#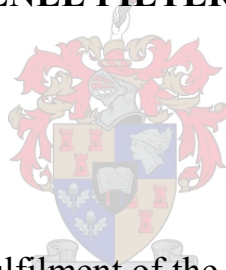 Control of bacterial pathogens associated with mastitis in dairy cows with natural antimicrobial peptides produced by lactic acid bacteria

by

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Thesis completed in partial fulfilment of the requirements for the degree of

Master of Science in Microbiology at the University of Stellenbosch

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March 2008

DECLARATION

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

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  Reneé Pieterse   Date

SUMMARY

Mastitis is considered to be the most costly disease affecting the dairy industry. Management strategies involve the extensive use of antibiotics to treat and prevent this disease. Prophylactic dosages of antibiotics used in mastitis control programmes could select for strains with resistance to antibiotics. In addition, a strong drive towards reducing antibiotic residues in animal food products has lead to research in finding alternative antimicrobial agents.

Streptococcus macedonicus ST91KM, isolated from bulgarian goat yoghurt, produces the bacteriocin macedocin ST91KM with a narrow spectrum of activity against Gram-positive bacteria. These include mastitis pathogens *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis* as well as *Lactobacillus sakei* and *Micrococcus varians*. Macedocin ST91KM is, according to tricine-SDS PAGE, between 2.0 and 2.5 kDa in size. The activity of macedocin ST91KM remained unchanged after 2 h of incubation at pH 2.0 to 10.0 and 100 min at 100 °C. The peptide was inactivated after 20 min at 121 °C and when treated with pronase, pepsin and trypsin. Treatment with α-amylase had no effect on activity, suggesting that the mode of action does not depend on glycosylation. Precipitation with 60 % saturated ammonium sulphate, followed by Sep-Pak C18 separation recovered 43 % of macedocin ST91KM. Amplification of the genome of strain ST91KM with primers designed from the sequence of the macedocin prescursor gene (*mcdA*) produced two fragments (approximately 375 and 220 bp) instead of one fragment of 150 bp recorded for macedocin produced by *S. macedonicus* ACA-DC 198. Strain ACA-DC 198 was not available. However, the DNA fragment amplified from strain LMG 18488 (ACA-DC 206), genetically closely related to strain ACA-DC 198, revealed 99 % homology to the *mcdA* of *S. macedonicus* ACA-DC 198 (accession

number DQ835394). Macedocin ST91KM may thus be a related bacteriocin described for *S. macedonicus*.

The peptide adsorbed equally well (66 %) to *L. sakei* LMG13558 and insensitive cells, e.g. *Enterococcus faecalis* BFE 1071 and FAIR E92, and *Streptococcus caprinus* ATCC 700066. Optimal adsorption of macedocin ST91KM was recorded at 37 °C and 45 °C and at pH of 8 - 10. Addition of solvents decreased adsorption by 50%, suggesting that the receptors to which the bacteriocin binds have lipid moieties. The addition of $MgCl_2$, KI and Na_2CO_3 completely prevented adsorption of macedocin ST91KM to the target cells, possibly due to competitive ion adsorption on the bacterial cell surface. The peptide has a bacteriocidal mode of action, resulting in lysis and the release of DNA and β-galactosidase. Atomic force microscopy of sensitive cells treated with macedocin ST91KM have shown deformation of the cell structure and developing of irregular surface areas.

Antimicrobial susceptibility patterns were evaluated against eighteen mastitis pathogens. All isolates tested were resistant to methicillin and oxacillin, but had minimum inhibitory concentrations (MICs) falling in the intermediate and susceptible range against erythromycin. *S. agalactiae* and *S. epidermidis* had the highest sensitivity to macedocin ST91KM. A teat seal preparation containing macedocin ST91KM effectively released bacteriocin inhibiting the growth of the bacterial pathogen. Macedocin ST91KM could form the basis for an alternative dry cow therapy to prevent mastitis infections in dairy cows, as it is effective against pathogens that display resistance to conventional antibiotic therapy.

OPSOMMING

Mastitis word beskou as die siekte wat die grootse ekonomiese verliese in die suiwel bedryf veroorsaak. Die beheermaatreëls vir die behandeling en voorkoming van die siekte berus hoofsaaklik op die graatskaalse gebruik van antibiotika. Die voortdurende profilaktiese gebruik van antibiotika in mastitisbeheerprogramme mag aanleiding gee tot die seleksie van stamme van patogene wat weerstandbiedend is teen antibiotika. Tesame hiermee het die vraag na voedselprodukte met laer antibiotiese residue gelei tot uitgebreide navorsing om alternatiewe antimikrobiese middels te vind en te ontwikkel.

Streptococcus macedonicus ST91KM, geïsoleer uit Bulgaarse jogurt, produseer 'n bakteriosien, macedocin ST91KM, met 'n nou spektrum van aktiwiteit teen Gram-positiewe bakterieë. Dit sluit die mastitispatogene *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus* en *Staphylococcus epidermidis*, asook *Lactobacillus sakei* en *Micrococcus varians* in. Volgens trisien-SDS PAGE is macedocin ST91KM tussen 2.0 en 2.5 kDa groot. Die aktiwiteit van macedocin ST91KM het onveranderd gebly na 2 h inkubasie by pH waardes van 2.0 – 10.0 en na 100 min behandeling by 100 °C. Die peptied was geïnaktiveer na 20 min by 121 °C en na behandeling met pronase, pepsien en tripsien. Behandeling met α-amilase het geen effek op die aktiwiteit gehad nie, wat aandui dat die werkswyse nie van glikosilering van die peptied afhanklik is nie. 'n Groot hoeveelheid (43 %) van macedocin ST91KM is herwin na presipitasie met 60 % versadigde ammoniumsulfaat, gevolg deur skeiding met 'n Sep-Pak C18 kolom. Die amplifikasie van die genoom van stam ST91KM met inleiers wat gebaseer was op die leesraam van die voorlopergeen van macedocin (*mcd*A), het twee fragmente geproduseer (ongeveer 375 bp en 220 bp) in plaas van een fragment van 150 bp wat vir macedocin ACA-DC 198 beskryf is. Isolaat ACA-DC 198 was nie beskikbaar nie. DNA fragment ge-

amplifiseer van stam LMG 18488 (ACA-DC 206), wat geneties naverwant is aan stam ACA-DC 198, het egter 99 % homologie met *mcd*A van *S. macedonicus* ACA-DC 198 (verwysing DQ835394) getoon. Macedocin ST91KM kan dus 'n verwante bakteriocin van *S. macedonicus* wees.

Die peptied het ewe sterk aan *L. sakei* LMG13558 en selle onsensitief vir macedocin ST91KM (*Enterococcus faecalis* BFE 1071 en FAIR E92, asook *Streptococcus caprinus* ATCC 700066) geadsorbeer. Optimale adsorpsie van macedocin ST91KM is by pH waardes van tussen 8.0 en 10.0 en temperature van 37 en 45 °C waargeneem. 'n Vyftig persent afname in adsorpsie na byvoeging van oplossmiddels soos etanol, metanol en merkapto-etanol is waargeneem, wat daarop dui dat die reseptore waaraan die bakteriosien bind moontlik lipied-agtig is. Die byvoeging van $MgCl_2$, KI en Na_2CO_3 het tot totale inhibering van adsorpsie aanleiding gegee, moontlik as gevolg van mededingende ioon-adsorpsie op die seloppervlak. Die vernietiging van die teikensel is bevestig deur die uitskeiding van DNA en β-galaktosidase na behandeling met macedocin ST91KM. Atoomkragmikroskopie van sensitiewe selle na behandeling met macedocin ST91KM toon deformasie en onreëlmatighede op die seloppervlak.

Die antimikrobiese sensitiwiteit van agtien mastitispatogene is bestudeer. Al die bakteriese isolate wat getoets is, was weerstandbiedend teen metisillien en oksasillien, terwyl die minimum inhibitoriese konsentrasies (MIK) vir eritromisien binne die gemiddelde tot vatbare meetgebied val. *S. agalactiae* en *S. epidermidis* het die hoogste sensitiwiteit teen macedocin ST91KM getoon. 'n Speenkanaal-seëlpreparaat wat macedocin ST91KM bevat het voldoende hoeveelhede bakteriosien vrygestel om die groei van patogene bakterieë te inhibeer. Macedocin ST91KM kan die basis vorm van 'n alternatiewe droë-koei behandeling om mastitis infeksies by suiwelbeeste te voorkom, aangesien dit blyk dat dit effektief is teen patogene wat weerstand toon teen normale antibiotiese behandeling.

BIOGRAPHICAL SKETCH

Reneé Pieterse was born in Randburg, Gauteng on 17 February 1977. She matriculated at Ladysmith High School, Ladysmith, in 1994. She obtained a N. Dip. in Biotechnology at Natal Technikon in Durban in 1998, after completing her experiential training at the Centre for Water and Wastewater Research at the technikon. She has been employed by the Western Cape Provincial Veterinary Laboratory in Stellenbosch since 1999 and has gained experience in clinical pathology, diagnostic bacteriology and PCR. While employed, she obtained a B.Tech. degree in Biotechnology (*cum laude*) through Natal Technikon in 2002.

ACKNOWLEDGEMENTS

PREFACE

This thesis is presented as a compilation of manuscripts written in accordance with the requirements of the Canadian Journal of Microbiology. Some repetition between chapters exists, as each manuscript is an individual entity. The literature review includes an overview of bovine mastitis and current trends in the management of this disease. The need for alternative treatment strategies, with reference to bacteriocins and their applications are discussed.

