°C (Herranz et al., 2001). *Lb. plantarum* could not produce any plantaricin at 37°C, but at 30°C. The production was highest at pH 6, at 30°C and lowest at pH 4.5 (Todorov et al., 2004). Hence, among other factors, the production and the activity of bacteriocins could be determined by the growth conditions of the producer cultures. The optimal growth temperature for maximal bacteriocin production becomes more crucial if a particular culture is used as a bacteriocinogenic culture.

Determining the molecular weight using SDS-PAGE showed that the peptides were smaller than 4.6 kDa. This result confirmed the molecular weight of enterocin P as determined in other studies using mass spectrometry (Cintas et al., 1997) and tricine SDS-PAGE (Park et al., 2003), to be 4.5 kDa. One study reported enterocin P to be 4.7 kDa (Mareková et al., 2007). Mass spectrometric results in this study showed that the closest bacteriocin was enterocin P from the species *Ent. faecium* and the predicted size was 7.5 kDa. No substantial conclusions could be made based on the mass spectrometry results alone since the method used, gel trypsin digestion, does not use the pure fraction of the mature peptide, but only the portion of the gel that showed an inhibition zone against a sensitive organism, *Lb. pentosus* DSM 20314. However, PCR

amplification of the enterocin P gene, sequencing reactions and BLAST search confirmed that all eight strains contained the enterocin P gene from *Ent. faecium*. No PCR was performed in order to confirm the potential presence of protein P54 as suggested by the mass spectrometric results. This was because protein P54 is not a bacteriocin and it has no record of antimicrobial activity. This protein was described as having a highly unusual sequence and is expressed in *E. coli* and *Ent. faecium* cell wall (Fürst et al., 1989).

Enterocin P is produced by *Ent. faecium* strains of different origins (Herranz et al., 1999; Cintas et al., 2000; Moreno et al., 2002; De Vuyst et al., 2003; Foulquié Moreno et al., 2003; Marekowá et al., 2003; Guti´errez et al., 2005). It is produced as a pre-peptide consisting of 71 amino acids and a signal peptide of 27 amino acids which produce a mature peptide of 44 amino acids after cleavage (Cintas et al., 1997; Gutiérrez et al., 2005a,b). The peptide is processed and secreted by a *sec*-dependent pathway and hence classified as a class IIc bacteriocin (Cintas et al., 1997; Gutiérrez et al., 2005a). This enterocin is active against both spoilage and pathogenic microorganisms (Herranz et al., 2001). The enterocin P produced by the *Ent. faecium* strains of this study were no exception since they showed strong antimicrobial activity against food and wine spoilage organisms.

Enterocin P tested in this study was heat stable as it remained active at 95°C (30 min), but lost 50% of its activity at 121°C (15 min). Enterocins like enterocin A produced by *Ent. faecium* MTCC 5153 (Badarinath and Halami, 2011) and other enterocins from *Ent. faecium* strains (Flariano et al., 1998; Park et al., 2003; Alvarado et al., 2005) retained activity at 100°C, but lost it after autoclave treatment at 121°C. However, enterocins from strains such as *Ent. faecium* 900 (Franz et al., 1996), *Ent. faecium* EF 9296 (Marciňáková et al., 2005) and *Ent. faecium* LR/6 (Kumar et al., 2010) were not inhibited by autoclaving at 121°C for 15 min. Enterocins from *Ent. faecium* strains isolated from rabbit faeces withstood heat treatments up to 100°C for 10 minutes (Simonová and Lauková, 2007). Bacteriocin production and heat resistance are among many factors that enable enterococci to be dominant in fermented products such as dairy foods or meat (Franz et al., 2007). Therefore enterococci producing bacteriocins have potential applications in various foods and fermented products (Aymerich et al., 2000; Callewaert et al., 2000; Giraffa, 2003; Hugas et al., 2003; Franz et al., 2007).

The pH tests showed that enterocin P was active over a broad pH range (2-10). The same phenomenon was observed with other bacteriocins produced by different *Ent. faecium* strains (Franz et al., 1996; Du Toit et al., 2000; Park et al., 2003; Marciňáková et al., 2005; Simonová and Lauková, 2007; Kumar et al., 2010). Enterocin samples showed sensitivity to pH 10 as compared to the lower pH treatments. This was evidenced by a significant reduction in activity observed at pH 10. Enterocin activity over a wide pH range make them potential natural preservatives for foods and fermented products where products are acidified or pH levels decreases due to natural LAB present (Franz et al., 1996). They also have potential application in oenological processes where pH levels are as low as 3.