CONTENTS

CHAPTER 1

INTRODUCTION

Mastitis is an inflammatory reaction of the mammary gland. It is a complex disease involving many factors and is primarily caused by microorganisms that gain entry into the teat canal and mammary glands (Philpot and Nickerson 1999). Clinical symptoms of this disease include inflammation and swelling of the teats and udders. In acute cases severe swelling accompanied by fever and loss of appetite is observed. Subclinical mastitis precedes clinical mastitis, where no visible symptoms occur and can only be diagnosed through regular monitoring and laboratory testing (Giesecke et al. 1994).

Mastitis is the most costly disease in the dairy industry (Petrovski et al. 2006). In South Africa, it is estimated that costs, including loss in production due to discarded milk, reduction in yield, expenditures for prevention and treatment, and culling, can be as much as R400 per cow annually (Giesecke et al. 1994).

The main etiological agents responsible for mastitis infections are divided into groups depending on the source of the organism involved. Contagious organisms found on the udder or teat surface are the primary source of infection between quarters. These include *Staphylococcus aureus, Streptococcus agalactiae*, *Corynebacterium bovis* and *Mycoplasma bovis*. Environmental pathogens occur in the immediate surroundings of the cow and include *Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus bovis, Enterococcus faecium, Enterococcus faecalis* and coliforms such as *Escherichia coli, Klebsiella pneumonia* and *Enterobacter aerogenes* (Schroeder 1997; Quinn et al. 1999;).

Contagious microorganisms are usually responsible for the highest incidence of mastitis. Treatment strategies employed to curb mastitis infection have resulted in a shift in the proportion of types of bacterial isolates. Improved farm management, teat disinfection before

and after milking, prompt treatment of clinical mastitis, culling of chronically infected cows and antibiotic dry cow therapy have resulted in a dramatic decrease in the number of *S. agalactiae* and *S. aureus* infections in the last fifty years (Bradley and Green 2001; Bradley 2002;). However, an increase in environmental pathogens such as *E. coli, Enterobacteriacae* and coagulase-negative staphylococci (CNS) has been recorded (Bradley 2002; Rajal-Schultz et al. 2004).

Treatment using antimicrobial agents can be administered either during lactation or during the dry period spanning 50 – 60 days (Giesecke et al. 1994). Antimicrobial infusions, containing slow-release antibiotic preparations are usually administered immediately after drying-off (Philpot and Nickerson 1994). An internal teat sealant can be used alone or with antibiotics which acts as a physical barrier to microorganisms entering the teat canal (Berry and Hillerton 2002). The presence of antibiotic residues in milk is an important consideration when treating mastitis during lactation. Treatment costs and the loss of production is a drawback for the use of antibiotics (Gruet et al. 2001).

In addition, regulations for organic farming favour alternate therapies (Shryock 2004). Prophylactic dosages of antibiotics used in mastitis control programmes could select for strains with resistance to antibiotics (Passantino 2007). Concerns that resistant strains could enter the food chain via contaminated food products, making treatment of human pathogens more challenging, favour the development of alternative antimicrobial agents (Shryock 2004).

Bacteriocins produced by lactic acid bacteria are generally regarded as safe (GRAS). These antimicrobial peptides are ribosomally synthesised. They differ from most antibiotics in that they usually have a narrow-spectrum of activity against closely related species (Jack et al. 1995). They often have a bactericidal mode of action, preventing cell wall synthesis and forming pores in the cell surface of sensitive strains. This results in an efflux of cytoplasmic

compounds that are required to maintain ion gradients, trans-membrane potential and the pH gradient across the membrane. Biosynthetic pathways such as ATP synthesis driven by proton motive force cease and cell death occurs (Cotter et al. 2005).

The aim of this study was to isolate and identify a bacteriocin-producing strain, active against mastitis pathogens. The study describes a novel bacteriocin, macedocin ST91KM, produced by *Streptococcus macedonicus* ST91KM. Parameters affecting the adsorption of the bacteriocin to target bacteria are evaluated. Due to widespread antibiotic resistance in pathogenic strains, the antibiotic resistance patterns of selected mastitis pathogens was determined as well as the effect of the bacteriocin ST91KM on sensitive strains. *In vitro* studies evaluating the possible administration of the bacterioicn for dry cow therapy in a teat seal preparation were carried out. Further studies, including the purification and determining of the amino acid sequence of the bacteriocin and possible *in vivo* studies should be carried out to fully explore the potential of this bacteriocin as a viable alternative for the treatment of bovine mastitis.

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CHAPTER 2

LITERATURE REVIEW

2.1   MASTITIS

2.1.1   Introduction

The general health and well being of individuals depends largely on meeting basic nutritional needs. Milk and fermented milk products such as cheese, cultured milks and yoghurt have formed an important part of daily nutrition, and the variety of products produced from milk has increased dramatically over the years, as modern food processing technologies have improved. An increase in global population coupled with the increasing demands for milk as an economic food and as an industrial raw food product has necessitated an increase in production by dairy farmers.

Current statistics indicate that the annual milk production in South Africa has increased steadily over the last 20 years from approximately 1700 million litres in 1985 to an estimated 2200 million litres in 2006. Consumption of dairy products has also increased at similar levels with a sharper increase in recent years, due primarily to a larger personal income base for individuals (Lactodata 2006).

In an commercial milking environment, dairy cattle need to be in perfect physical condition to maintain a high level of milk production. The risk of lesions and infections that develop in modern dairy farming has consequently increased. Low milk production has been attributed to a large extent to the control of diseases in dairy cattle, of which mastitis accounts for the largest economic losses on dairy farms in many countries in the world, including the USA, United Kingdom, Europe, Australia and South Africa (Giesecke et al. 1994; Petrovski et al. 2006).

Improving udder health and decreasing the incidence of udder infection and inflammation in dairy herds, will result in increased milk production as huge losses are

directly or indirectly incurred through loss of milk during treatment periods, culling of cows and death of clinically infected cattle. Mastitis control programmes addressing various aspects of dairy farming such as feeding practices, animal husbandry, hygiene and general health care can contribute towards reducing the incidence of udder infections. Treating infection with antimicrobials can, in conjunction with good farming practices, assist in this endeavour to eliminate, or at least decrease, the incidence of mastitis infection within a dairy herd.

2.1.2   Classification of the types of mastitis

"Mastitis" describes an inflammatory reaction in the mammary gland. The term comes from the Greek derived word elements *masto-* referring to the mammary gland and *-itis* meaning – "inflammation" (Blood and Studdert 1999). Although "mastitis" could technically be used to describe any udder injury that may result in inflammation, it is generally accepted that the causative agents for the inflammatory reaction are microorganisms that have gained entry into the teat canal and mammary tissue (Philpot and Nickerson 1999).

The extent of the infection that occurs as microorganisms multiply and proliferate within the mammary tissue determines the type of mastitis affecting the cow udder.

Clinical mastitis occurs when a visible sign of inflammation is observed in the udder of the cow or the teats. The clinical case could be **subacute**, where the symptoms are very mild and may only be accompanied by slight swelling of the udder and the presence of flakes in the milk. An **acute** or **peracute** case of clinical mastitis may occur where a sudden onset of symptoms such as severe inflammation of the teats, fever, loss of appetite, dehydration and even death occurs. Clinical mastitis will eventually lead to **chronic** mastitis if not treated. In cases of chronic mastitis, the cow will suffer from a constant infection oscillating between subacute and acute clinical mastitis. A permanent change in the udder may occur with the

presence of scar tissue and a change in the shape and size of the glandular tissue (Giesecke, et al. 1994; Philpot and Nickerson 1999).

Subclinical mastitis precedes clinical mastitis and is more subtle in that no signs of infection are visible. Most dairy herds will have cows with subclinical mastitis. The only way to detect the presence of infecting microorganisms invading the teat canal and udder tissue is to monitor the inflammatory response of the cows, i.e. quantification of the somatic cells (leukocytes and discarded epithelial cells) in the milk (Giesecke et al. 1994; Philpot and Nickerson 1999).

Subclinical mastitis is considered more severe than clinical mastitis, as early detection is impossible without regular monitoring. Cows with subclinical mastitis harbour a constant reservoir of pathogens that could lead to severe udder infection and spreading to other cows (Philpot and Nickerson 1999).

2.1.3   Economic implications of mastitis

The implication of mastitis has been well researched and documented for many years. The overall conclusion is that the disease is economically the most important in the dairy industry, especially in developed countries (Petrovski et al. 2006). Losses incurred by the industry are often underestimated as farmers may only consider the obvious losses due to clinical cases of mastitis. Subclinical mastitis may be present without the farmer realising it and the resulting decrease in milk production and poor milk quality may not be noted. In the USA the actual economic losses incurred are calculated, taking into account various direct and indirect costs. Herds monitored over a 12-month period in 1993 showed losses ranging from $161 to $344 per lactating cow/year. This led to an average annual loss estimated at $2 billion (Morin, et al., 1993). Schroeder (1997) reported similar losses in 1997 ($185 per cow annually and a total cost of $1.8 billion). Giesecke et al. (1998) estimated a loss of R150 per

cow/year in South Africa in 1978 and ten years later in 1987/88, the estimated cost had risen to approximately R400 per cow/year.

The reasons given for direct and indirect losses caused by mastitis infections are summarised in a review by Petrovski et al. (2006). Direct losses include treatment costs (veterinary fees and drugs), milk that is discarded due to poor quality, or milk lost during the required withdrawal period before and after drug administration, labour costs incurred by workers having to attend to sick animals, animal fatalities or euthanasia. Repeated cases of infection will amplify the costs incurred. The indirect costs need to be highlighted as dairy farmers often underestimate these, as a decrease in milk yield may be gradual and if a subclinical case of mastitis is present, will go unnoticed and may lead to more serious losses. As the cow responds to the presence of invading microorganisms, energy will be directed towards immune response and away from milk production and discomfort and pain will lead to a loss of appetite and reduced food intake, thereby lowering energy intake for milk production (Petrovski et al. 2006).