Proteolytic enzyme treatments with lysozyme and lipase could not inhibit enterocin activity. This indicated that their antimicrobial activity was independent of a lipid or carbohydrate moiety (Franz et al., 1996; Park et al., 2003). This result agreed with what was previously reported on lipase-treated enterocins

(Franz et al., 1996; Du Toit et al., 2000; Park et al., 2003; Kumar et al., 2010). α-Chymotrypsin and proteinase K inactivated enterocin P, which indicated that the compound was proteinaceous in nature and activity was independent of organic acids. Similar results were reported on other enterocin studies (Franz et al., 1996; Flariano et al., 1998; Du Toit et al., 2000; Marciňáková et al., 2005; Kumar et al., 2010; Badarinath and Halami, 2011). However, proteinase K could not inactivate a bacteriocin-like substance produced by *Ent. faecium* UQ31 (Alvarado et al., 2005). Papain also inhibited enterocin activity against a sensitive indicator organism. This result is similar to that reported by Du Toit et al. (2000) and Kumar et al. (2010) but different to that of Franz et al. (1996). Lyticase and catalase could not inhibit enterocin activity. This mean that the antimicrobial activity was not related to hydrogen peroxide.

Bacteriocin production by #16.3 and 128.1 strains coincided with the exponential growth phase which occurred after 6 hours of incubation at 30°C. This trend was reported in other studies on enterocin production at logarithmic growth phase (Franz et al., 1996; Ferreira et al., 2007; Simonová and Lauková, 2007; Kumar et al., 2010), which is an indication of primary metabolite kinetics according to the authors. The highest production of 400 AU/ml was observed after 8 hours and was maintained for several hours (46 hours) of stationery phase. This trend was also reported in other enterocin studies (Franz et al., 1996; Ferreirra et al., 2007; Kumar et al., 2010). The gradual decrease in activity that followed after 54 hours of incubation could be linked to the degradation caused by the proteolytic enzymes accumulating in the medium, or to a drop in culture pH (Torri Tarelli et al., 1994). Re-adsorption of bacteriocins to the cell surfaces at low culture pH could also be a contributing factor to the decrease in bacteriocin production and activity (Franz et al., 1996).

The bactericidal effect of the #16.3 and 128.1 partially pure CFS against the sensitive culture of *Lb. pentosus* DSM 20314 was clearly demonstrated by complete inhibition of growth for most of the experimental period, while the control cell counts increased exponentially throughout the experiment. This confirmed that the partially pure CFS are proteinaceous in nature and have the same bactericidal effect as demonstrated by other enterocins from *Enterococus* strains of various origins (Franz et al., 1996; Du Toit et al., 2000; Kumar et al., 2010).

The current study screened 155 bacterial isolates from the South African red wines undergoing malolactic fermentation. Eight isolates of an *Ent. faecium* strain were identified to be bacteriocin producers and their bacteriocin was identified and characterised to some extent. To our knowledge, this is one of the few studies that isolated *Enterococcus* species from wine (Capozzi et al., 2011; Kačániová et al., 2012). In their study, Capozzi et al. (2011) could not identify any *Enterococcus* from the grapes used in the wineries but isolated an *Ent. faecium* strain in the wines undergoing malolactic fermentation. The authors suggested that contamination by *Ent. faecium* could be associated with the winery equipment or practices, and/or the water used to clean fermentation tanks.

However, enterocins have potential applications in wine preservation against spoilage organisms. This is due to their activity at a broad pH range which could enable tolerance at low pH conditions in a wine environment. They also showed broad spectrum of antimicrobial activity against food pathogenic bacteria

and spoilage bacteria found in food and wine. Efficacy of bacteriocins in complex food systems is affected by numerous factors which include both chemical composition and physical conditions (Cleveland et al., 2001). Hence, some bacteriocins may show antimicrobial activity in laboratory broth media, but not when tested in complex food environments. It then becomes crucial to test the promising bacteriocins under both laboratory conditions and in real food conditions where they will interact with many influencing factors. In case of the enterocin P from this study, testing it under small scale winemaking conditions should be a necessary test for its effectiveness against spoilage bacteria.

3.6. Conclusion

This study has identified an *Ent. faecium* strain, usually not associated with wine, to produce enterocins. The partially characterised enterocins could further be evaluated for potential applications in wine preservation against spoilage bacteria. This could be achieved by testing purified or semi-purified enterocins individually and as part of hurdle technology where more than one preservation strategies are employed in order to maximize the inhibition of spoilage microrganisms. The producer strain could also be tested as starter or co-culture in vinification. Given the few reports on the production of bacteriocins by oenological bacteria and on the presence of bacteriocins in finished wines, further research following this preliminary study could lead to better understanding of wine *Enterococcus* strains and their potential in wine preservation.

3.7. Acknowledgments

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CHAPTER 4

General Discussion & Conclusions

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CHAPTER 4

4.1 General discussion

Over the past decades, bacteriocins have become a subject of research interest due to the rising demand to reduce chemical preservatives, consumer bias towards these preservatives and subsequent demand for less processed foods and legislative issues (Du Toit and Pretorius, 2000; Cleveland et al., 2001; Enrique et al., 2007). These proteinaceous substances are of significant importance in the food industry, since they prevent the growth of many food spoilage and pathogenic bacteria, including: *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum* (Hernandez et al., 2005; Ruiz-Larrea et al., 2007). Many bacteriocins have been identified and characterised already (Hernández et al., 2005), but there are few reports on bacteriocins produced by LAB of oenological origins or their presence in the finished wines (Navarro et al., 2000; Rojo-Bezares et al., 2007a; Saenz et al., 2009).

The present study has evaluated wine-isolated bacterial strains for bacteriocin production. The main aim was to identify lactic acid bacteria (LAB) isolates other than the previously identified *Lactobacillus plantarum* strain (isolated at the IWBT) that produce bacteriocins. This was achieved by screening several wine LAB isolates for bacteriocin production, identifying the producers using both genotypic and phenotypic methods and characterising the bacteriocins they produce using both biochemical and physiological characteristics.

This involved the screening of 155 LAB isolates from the IWBT culture collection previously isolated from South African red wines undergoing spontaneous malolactic fermentation. Only five percent (5%) of the screened isolates were identified as presumptive bacteriocin producers as evidenced by clear zones of inhibition against a few selected sensitive organisms. From a wine environment, Morgan et al. (2003) screened 170 LAB wine isolates from Pinotage, Merlot and Cabernet Sauvignon wines cultivated in the Western Cape region. From these isolates 15% were bacteriocin producers. Two of the producers that showed stable bacteriocin production and activity were identified to be *Lactobacillus paracasei* and *Lb. brevis*, respectively. Knoll et al. (2008a) screened 330 wine LAB isolates from South African red wines undergoing alcoholic and malolactic fermentations and only 8% were identified as producers. They belonged to *Lb. plantarum*, *Lb. paracasei*, *Lb. hilgardii* and *O. oeni*.

Bacteriocin-producing isolates were identified to a species level using both phenotypic and genotypic methods. The physiological tests, biochemical and sugar fermentation tests putatively identified all seven isolates to be enterococci. The seven isolates tested (#3, 6, 16.3, 35.3, 60.3, 112.4 and 128.1) were further identified through PCR amplification and sequencing analysis of the 16S rRNA gene to be *Ent. faecium*. RAPD-PCR confirmed the similarity of these isolates to each other as they all gave the same gene profiles. The combination of both genotypic and phenotypic methods to identify bacterial strains, particularly Gram-positive cocci, is essential as phenotypic methods on alone may not be sufficient for species

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identification (Wijaya, 2003). Hence complementing phenotypic methods with better distinguishing genotypic methods gave more confidence to the result.

This is not the first study to report on the presence of *Enterococcus* in wine. Capozzi et al. (2011) isolated *Ent. faecium* from red wines undergoing malolactic fermentation. To our best knowledge, this study is therefore the second report on the isolation of *Enterococcus* from wine. This implies that wine is not a common habitat for enterococci and that it cannot be excluded that its presence in the wine could possibly be due to contamination during the winemaking process from e.g. human handling or contaminated water.

Peptides size was determined by tricine-SDS-PAGE to be 4.5 kDa. This result is in agreement with previous studies which also determined enterocin P to be 4.5 kDa in size (Cintas et al., 1997; Park et al., 2003). However, some studies have reported enterocin P to be 4.7 kDa (Mareková et al., 2007). Park et al. (2003) mentioned that the molecular weight of the enterocins (A, B and P) produced by *Ent. faecium* JCM 5804^T in their study was an approximation since size determination by SDS-PAGE was not accurate. The inhibition zone formed on the gel overlaid with a sensitive culture was between 3496 Da and 6500 Da. Crude bacteriocin extracts used for SDS-PAGE assay in their study could possibly be another reason to explain the inaccuracy of the molecular weight of enterocins. The current study determined the molecular weight of the partially pure peptides after isolation with ammonium sulphate precipitation, dialysis and freeze drying. Further purifying the peptides before determining the molecular weight could possible increase the accuracy of the molecular size obtained. Different purification methods that have been reported previously include hydrophobic or electrostatic interactions (Stoffels et al., 1993), acid extraction and RP-HPLC (Daba et al., 1994), ammonium sulphate precipitation and reversed-phase chromatography (Joosten et al., 1996), pH-mediated cell adsorption-desorption and semi-preparative RP-HPLC (Elegado et al., 1997) and ethanol precipitation, preparative isoelectric focusing and ultrafiltration (Venema et al., 1997).