The quality and composition of milk will also be affected by mastitis infection. Consumers are well informed and demand milk and milk products of an exceptionally high standard. Various governmental bodies world-wide demand specific levels of quality to ensure adequate nutrition and consumer safety with regard to the presence of drug residues in the milk and the presence of infecting microorganisms (Giesecke et al. 1994). It follows that if the total bacterial count in milk increases, the number of microorganisms not killed by pasteurisation also increases. Monitoring the somatic cells (neutrophils, macrophages and epithelial cells) in the milk gives an indication of the total bacterial count in milk. For example, pasteurised milk that is processed from raw milk containing more than 250 000 somatic cells/ml will have a longer shelf life than raw milk with a somatic cell count of more than 500 000 somatic cells/ml (Philpot and Nickerson 1999).

The South African Department of Health follows guidelines that were implemented by the International Dairy Federation (IDF) in 1967, stating that bulk raw milk which is intended for further processing should not be used or sold if it contains an average of 500 000 or more somatic cells/ml of bovine milk (South Africa 1997: 1555). South African regulations also stipulate the maximum allowable limits of substances, including antibiotic residues in foodstuffs, as published in the Government Notice, No. R 1809 of 3 July 1992.

Farmers are penalised if market milk is of an inferior quality or contains drug residues and has higher somatic cell count levels per millilitre of milk as set forth by government regulations (Petrovski et al. 2006).

2.1.4   Mastitis-causing pathogens

The main etiological agents responsible for mastitis infections can be divided into different groups of organisms depending on the source of the organism involved. These include contagious pathogens, environmental bacteria, opportunistic bacteria and other organisms that less frequently cause mastitis less frequently (Philpot and Nickerson 1999).

2.1.4.1 Contagious organisms

Contagious microorganisms are usually found on the udder or teat surface of infected cows and are the primary source of infection between uninfected and infected udder quarters, usually during milking. The organisms that fit into this category include: *Staphylococcus aureus* (coagulase-positive staphylococci), *Streptococcus agalactiae* and the less common sources of infection caused by *Corynebacterium bovis* and *Mycoplasma bovis* (Philpot and Nickerson 1999; Quinn, et al. 1999).

2.1.4.2 Environmental organisms

Environmental pathogens are found in the immediate surroundings of the cow, such as the sawdust and bedding of housed cows, the manure of cattle and the soil. Bacteria include streptococcal strains other than *S. agalactiae,* such as *Streptococcus. dysgalactiae*, *Streptococcus uberis* and *Streptoccous bovis*; *Enterococcus faecium* and *Enterococcus faecalis* and coliforms such as *Escherichia coli*, *Klebsiella pneumonia* and *Enterobacter aerogenes* (Quinn et al. 1999; Schroeder 1997). Mastitis caused by environmental organisms is essentially opportunistic in nature and becomes established if the immune system of the host is compromised or if sanitation and hygiene is not adequately practiced (Schukken et al. 2005).

2.1.4.3 Opportunistic organisms

Opportunistic pathogens result in mild forms of mastitis and include coagulase-negative staphylococci. The coagulase test correlates well with pathogenicity and strains that are coagulase-negative are generally regarded as non-pathogenic (Quinn et al. 1999). These staphylococci occur commensally and may be isolated from milk but usually illicit a minor immune response in cattle and infections caused are slight. They include *S. epidermidis*, *S. saprophyticus* (Quinn et al. 1999; dos Santos Nacimento et al. 2005), *S. chromogenes* (De Vliegher et al. 2003) and *S. simulans* (dos Santos Nacimento et al. 2005).

2.1.4.4 Other organisms

Many other bacteria and even yeasts may be responsible for causing mastitis, but are less common and occur if conditions in the environment change to increase exposure to these organisms. A condition known as "summer mastitis" occurs mostly in European countries in the summer months when wet, rainy conditions prevail. The source of infection is usually

traced to an increase in exposure of the cows to flies in pastures that transmit infecting *Arcanobacterium pyogenes* and *Peptostreptococcus indolicus* strains and is more common in non-lactating cows (Sol 1984; Quinn et al. 1999).

Mastitis caused by *Pseudomonas aeruginosa* is often traced to contaminated water sources and will result in a condition similar to coliform mastitis infections where endotoxemia occurs (Philpot and Nickerson 1999; Quinn et al. 1999).

Nocardia asteroides causes severe cases of mastitis resulting in fibrosis and permanent damage to mammary tissues (Quinn, et al. 1999). Treatment is usually ineffective and a high mortality rate occurs. The source of the infection caused by *Nocardia asteroides* is usually from the soil and could be prevented by ensuring that effective sanitation measures are enforced before treatment with intramammary infusions (Philpot and Nickerson 1999).

Less common causes of bovine mastitis include *Bacillus cereus,* resulting in peracute and acute mastitis and also the human pathogens *Streptococcus pyogenes* and *S. pneumonia* that causes acute mastitis and is accompanied by fever symptoms in the host (Quinn et al. 1999).

2.1.4.5 Current aetiology of mastitis

Contagious organisms have usually been responsible for the highest incidence of both clinical and sub-clinical cases of mastitis. Bradley (2002) sites the changes that have occurred in the United Kingdom from 1967, where *S. aureus* and *S. agalactiae* were primarily responsible for the highest number of clinical mastitis cases in dairy herds. Three decades later in 1998, after the implementation of control strategies in the late sixties, the number of incidences of contagious pathogens responsible for clinical mastitis decreased significantly, accounting for only 10 % of cases. *E. coli* and *Enterobacteriacae,* however, were responsible for 34.7 % and 40.9 %, respectively, of all cases (Bradley and Green 2001).

Adequate mastitis control strategies have thus played a key role in reducing contagious cases of mastitis. It would appear however, that as contagious pathogens were reduced, opportunistic and environmental pathogens seemed to play a greater role in causing persistent infections (Bradley 2002). The importance of the correct diagnosis and identification of the aetiological agent causing inflammation in the udder tissue is essential in determining the treatment strategies. It is also important to understand the history of mastitis incidence within a herd over a period of time and to understand the different periods when a cow may be at higher risk for infection. For example, cows are especially susceptible to mastitis during the periparturient period (just before and after calving) and at drying off - due to structural changes occurring in the mammary gland. A decrease in the number and functionality of white blood cells caused by interactions with specific hormones during these periods results in a compromised defence system (Oliver and Sordillo 1988; Vangroenweghe et al. 2005).

2.1.5   Infection

2.1.5.1 Mammary structure

Fig. 1 (A) shows a schematic representation of an udder quarter (Shroeder 1997). Each quarter is composed of the milk-producing tissue or alveoli that lead into the lactiferous ducts, gland cistern, teat canal and finally the teat opening or duct. The alveoli (B) are lined with epithelial cells that become specialised during the gestation period, before calving, and after calving. These specialised cells produce colostral and lacteal secretions and finally, milk. Connective tissue and muscle cells support the alveoli glands and contract and squeeze milk from the alveoli during milking (Giesecke et al. 1994; Philpot and Nickerson 1999).
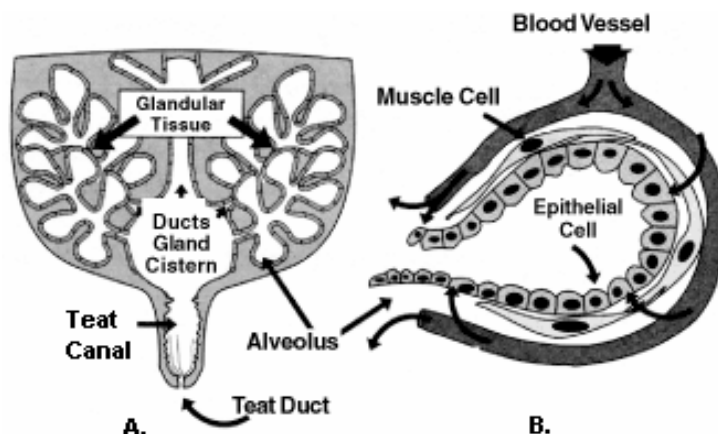
Fig. 1. Schematic representation of the bovine mammary gland (A) and detailed structure of each alveoli sac (B) (Schroeder 1997).

2.1.5.2 Invasion, infection and immune response of mammary gland

To treat mastitis infections effectively, it is important to understand the invasive patterns of the different pathogenic bacteria and the host immune response to these pathogens. Mastitis occurs when microorganisms enter via the teat opening or duct and are able to overcome the immune system, multiply and establish within the teat canal and the mammary tissue. Invasion of the udder most likely occurs between milking periods. This is when microorganisms are present on the outer surface of the udder, on milking machines, or on the hands of workers. The opening of the teat canal has sphincter muscles that provide a physical barrier from the outside and is able to maintain a tight closure of the opening (Philpot and Nickerson 1999). In addition, the teat canal is also lined with keratin which is a waxy substance derived from squamous epithelial cells. The keratin not only acts as a barrier between invading organisms and the gland cistern, but also contains bacteriostatic anti-microbial agents (Sordillo and Streicher 2002). This physical barrier can be compromised through trauma incurred, or microorganisms can simply be propelled through the teat canal during the use of milking machines (Philpot and Nickerson 1999). These anatomical factors

are the first line of defence against colonisation and form part of the innate or non-specific immune response in the mammary gland (Oviedo-Boyso et al., 2006).

The second part of innate immunity occurs if the physical barriers have been overcome and microorganisms invade the treat canal and colonise the epithelial linings of the gland cisterns. These are cellular factors and include neutrophils, macrophages and lymphocytes. The macrophages recognise the invading pathogens and initiate the inflammatory response. Pro-inflammatory cytokines induce neutrophil recruitment to the mammary gland. The main function of neutrophils is to kill bacterial pathogens through phagocytosis and to produce antibacterial peptides known as defensins (Oviedo-Boyso et al. 2006).