The enterocins produced by the *Enterococcus* strains from this study demonstrated a proteinacious nature, a high degree of heat tolerance and activity over a wide pH range. These biochemical characteristics are similar to what has been previously reported by other authors on different enterocins studied (Franz et al., 1996; Du Toit et al., 2000; Park et al., 2003; Marciňáková et al., 2005; Badarinath and Halami, 2011). The result from these tests allows these peptides potential application to the food fermentation and wine industry where characteristics such as heat stability and pH stability are a requirement (Daeschel et al., 1991; Callewaert et al., 2000; Franz et al., 2007).

Bacteriocin production kinetics and mode of action are important features of any bacteriocin. This study has determined the production kinetics of enterocins over a period of 72 hours where bacteriocin production began at the mid exponential phase and was maintained for most of the stationery phase before it declined. The growth and production trend observed in this study has been reported on other enterocin-producing *Enterococcus* (Franz et al., 1996; Du Toit et al., 2000; Ferreira et al., 2007) strains as well. Production kinetics determines whether bacteriocins are a product of the primary or secondary metabolites of the bacterial cells. Enterocin production by the *Enterococcus* strains from this study followed the path of primary metabolite kinetics. The enterocins also demonstrated a bactericidal (instead of a bacteriostatic)

nature against a sensitive organism. These result was not different from what was observed with other enterocins previously studied (Franz et al., 1996; Du Toit et al., 2000).

4.2. Future Prospectives

Direct screening of microbes for antimicrobial peptides through culturing methods is not optimal since a vast majority of non-culturable microbes with great potential could be missed. For example, hundreds of LAB in this study could not be screened, because they could not grow in the liquid media. Growth conditions might not have been optimal for this isolates to grow. Only a low percentage grew and ninenty-five percent (95%) of those were screened against sensitive organisms to be non-producers. However, this might not be the case because putative sensitive organisms were used. It could have been that some isolates belonged to the same species as putative sensitive organisms and hence could not demonstrate any activity against them. It could also be that the growth conditions were not optimal for bacteriocins production since it requires specific growth conditions. All these factors put together mean that the screening method used in this study was not the best high-throughput method to screen for potential bacteriocin producers. Hence future screenings should also employ high-throughput methods such as metagenomics and bioinformatics as they are more accurate and also increase the scope of screening.

The preliminary work done on this study could be extended for a broader understanding of the identified *Ent. faecium* strain isolates and the enterocin they produce. This could be done by testing the isolates in wine synthetic media or small scale winemaking to determine antimicrobial activity against spoilage bacteria in a complex wine medium. Some bacteria strains and/or their bacteriocins have antimicrobial activity against selected indicator organisms on the nutrient rich media such as laboratory broth. However, in a complex environment such as wine, their activity can be inhibited due to a number of influencing factors (Abee et al., 1995; Cleveland et al., 2001). Knoll et al. (2008b) investigated the production of bacteriocins in laboratory MRS medium, synthetic wine medium and in wine must. In their study, phenolic compounds and polyphenols had no negative effects on the stability and activity of nisin and pediocin PA-1. However, previous studies had reported the decrease of bacteriocins activity as a result of these compounds in red wines (Daeschel et al., 1991; Bower et al., 1992).

Future research could also focus on screening for enterocin structural genes from the producer strains with PCR. The expression of these genes could be investigated under winemaking conditions to determine how vinification stages affect expression levels. In future, if expression levels are not affected negatively, the genes could be expressed in suitable commercial malolactic fermentation (MLF) starter cultures. This could possibly confer antibacterial activity to MLF strains and enable them to perform dual purposes of being starter cultures for secondary fermentation as well as inhibiting the growth of wine spoilage bacteria. Miller (2010) investigated the expression of plantaricin genes under winemaking conditions and found that these particular *Lb. plantarum* strains had the potential to produce plantaricin in wine. Knoll et al. (2008a) heterologously expressed *nisA* in *Lb. plantarum* strains in order to enhance their

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antimicrobial activity. Under certain conditions, the transformed strains showed improved levels of antimicrobial activity when compared to the parental strains.

4.3 Concludig Remarks

This study is among the few that have been done to identify and characterise bacteriocins produced by LAB of oenological origin. To our knowledge, very few studies have identified the presence of *Enterococcus* in wines undergoing malolactic fermentation. However, it is the first to record the production of bacteriocins by wine isolated *Enterococcus*. Thus there may be a need for future studies to screen for *Enterococcus* strains in wine environment and to evaluate them for bacteriocin production. Enterocins could also be evaluated for the potential application in wine preservation as part of hurdle technology.

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