Early responses to mastitis infection are controlled by innate immunity, which is non-specific and will react to any invading microorganisms (Sordillo and Streicher 2002). Acquired immunity, however, recognises specific antigenic determinants of a pathogen. These antigenic determinants can be lipopolysaccharide, peptidoglycan and lipoteichoic acids on the surface of the pathogenic bacteria. Recognition of these antigenic sites will determine the response of the immune system and if there is a repeated exposure to the pathogen, the acquired immune response is more rapid (Oviedo-Boyso et al. 2006).

The type of infecting pathogen may illicit different immune responses in the mammary gland. Pathogenic bacteria have developed many strategies to invade and overcome immune responses (Hornef et al. 2002). Mastitis-causing pathogens can adhere or attach to tissue and are therefore not easily removed from the teat canal even during lactation (Philpot and Nickerson 1999). It has even been suggested that the ability of mastitis-causing pathogens to form biofilms on epithelial cells may be responsible for recurrent mastitis infections as the pathogens become inaccessible to antibiotic treatment (Melchior et al. 2006).

As the host immune system responds to invading pathogens, the somatic cells, consisting of mostly neutrophils, macrophages and epithelial cells move through the udder

tissues towards the site of infection. The presence of somatic cells, pathogens and toxins around the mammary tissue may cause unaffected alveoli to revert to a resting state, known as involution. The glandular ducts through which the milk should drain can also become clogged due to tissue damage and the presence of somatic cells (Philpot and Nickerson 1999).

Table 1 summarises the type of mastitis infection that occurs when pathogens invade the teat canal and mammary tissue. Some pathogens are well adapted for the udder tissue environment and are the primary source for recurrent intramammary infections, especially contagious mastitis caused by *S. aureus* and *S. agalactiae*. Most microorganisms, including *S. uberis* (Almeida et al. 2005), *S. dysgalactiae* (Almeida and Oliver 1995) and *E. coli* (Dopfer et al. 2000; Dogan et al 2005) adhere to and internalise into epithelium cells. Persistence of the pathogen in the tissue may vary, some are easily destroyed by the host immune system while others such as *S. aureus* are well-adapted and cause serious injury within the mammary tissue, producing virulence factors that disarm the host immune systems cells (Almeida et al. 1996; Haveri et al. 2005).

E. coli and other coliform pathogens are not only able to adhere to and invade epithelium (Dopfer et al. 2000) but are also able to multiply rapidly in the gland cistern, which elicits a rapid inflammatory response that destroys a large number of the invading pathogens. However, upon cell lyses endotoxins are released causing severe toxaemia in the blood stream of the cow (Philpot and Nickerson 1999; Quinn et al. 1999).

Table 1.Characteristics of common mastitis-causing pathogens, invasiveness and infection

Pathogen	Type of mastitis	Infection
S. agalatiae	Mostly subclinical, but also clinical, recurrent and chronic if treatment is not effected soon enough	Highly contagious. Primarily infect duct system and lower portion of the udder on the surface of epithelium. Causes injury and scarring to duct system and clogging results in accumulation of milk in ducts and reduction in milk production. Involution occurs (Philpot and Nickerson 1999).
S. dysgalactiae	Clinical acute	Environmental source. Bacterium can adhere to and be taken up into cells without losing viability and therefore persist in tissue and may be protected from antibiotic therapy. Bacterium does not cause severe permanent injury to epithelial tissue (Calvino and Oliver 1998).
S uberis	Clinical acute	Environmental source. Able to adhere to and is taken up by epithelium cells and persist intracellularly for extended periods. Responsible for chronic infection but does not cause severe tissue injury. One of the most commonly isolated organisms during non-lactating period (Tamilselvam et al. 2006)
S. aureus	Subclinical, clinical or chronic, in severe cases gangrenous mastitis	Highly contagious. Bacterium adheres invades the deeper tissue of the alveoli where it becomes encapsulated by fibrous tissue and abscesses form, thus walling-off the bacterium. Involution occurs. In severe cases, toxins can cause blood vessel constriction and clotting cutting off blood supply to tissue resulting in gangrenous mastitis (Philpot and Nickerson 1999).
E. coli and other coliform bacteria	Acute clinical (toxaemia) mastitis, may develop chronic mastitis	Environmental, fairly common due to high incidence of bacteria on host and environment. Bacteria invade tissue in teat and gland cistern. Tissue damage occurs in teat cistern, gland cistern and large ducts. Large influx of somatic cells through damaged tissue results in formation of clots in the milk. Usually no long-term effects to alveoli occur and host immune system often clears up infection. (Philpot and Nickerson 1999)

2.1.5.3 Diagnosis of mastitis

Regular monitoring of dairy herds is essential to allow for early detection and treatment of mastitis. Physical inspections of the udder and secretions should be done to determine if any clinical symptoms are present. These will include hard, swollen udder quarters, redness, or tissue that feels warm to the touch due to acute inflammation in the udder (Philpot and Nickerson 1999). Symptoms may vary, depending on the severity of the clinical mastitis present. It can range from a slight palpitation of the empty udder, which may indicate a hardening of the gland tissue, causing sub-acute mastitis, through to peracute mastitis, where the cows show visible signs of illness, dehydration, fever and a rapid physical deterioration that can lead to death within hours (Giesecke et al. 1994).

Milk samples should be submitted for a number of laboratory tests to aid in the diagnosis of mastitis. Monitoring should include regular sampling of a bulk milk sample on a monthly basis for somatic cell counts (SCC). Most researchers and veterinarians will agree that the normal SCC in a bulk milk sample should be less than 200 000 cells/ml (Philpot and Nickerson 1999; Quinn et al. 1999). An elevation in the SCC is an indication that inflammation is present within the udder as the immune system responds to the presence of invading organisms. If the SCC exceeds 300 000 cells/ml, the farmer would need to contact a veterinarian for follow-up examination to determine where the problem exists within the herd. Individual SCC's can then be done on specific cows (and udder quarters) in the herd. If the SCC on the composite sample exceeds 500 000 cell/ml; both clinical and subclinical mastitis is likely to be present within in the herd (Philpot and Nickerson 1999). The presence of subclinical mastitis in a herd can only be detected if regular SCC monitoring is done, as visible symptoms may not be present (Giesecke et al. 1994).

Microbiological investigations should be performed on individual milk samples with a SCC count greater than 200 000 cells/ml (Western Cape Department of Agriculture 2007).

These are to be performed on milk samples that have been collected aseptically to ensure that the potential pathogens can be identified from milk that comes from within the mammary gland and not from the udder surface. Samples must also be obtained before antimicrobial agents have been administered to ensure that all potential pathogens are isolated. The milk samples are streaked onto blood agar as well as MacConkey agar plates and pure cultures are identified using biochemical tests. If *Mycoplasma bovis* is suspected to be a pathogen, specialised sampling techniques and growth medium are required (Quinn et al. 1999).

Once the major pathogens have been isolated and identified, an "antibiogram" can be done to determine the antimicrobial sensitivity profile of the pathogens (Quinn et al. 1999). This may be necessary to assist the veterinarian in administering the most effective antimicrobial agent in a responsible manner, thereby reducing the risk of antibiotic resistance developing in pathogens.

Research has also been conducted to develop alternative techniques to aid in mastitis diagnosis. These include molecular techniques such as real time multiplex PCR which offers the advantage of faster turnaround time and more accurate identification of pathogens (Gillespie and Oliver 2005). This technique however requires more specialised training and will likely not be affordable for routine testing of samples at present.

2.1.6 Mastitis control strategies

The "five point plan for mastitis control" has been the gold standard for control strategies for many years (Giesecke et al. 1994), and has been successful in reducing the incidence of mastitis. The strategy addresses areas where the risk of infection is the greatest and promotes the use of treatment at specific times. The five points listed by Giesecke et al. (1994) include:

- Teat disinfection after milking

- Proper hygiene and milking procedures and adequate milking equipment

- Culling of chronically mastitis cows

- Antibiotic dry-cow therapy

- Prompt treatment of clinical mastitis during dry period and during lactation

The first three can be described as farm management related areas and the last two as specific treatment actions involving the use of antimicrobial agents. The NMC (formerly the National Mastitis Council), a non-profit organisation based in the USA recommends a similar strategy, but strongly suggests that other management areas also form part of the control program. These include good record keeping of clinical mastitis cases and treatment times, outcome of treatments and various other records of SCC to monitor the incidence of subclinical mastitis. Planning and regular reviewing of the strategy is also recommended and adequate communication between the farmer, veterinarian and staff is necessary so that the strategies can be implemented practically (NMC, 2007).

2.1.6.1 Farm management

A strategy to control mastitis must be practical and economical. The primary goal would be to reduce the rate of new infections and the duration of current infections within a herd. It would also be essentially important to maintain normal udder health ensuring that the natural immune response in the cow can resist and fight disease while still producing the required level of milk (Philpot and Nickerson 1999).

Control strategies need to target every facet and process of dairy farming and can begin with maintaining good hygiene practices in the environment. The holding yards or stalls should be kept clean and dry. The water supply should be adequate and free of coliform bacteria and equipment should be maintained and sanitised between milking (Giesecke et al.

1994).   The welfare of animals is becoming increasingly important in modern dairy production as consumers become more concerned about the manner in which farm animals are treated. The Farm Animal Welfare Council in the UK has defined "the five freedoms" of animals, which highlight issues relating to the treatment and management of animals. The advantage of implementing such quality control measures within the herd would ensure that dairy cows are free of a stressful environment, injury, pain, hunger and discomfort, which in turn would promote a healthy immune system and udder health in general (Sandgren and Ekman 2005).

The milking practice is of paramount importance as this is most often the route of infection. The udder should be prepared before milking by washing the teats, followed by disinfection and drying with clean paper towels. If the teat area is dripping with water from run-off of areas that were heavily soiled it could lead to pathogens gaining access to the teat canal. Milker's hands should also be disinfected to prevent the transfer of pathogens. Post milking treatment is also important and all cows should be treated with a teat dip disinfectant to reduce the risk of infection (Giesecke et al. 1994; Philpot and Nickerson 1999).

Monitoring SCC on a regular basis and follow-up investigations give an indication of the success of good animal husbandry and hygiene practices. It therefore forms an integral part of mastitis control strategies and assists in diagnosis and treatment.

The elimination of mastitis in a herd may require the culling of cows that are incurable or are so severely infected that the mammary tissue has been scarred and damaged to the extent that the tissue no longer functions (Giesecke et al. 1994).

2.1.6.2 Treatment

A cow may spontaneously recover from mastitis, but this will usually occur in mild cases of subclinical mastitis. Theoretically, the mechanism by which a cow recovers from

infection without treatment can be capitalised upon to produce a vaccine (Philpot and Nickerson 1999). Research in this area continues and some vaccines such as *E. coli* J5 can reduce the number and severity of coliform mastitis cases by 70 – 80 % (Crist et al. 1997). Recent technology has focused on a DNA vaccine that expresses virulence factors *in vivo* and is primarily targeted against *S. aureus* mastitis, as antibiotic therapy is usually less effective against this pathogen (Talbot and Lacasse 2005; Zecconi 2005).

Antimicrobial agents can be administered either during lactation or during the dry period. Treatment during lactation will be necessary if clinical mastitis is present, whereas dry cow therapy can be used to treat existing infections and can also be administered in a prophylactic manner to prevent new infections from developing during this period. A cow will usually lactate for a period of approximately 300 days per year and have a dry period of between 50 to 60 days. The most vulnerable period when new mastitis infections occur is at the end of the lactation period and again just before the start of the next lactation period (Giesecke et al. 1999). This can be attributed to hormonal and structural changes occurring in the mammary tissue which affects the immune system as the cow prepares for calving or for the drying-off stage (Oliver and Sordillo 1988; Vangroenweghe et al. 2005).

2.1.6.2.1 Dry cow therapy

Dry cow therapy is as much a management issue as it is a treatment issue. The manner in which the cows enter this period is important and the way in which the housing conditions and nutrition is handled impacts on the success of the treatment itself. The energy intake of the cows should be lowered to reduce milk production towards the drying-off stage and then, as soon as drying-off occurs, they need to be treated immediately with either antimicrobial infusions (containing slow release antibiotic preparations) or with internal teat sealant products (NMC 2006). Antimicrobials will be required if an existing infection is present,

whereas an internal teat sealant can be used alone if no infection is present. Commercially available teat sealants such as Orbeseal® (Pfizer Animal Health) are approved for use in North America and Europe, but are not available in South Africa (Van Dijk 2007).

The teat sealant is composed of an inert salt (bismuth subnitrate) in a paraffin base. The paste is infused into the teat of each quarter using a sterile syringe. After drying-off, the product is stripped out at first milking (Pfizer Animal Health 2004). To ensure that other pathogens are not introduced into the teat along with the teat sealant, trained personnel should perform the administration of the product. Fig. 2 shows the position of teat sealant product within the teat canal.

Teat Sealant Orbeseal® ⟶
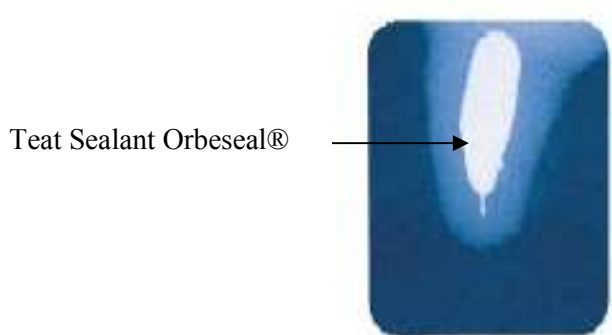
Fig. 2. Internal teat sealant Orbeseal® (bismuth subnitrate in an oily base) in the teat canal (Pfizer Animal Health 2004)

The teat sealant forms an impermeable plug as it lines the teat canal and results in a physical barrier against invading microorganisms through the teat opening, thereby preventing new infections during the dry period. Research has shown that the internal teat sealant (Orbeseal®, Pfizer Animal Health) is effective in reducing the infection rate when compared to untreated cows (Berry and Hillerton, 2002). A recent study also demonstrated the benefit of administering Orbeseal® (Pfizer Animal Health) along with an antibiotic infusion (Orbenin® Extra Dry Cow, Pfizer Animal Health) containing cloxacillin. The use of the teat sealant and the antibiotic infusion performed slightly better in preventing clinical

mastitis in the dry period compared with using only the antibiotic infusion (Bradley et al. 2005).

2.1.6.2.2   Lactation therapy

The use of antimicrobials during lactation must be carefully considered. Only cases of clinical mastitis and some specific cases of subclinical mastitis, where the quality and production of the milk is severely affected, are treated. Mastitis caused by *S. agalactiae* can be treated most readily during lactation and has a high cure rate (90-95 %). Mastitis caused by S. *aureus* has the lowest cure rate and along with environmental streptococci should be treated during the dry period (Philpot and Nickerson 1999).

An important consideration for treatment during lactation is the presence of antibiotic residues in the milk. A waiting period is required for the duration of the treatment and for a given period after treatment where milk and meat products need to be withheld to ensure that the level of antibiotics present in the product meets the legislative requirements. The withdrawal period and the type of product that is administered vary in different countries (Gruet et al. 2001). The maximum allowable limit of veterinary antibiotics in meat and milk products in South Africa is specified in the Government Gazette Notice No. R 1809 of 3 July 1992. The cost of treatment and the loss of milk during the withdrawal period are important in determining the type of product used and the manner in which it is administered. The withdrawal period for milk products marketed in South Africa during lactation varies between 1 and 4 days (Table 3, van Dijk 2007). A product is considered excellent if it has a high cure rate and a minimum withdrawal period (Gruet et al. 2001).

2.1.6.3 Efficacy of drug delivery

The administration of drugs can be done either directly into the teat canal, as previously described for dry cow therapy, in the form of intramammary infusions, but can also be given parenterally by intravenous or intramuscular injection (Philpot and Nickerson 1999). The route of choice for subclinical mastitis is usually by intramammary infusion; and in the case of severe acute clinical mastitis, a combination of parenteral and intramammary treatment is usually necessary (Ziv 1980).

To be effective, the drug has to exert specific antimicrobial activity at the site of infection (Gruet et al. 2001) and must have certain characteristics to be an effective agent in the mammary tissue. The pH of blood plasma is 7.4. The pH of milk varies between 6.4 and 6.6, but increases to 7.4 in the case of an infection. Most antibiotics are weak organic acids or bases and exist in both an ionised and non-ionised form in varying proportions in blood and milk, depending on the change in pH of the environment. Drugs that are administered parenterally must pass from the circulatory blood system and into the milk and milk tissue via lipid membranes. The active fraction of the drug must be in a non-ionised, non-protein bound, lipid-soluble form to pass this blood-to-milk barrier (Ziv 1980).

Antibiotics that are administered via the teat opening must reach the site of infection in the teat canal or upper cistern, but often the distribution is uneven and diffusion through the mammary ducts where severe inflammation and swelling is present may block the movement of the therapeutic agent (du Preez 2000). Added to this, most pathogens have the ability to invade the epithelium tissue. In the case of *S. aureus* infection, interaction with antibiotics is prevented by the formation of fibrous scar tissue. The scar tissue may also have no blood supply, rendering intramuscular or intravenous drug therapy less effective (Philpot and Nickerson 1999). Some bacteria may also evade interactions with antibiotics once engulfed by macrophages, where they remain active within the leukocyte and can cause recurrent

infections once the antibiotic has been eliminated from the area (Philpot and Nickerson 1999). The formation of biofilms within the teat canal as bacteria adhere to bacteria on the epithelium surface may also contribute to the ineffectiveness of local intramammary infusions (Melchior et al. 2006).

The type of drug used to treat an infection can be determined once an accurate diagnosis has been made and the pathogens identified. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a drug that prevents the growth of a specific pathogen (NCCLS 2002). Antimicrobial disk diffusion tests are performed on the pathogens isolated from mastitic milk samples to determine the drug sensitivity profile of the pathogens. The veterinarian is then able to select the most effective drug for treatment (Philpot and Nickerson 1999). The ideal drug should have the lowest MIC against the majority of udder pathogens. No single drug can, however, be effective against all pathogens and most need to be used in combinations and in different formulations to increase efficacy and bioavailability within the udder tissue (Ziv 1980; Gruet et al. 2001).

2.1.6.4 Types of antimicrobial agents

Commonly used remedies available in South Africa for dry cow and lactation therapy, the recommended withdrawal period (Van Dijk 2007) and the possible activity spectrum of mastitis pathogens (Du Preez 2000) are shown in Table 2 and 3. The antibiotic groups and antimicrobials used in these remedies have different mechanisms of action and many new semi-synthetic compounds have been developed to counter the threat of antimicrobial resistance. The majority of antibiotics used are broad-spectrum antibiotics acting against Gram-positive and Gram-negative bacteria (NCCLS 2002).

β-lactam Penicillins (penicillins, ampicillin, cloxacillin, amoxycillin, nafcillin, methicillin) and **β-lactam Cephalosporins** (cephalexin, cefuroxime, cephapirin) inhibit cell

wall synthesis by preventing the formation of cross-links between polysaccharide chains in the cell wall. Many staphylococcal strains produce the enzyme penicillinase, which acts by breaking the β-lactam ring structure of the antibiotic and are therefore resistant. Penicillinase-resistant penicillins such as cloxacillin are specifically used to treat the penicillinase-producing, methicillin-susceptible staphylococci (NCCLS 2002).

Clavulanic acid inhibits the activity of penicillinase produced by staphylococcal strains. Combined with β-lactam antibiotics such as amoxicillin it can eliminate β-lactamase activity by pathogens and improve susceptibility to the antibiotic (Soback and Saran 2005).

Tetracyclines such as oxytetracycline inhibit protein synthesis by binding to the 30S ribosomal sub-unit and interfere with amino-acyl-tRNA binding. Tetracycline is bacteriostatic and usually more active against Gram-positive organisms (NCCLS 2002). Oxytetracycline is an irritant and should therefore not be administered as an infusion, but rather intravenously (du Preez 2000).

Aminoglycosides (streptomycin, neomycin) inhibit protein synthesis by binding to the 50S ribosomal sub-unit and inhibits peptide chain elongation. Aminoglycosides are mostly active against Gram-negative bacteria and are often formulated together with β-lactam penicillins (NCCLS 2002).

Polymixin B is an antimicrobial compound that binds to the cell membrane and disrupts its structure and permeability properties. It is the antimicrobial drug of choice for infections caused by *P. aeruginosa* (Du Preez 2000).

Macrolide antibiotics (tylosin, lincomysin, erythromycin) are effective in treating Gram-positive udder infections both by parenteral and intramammary administration (Du Preez 2000). They are bacteriostatic and thus act in conjunction with the host immune system to fight infection. The mechanism of action is to inhibit protein synthesis by binding to the 50S ribosomal sub-unit to prevent peptide elongation (Prescott et al. 1996).

Table 2. Recommended remedies for dry cow treatment, withdrawal period and activity spectrum (Du Preez 2000; Van Dijk 2007).

Remedy	Milk withdrawal period	Antibiotic Composition	Activity Spectrum (if sensitive)
Bovaclox DC	30 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Cephudder	21 days	Cephapirin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Cepravin DC	4 days	Cephalexin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Curaclox DC	2.5 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Curaclox DC XTRA	4 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Dispolac DC	None specified	Penicillin, dihydrostreptomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Arcanobacterium pyogeness*
Dri Cillin	2.5 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Masticillin DC	28 days + 10 milkings after calving	Cloxacillin	*S. aureus*, streptococci
Masticlox DC	2.5 days	Cloxacillin	*S. aureus*, streptococci
Masticlox Plus DC	None specified	Cloxacillin, ampicillin	*S. aureus* streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Masticlox Plus DC EXTRA	4 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Nafpenzal DC	3 milkings	Penicillin, dihydrostreptomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Arcanobacterium pyogenes*
Neomastitar DC	5 weeks	Penicillin, neomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Noroclox DC	2.5 days	Cloxacillin	*S. aureus*, streptococci
Noroclox DC EXTRA	2.5 days	Cloxacillin	*S. aureus*, streptococci
Orbenin EXTRA DC	4 days	Cloxacillin, blue trace dye	*S. aureus*, streptococci
Pendiclox DC	24 hours after blue colour disappears	Cloxacillin, ampicillin, blue tracer dye	*S. aureus,* streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Penstrep DC	24 hours after blue colour disappears	Penicillin, dihydrostreptomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Arcanobacterium pyogenes*
Rilexine 500DC	4 weeks	Cephalexin, neomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)

Table 3. Recommended remedies for lactating cow treatment, withdrawal period and activity spectrum (du Preez 2000; van Dijk 2007; IVS Desk Reference 2005/2006).

Remedy	Milk withdrawal period	Antibiotic Composition	Activity Spectrum (if sensitive)
Cloxamast LC	3 days	Cloxacillin, ampicillin	Septic mastitis. *S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Curalox LC	3 days	Cloxacillin, ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Dispolac RX 4	24 hours after blue colour has disappeared	Penicillin, dihyrostreptomycin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*
Lactaclox	2.5 days	Cloxacillin	*S. aureus*, streptococci
Lactaciliin	3 days	Ampicillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Lincocin Forte	2.5 days	Lincomycin, neomycin	*Staphylococcus aureus,* streptococci
Mastijet Forte	4 days	Oxytetracycline, neomycin, bacitracin, cortisone	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Nafpenzal MC	6 milkings in treatment + 3 milkings after treatment	Penicillin, dyhrostreptomycin, nafcillin	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Arcanobacterium pyogenes*
Noroclox QR	24 hours after blue colour has disappeared	Cloxicillin, blue tracer dye	*S. aureus*, streptococci
Pendiclox Blue	24 hours after blue colour has disappeared	Cloxicillin, ampicillin, blue tracer dye	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.)
Penstrep 300 D	24 hours after blue colour has disappeared	Penicillin, dihydrostreptomycin, blue tracer dye	Acute mastitis. *S. aureus*, streptococci, soliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus, Arcanobacterium pyogenes*
Rilexine LC	4 days	Cephalexin, neomycin, cortisone	Acute & chronic mastitis
Spec Form Forte	3 days	Penicillin, dihydrostreptomycin, novobiocin, polymyxin B, cortisone	Acute or chronic mastitis. *S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Pseudomonas aeruginosa, Arcanobacterium pyogenes*
Streptocillin	24 hours after blue colour has disappeared	Penicillin, dihyrostreptomycine, blue tracer dye	*S. aureus*, streptococci, coliforms (*E. coli* & *Klebsiella* spp.), *Clostridium perfringens*, *Bacillus cereus*, *Arcanobacterium pyogenes*

2.1.7 Antibiotic resistance and human health

Many bacteria develop resistance mechanisms, enabling them to inactivate antimicrobial compounds in their environment. Genetic exchange between similar or different bacterial species may result in the spread of resistance genes (Prescott et al. 1996). Pathogens in animals that are used for food products pose one of the greatest risks for human health, as this is a major route for the transfer of bacteria from animals to humans (Mevius et al. 2005). The incorrect use of antibiotics in the treatment of diseases such as mastitis and for use in feed as growth promoters has led to the assumption that antibiotic resistance in bacteria could become more widespread because of the transmission of resistant zoonotic and non-zoonotic bacteria via food. This could have a serious impact on the treatment of bacterial pathogens causing disease in humans if similar antibiotics are used (Shryock 2004). Pathogens associated with mastitis can also infect humans, e.g. food poisoning by *S. aureus*. The sources of many outbreaks of food poisoning in France were due to the growth of *S. aureus* in raw and processed dairy products (Kérouanton et al. 2007). Another mastitis causing pathogen, *S. agalactiae,* causes septicaemia and neonatal meningitis (Petrovski et al. 2006).

Veterinarians are ethically required to administer antibiotics in such a manner as to protect animals and prevent the spread of disease. In addition, the spread of zoonoses to humans must also be prevented and the human food safety must be ensured (Passantino 2007). Antibiotics have been used for many years to eliminate bacterial pathogens causing disease. In the case of mastitis, it is important to note that antibiotic therapy cannot be relied upon to reduce the incidence of mastitis as a stand-alone anti-mastitis action. A good example is Norway and Sweden who have embarked on mastitis control strategies in which one of the core aims in reducing mastitis was also to reduce the use of antibiotics in all animal productions by 25 % (Ekman and Østerås 2003; Østerås and Solverod 2005).

The question as to whether widespread use of antibiotics to treat mastitis in dairy cattle has led to antibiotic resistance remains unanswered. In a review by Erskine et al. (2004), trends in resistance to antimicrobial drugs were investigated and although many examples of antimicrobial resistance have been recorded over the last three decades, results are not consistent. Comparisons between studies are difficult, primarily because different methods were used to determine susceptibility of pathogens to antibiotics. The recommended method of minimum inhibitory concentration (MIC) by the NCCLS (2002) should be used to determine the emergence of resistance patterns. The isolation and genetic typing of bacteria is also essential in tracking resistance and relatedness to similar human pathogens (Mervius et al. 2005).

Methicillin (oxacillin) resistance by *S. aureus* is of primary concern due to the emergence of hospital-acquired infections caused by methicillin resistant *S. aureus* (MRSA). The transmission of MRSA occurs mostly within a hospital environment, but community acquired MRSA has also emerged (Mervius et al. 2005). Montioring MRSA in bovine populations and specifically in food products such as milk, is important to determine how closely related MRSA isolates are between bovine and human populations. A report by Lee (2003) demonstrated the importance of monitoring MRSA from animal origin food sources and found that six bovine milk isolates were very closely related to a human MRSA isolates, suggesting the plausibility of the transfer of MRSA via food.

Macrolide antibiotic resistance is found in bovine isolated streptococci (Loch et al. 2005), coagulase-positive *S. aureus* (Khan et al. 2000) and in coagulase-negative staphylococci (Lüthje and Schwarz 2006). Macrolide antibitotics such as lincomycin are still used in both lactation and dry cow therapy and as a result may add selective pressure for resistant strains, especially in dry cow therapy where long-term exposure to antibiotics occurs (Mervius et al. 2005).

2.1.8   What are the alternatives?

The risks involved in the treatment of mastitis has been discussed in terms of the development of antibiotic resistance, but from a commercial standpoint, milk products containing specific levels of antibiotic residues cannot be sold for human consumption. Processing of milk for cheese and yoghurt manufacture is also affected as bacterial starter cultures are inhibited and the quality of the product produced is generally compromised (Miles et al. 1992). Completely eliminating the use of antibiotics for the treatment of mastitis is unlikely, as modern intensive farming practices and high demand dictate rapid and intensive treatment strategies, which involve the use of antibiotic therapy in both lactation and dry periods. The ultimate goal would be to reduce the use of antibiotics. This could primarily be achieved through better management and hygiene practices and legislation enforcing a reduction in the indiscriminate use of antibiotics for treatment and for growth promotion, as was done in Nordic countries in 1980's (Ekman and Østerås 2003). Improving host defences can result in rapid elimination of new infections. Supplementing of selenium and vitamin E and improving general nutrition during high-risk periods such as periparturient and drying-off periods can increase host defence mechanisms (Moyo et al. 2005).

Vaccination is one of the new emerging technologies aimed at improving resistance to infections, especially *S. aureus* and coliform infections (Miles et al. 1992). *E. coli* J5 vaccines (UPJOHN J-5 BACTERIN™, Escherichia Coli Bacterin J-5 Strain, Pfizer Animal Health) are core antigen vaccines that can reduce the number and severity of clinical cases caused by coliform pathogens. Crist et al. (1997) report a 70 – 80 % reduction in clinical cases after vaccinating at drying off, 30 days before calving and at calving. Another mechanism aimed at stimulating host cell immunity has been proposed using a polysaccharide sugar known as "Poly-x" from yeast. Preliminary reports showed a decrease

in the rate of new infections during the dry period after the administration of the "Poly-x" sugar (Suszkiw 2006).

Antimicrobials are still, however, necessary to combat bacterial pathogens and a solution could be to focus research into the development of other antimicrobial agents that offer some advantages over the antibiotics that are currently in use. In a review by Shryock (2004), the future use of antibiotics in treating animals, especially food animals, is shifting towards alternatives because of food safety concerns as well as business factors. This resulted in directing funding away from antibiotic research to new anti-infective technologies. Another driving force in exploring alternatives to antibiotics is the move towards organic dairy farming, which prohibits the use of antibiotics. Some of the new technologies include antimicrobial peptides produced by plants, animals and insects; bacteriocins produced by bacteria, and bacteriophages to treat bacterial disease in animals and humans.

Bacteriocins are produced by microorganisms naturally and are antagonistic towards bacteria in their environment as a natural defence mechanism for survival. They offer an advantage over antibiotics in that they target very specific organisms (usually closely-related species). The benefits of using bacteriocins in treating mastitis infections needs to be explored, but one factor that drives research into the use of bacteriocins is that they are generally regarded as safe for humans. Bacteriocin residues in milk and milk products would not carry the same level of risk in terms of milk quality, processing for cheese and yogurt and food safety issues (Miles et al. 1992). Current research and applications into the use of bacteriocins in the food industry, medical and veterinary fields and their use for mastitis treatment will be discussed.

2.2 BACTERIOCINS – EXPLORING ALTERNATIVES TO ANTIBIOTIC TREATEMNT

2.2.1 Introduction

The study of the antibacterial properties of peptides that became known as colicins began in 1925 when one strain of *E. coli* produced an antagonistic effect against another *E. coli* culture (Gratia 1925). The antibiotic effect between other enteric bacteria was also reported by Fredericq and Levine (1947) and further research into these proteinaceous molecules centred on colicins that were active against *E. coli* and various other members of the family *Enterbacteriaceae*.

Colicin-like molecules produced by Gram-positive bacteria have also been studied extensively since the first report of nisin produced by *L. lactis* subsp. *lactis* (Rogers 1928). The term "bacteriocin" was used to describe these antibiotic substances as not all were produced by coliform bacteria (Jacob et al. 1953) and according to Tagg et al. (1976), were defined as ribosomally synthesized polypeptides that usually possess a narrow spectrum of antibacterial activity against bacteria of the same or closely related species. Jack et al. (1995) however noted some discrepancies in this definition in that some bacteriocins (or bacteriocin-like substances) have a broader spectrum of activity and some are even active against Gram-negative species.

2.2.2 Classification of bacteriocins

Klaenhammer (1993) classified bacteriocins on the structure and mode of action of the peptide and predominantly included those produced by lactic acid bacteria (LAB). Four distinct classes were identified: class I, small lantibiotics (<5 kDa), that contained the amino acids lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine; class II, small

(<10 kDa), heat-stable, non-lanthionine containing peptides; class III, large (>30 kDa), heat-labile proteins and class IV, consisting of complex bacteriocins containing carbohydrate or lipid moieties that were required for bacteriocin activity.

Class I lantibiotics were further divided into type A and B, where type A consisted of peptides that were elongated with a net positive charge and acted on bacterial cell membranes. Type B lantibiotics were globular and had either a net negative charge or no charge at all and their mode of action was related to the inhibition of specific enzymes.

Class II bacteriocins were sub-divided into 3 groups: class IIa bacteriocins had consensus in their N-terminal amino-acid sequence and were significant in that they were active against the food spoilage strain, *Listeria* (Ennahar et al. 2000). Class IIb bacteriocins were composed of two peptides that were both required for its activity. Lastly, class IIc bacteriocins included all other bacteriocins not classified into classes IIa and IIb (Chen and Hoover 2003).

More recently a new classification system was proposed by Cotter et al. (2005a) where only 2 classes of bacteriocins were characterised, dividing the lanthionine (lantibiotics) and non-lanthionine containing bacteriocins into class I and class II, respectively. The lanthionine-containing bacteriocins were similarly classified into elongated amiphilic cationic bacteriocins and globular lantibiotics. The peptides were further divided into 11 sub-groups based on their unmodified peptide sequences (Cotter et al. 2005b).

Class I lantibiotics were described as peptides that underwent post-translational modification, acting against sensitive bacteria through the formation of pores and prevention of peptidoglycan synthesis. Class II non-lanthionine containing bacteriocins had no post-translational modification and acted by causing leakage of molecules from target bacteria after permeabilisation of the cell membrane. Sub-classes were characterised as class IIa pediocin-like or *Listeria*-active, IIb were the two-peptide bacteriocins that required the

combined activity of both peptides and IIc bacteriocins had a covalently linked N- and C-termini resulting in a cyclic structure. A class IId was added and included non-pediocin single linear peptides such as lactococcin A (Cotter et al. 2005a).

Those bacteriocins forming part of class III were designated as "bacteriolysins". Bacteriocins of Class IV, containing non-protein moieties, were excluded from the classification, since little research verifying their activity was demonstrated (Cotter et al. 2005a).

2.2.3   Applications of bacteriocins

The antibacterial activity of bacteriocins has resulted in research into the practical applications thereof and can be broadly divided into two focus areas: food production and preservation, by preventing the growth of unwanted or disease-causing organisms and secondly, medical and veterinary applications. Traditionally, antibiotics have been administered to prevent and treat disease. However, with the widespread development of antibiotic drug-resistant strains, the importance of alternative antimicrobials is becoming increasingly urgent and bacteriocin-producing organisms could be considered as an important source of antimicrobial agents in the medical and veterinary fields. The important role that bacteriocins continue to play in food production and clinical applications will be discussed.

2.2.3.1 Application in food production

The interest in bacteriocins produced by LAB stemmed from the obvious potential use of these bacteriocins in the preservation of food. LAB have been used for centuries in the production of fermented food products such as cheese, yoghurt and meat. Bacteria that are safe to consume are referred to as GRAS (generally regarded as safe) and have been the

target of much research in the prevention of food spoilage in fermented and other food products (Jack et al. 2005).

The application of bacteriocins in food preservation usually falls within one of three approaches. Firstly, the purified bacteriocin as a food additive is applied to a food product or packaging. Secondly, the starter culture, producing a bacteriocin has attributes necessary to produce the fermented product and antibacterial properties for food preservation. Finally, a previously fermented food product produced by bacteriocin-producing bacteria is added as an ingredient to the processed food (Schillinger et al. 1996).

Researchers most often target pathogenic bacteria commonly associated with food spoilage or food-borne illness to determine the potential application of a bacteriocin. *Listeria monocytogenes* is associated with food-borne outbreaks of disease and is particularly hardy. It can grow over a wide pH range (4.1 – 9.6) and temperature range (0 – 45 ° C), is resistant to desiccation and can grow anaerobically and therefore will not easily be destroyed in food processing (Chen and Hoover 2003). Other bacteria that pose a problem in food production include *Clostridium* spp. The bacterial spores contaminate milk from the environment and pose a serious problem in the ripening process of hard cheeses, such as Emmental, Beaufort and Comté, as a result of late blowing (Das Gupta et al. 1989). The commercial value of the cheese is lost due to bursting, splitting and rancid flavours caused when the contaminating *Clostridium* bacteria, primarily *Clostridium tyrobutyricum*, ferment lactic acid to butyric acid, acetic acid, carbon dioxide and hydrogen (Le Bourhis et al. 2007).

Many bacteriocins produced by Gram-positive bacteria have been characterised and may find application in food production. A few examples include the bacteriocins nisin, lacticin 3147 and lacticin 481 produced by *L. lactis* subsp. *lactis* (Delves-Broughton 1990; Piard et al. 1992; Ryan et al. 1996); bacteriocins produced by *Streptococcus thermophilus*, including thermophilin 81 (Ivanova et al., 1998), thermophilin 13 (Marciset et al. 1997),

thermophilin 580 (Mathot et al. 2003) and macedocin, produced by *Streptococcus macedonicus* ACA-DC 198 (Georgalaki et al. 2002).

2.2.3.2 Application in medical and veterinary fields

Bacteriocins, by definition usually only target closely related species; they could offer an advantage over antibiotics in that treatment could be targeted against specific pathogenic organisms. Bacteriocins, identified for potential use as antimicrobials include lantibiotics produced by Gram-positive lactic acid bacteria, and colicins and microcins, produced by Gram-negative bacteria (Gillor et al. 2005). Applications are widespread, ranging from topical applications in the treatment of skin infections to the treatment of inflammation and ulcers. Commercial products are currently available for the treatment of mastitis in dairy cattle and will be discussed in more detail. Table 4 summarises some of the potential applications of some bacteriocins in the medical and veterinary field. Most testing for clinical applications have been carried out in animal models, however the bacteriocin nisin has already undergone human clinical trials for the treatment of peptic ulcers caused by *Helicobacter pylori* (Hancock 1997). Bacteriocins produced by Gram-negative bacteria can be advantageous in that they can be used to target other pathogenic Gram-negative strains. Bacteriocins produced by Gram-positive LAB are not active against Gram-negative strains without pre-treatment strategies to compromise the integrity of the outer membrane (Cotter et al. 2005a). For example, nisin, after treatment with ETDA, citrate and lactate, was shown to be effective against *Salmonella typhimurium* and *E. coli* 0157:H7 (Cutter and Siragusa 1995). In contrast, colicins produced by Gram-negative *E. coli* are naturally active against other *E. coli* strains as well as some Salmonella strains (Braun et al. 1994). Microcins produced by enteric bacteria, usually target strains in the family *Enterobacteriaceae* (Moreno et al. 1995).

Bacteriocins produced by Gram-positive strains can substitute antibiotics such as ionophores routinely applied as feed additives for livestock animals, such as cattle. The ruminal bacterial populations of Gram-positive bacteria that produce excessive fermentation products, such as methane and ammonia, can be inhibited, without the dangers and perceived risks of antibiotics in feed rations (Russell and Mantovani 2002).

Table 4. Potential medical and veterinary applications of some bacteriocins

Bacteriocin	Producer	Potential use	Reference
Gram- positive bacteria			
Nisin	*L. lactis* subsp. *lactis*	Treat peptic ulcer disease Antimicrobial activity in medical devices such as catheters Treat *S. pneumonia* infections Treat mastitis in cattle Vaginal contraceptive agent	Hancock (1997) ; Bower et al. (2002) ; Goldstein et al. (1998) ; Sears et al. (1992) ; Reddy et al. (2004)
Lacticin 3147	*L. lactis* subsp. *lactis*	Treat mastitis in cattle	Ryan et al. (1998)
Galliderm	*Staphylococuccus gallinarum*	Treat skin infections such as acne	Kellner, et al. (1988)
Epidermin	*S. epidermidis*	Treat skin infections such as acne	Allgaier et al. (1986)
Mutacin B-Ny266	*Streptococcus mutans*	Bacterial infection caused by methicillin-resistant staphylococci	Mota-Meira et al. (2005)
Tomicid	*Streptococcus* sp. Thom-1606	Streptococcoal respiratory infections (Scarlet Fever) in children	Golshmid and Landsman (1989); Briko and Zhuravlev (2004)
Gram-negative bacteria			
Microcins J25 and 24	*E. coli*	Treat *E. coli* and salmonella infections in chickens	Sable et al. (2000); Wooley et al (1999)
Colicins E1, E4, E7, E8, K &S4	*E. coli*	Treat haemorrhagic colitis and haemolytic uremic syndrome cause by *E. coli* 0157:H7	Jordi et al. (2001)

2.2.4   Bacteriocins used in the treatment of mastitis

2.2.4.1 Introduction

The most economically costly disease in cattle is mastitis. As a result the dairy industry could benefit greatly from the development of safe antimicrobial agents and bacteriocins could be an attractive alternative to antibiotics. The treatment of mastitis has been a target of research since the inception of scientific research into the applications of bacteriocins (Taylor et al. 1949). To date, only the lactococcal bacteriocin, nisin, has been developed for commercial application and the lantibiotic, lacticin 3147, has been extensively researched for dry cow therapy. Applications for prevention and treatment using these lactococcal bacteriocins will be discussed in detail below.

Other bacteriocins that are active against mastitis pathogens have also been investigated. Researchers have targeted staphylococci and streptococci isolated from the normal flora of the teat canal and other areas as these could be a source for bacteriocins to treat mastitis pathogens. The potential applications for these bacteriocins will also be discussed.

2.2.4.2 Lacotococcal bacteriocins

2.2.4.2.1   Nisin

Classification and mode of action

Nisin was the first bacteriocin applied to the preservation of food products and was approved for use in pasteurised processed cheese spreads in 1988 by the FDA (Delves-Broughton 1990). Nisin is classified as a class Ia lantibiotic (Klaenhammer 1993) and is a 34 amino acid peptide (3488 Da). Nisin has a dual mode of action, which essentially involves the prevention of cell wall synthesis and pore formation, leading to cell death. The precise mechanism involves binding to lipid II molecules (Undecaprenyl-pyrophosphate-MurNAc(pentapeptide)-GlcNAc) located in the cell membrane of the target cells. Lipid II is

the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall and when nisin binds to lipid II, it prevents the transfer of the peptidoglycan across to the cell wall (Cotter et al. 2005a). The process of pore formation is initiated in the membrane of the target cell after docking at lipid II occurs and results in the efflux of cytoplasmic compounds that are required to maintain ion gradients, thereby affecting trans-membrane potential and the pH gradient across the membrane. Biosynthetic processes such as ATP synthesis driven by proton motive force cease and cell death occurs (Rhur and Sahl 1985; Sahl 1991).

Nisin has a wide spectrum of activity against Gram-positive bacteria, including species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (Cintas et al. 1998). Nisin is also active against *L. monocytogenes* and its efficacy against this food pathogen in raw meat products have been evaluated by Pawar et al. (2000), as well as in dairy products (Bhatti et al., 2004). Nisin has also been applied to cheese products to control the growth of spores produced by *Closrtidium tyrobutyricum* (Schillinger et al. 1996; Rilla et al. 2003).

Mastitis treatment

Sears et al. (1992) investigated the use of a nisin-containing germicidal formulation in preventing mastitis in cattle. Teat sanitisers are routinely used before and after milking cows to prevent the introduction of pathogens into the teat canal, which could lead to intramammary infections. The study compared the nisin-based formulation (Ambicin® N, Applied Microbiology, Inc., New York, NY) with that of conventional chemical treatments such as iodines and chlorohexidines. Initial performance data for a nisin-based teat sanitizer (Amibicin N®) showed a significant reduction in pathogen in experimentally challenged teat surfaces after 1-minute exposure to the germicidal formulation (Table 5). The formulation also showed little potential for skin irritation after repeated exposure in contrast to 1 %

iodophore and 5 % chlorohexidine digluconate preparations. Table 6 shows the skin irritation data reported by Sears et al. (1992). Dermal irritation scores indicated the degree of redness or scab formation, with a score of <1.0 indicating a product with little or no potential for irritation. Products with a score of ranging from 3.0-4.9 would have the potential to cause severe irritation.

Table 5. Performance data for nisin-based germicidal teat sanitizer (Sears et al. 1992)

Mastitis-causing organisms	Reduction using Ambicin N®
S.aureus	61.8 %
S. agalactiae	98.6 %
E. coli	85.5 %
S. uberis	67.1 %
K. pneumoniae	76.5 %

Table 6. Comparative skin irritation to rabbit skin after exposure to teat sanitizer

	Dermal irritation scores	
Teat sanitizer	Single application (72 hr after application)	Multiple application (72 hr after the last of 7 daily applications)
Amibicin N® (nisin-based sanitizer, 1x concentration)	0.21	0.30
Amibicin N® (nisin-based sanitizer, 12x concentration)	0.09	0.04
1 % Iodophor	0.5	3.34
5 % Clorohexidine digluconate	0.38	2.34

Contamination of milk with a sanitizer chemical based product is a concern if it is not completely removed before milking. Using bacteriocin-based sanitizers or products would be advantageous in that complete removal of the product would not necessarily be required.

In addition to Ambicin®, two other nisin-based products, namely Wipe-Out® Dairy Wipes and Mast Out® were developed by Immucell Corporation (Cotter et al. 2005a). Mast

Out® was used in January 2004 in initial field trials involving 139 cows with subclinical mastitis. Significant cure rates were reported and the product was subsequently licensed to Pfizer Animal Health for further development and distribution (Immucell Corporation 2004). The product has however not been made available by Pfizer Animal Health and no further trial results have been reported.

2.2.4.2.2   Lacticin 3147

Classification and mode of action

Lacticin 3147 is produced by *L. lactis* subsp. *lactis* DPC3147 and was first isolated from Irish Keffir grain (Ryan et al. 1996). As with nisin, it is also classified as a Class 1a lantibiotic, but it differs from nisin in that it is a two-peptide lantibiotic, requiring both the LtnA1 and LtnA2 peptides for full activity. The mode of action of lacticin 3147 is similar to that of nisin in that it results in the inhibition of cell synthesis and pore formation in the target cell (Wiedeman et al. 2006).

The primary structure of the lacticin A1-peptide, LtnA1, consists of 30 amino acids (3306 Da) and has a lanthionine-bridging pattern resulting in a globular structure similar to class Ib lantibiotics such as mersacidin. The LtnA2 peptide consists of 28 amino acids (2847 Da) and is an elongated peptide. Wiedeman et al. (2006) proposed a three-step model to describe how both peptides are involved for antibacterial activity of lacticin 3147. Fig. 3 illustrates this process. LtnA1 first binds to lipid II (i), thereby inducing a conformation that facilitates the interaction with LtnA2. This enables the formation of a two-peptide-lipid II complex (ii). When bound to the complex, LtnA2 is able to adopt a transmembrane conformation that results in the formation of a defined pore and the release of ions across the membrane (iii). In an earlier study, McAuliffe et al. (1998) reported that the pore formation resulted in the efflux of potassium ions and inorganic phosphate, resulting in the dissipation

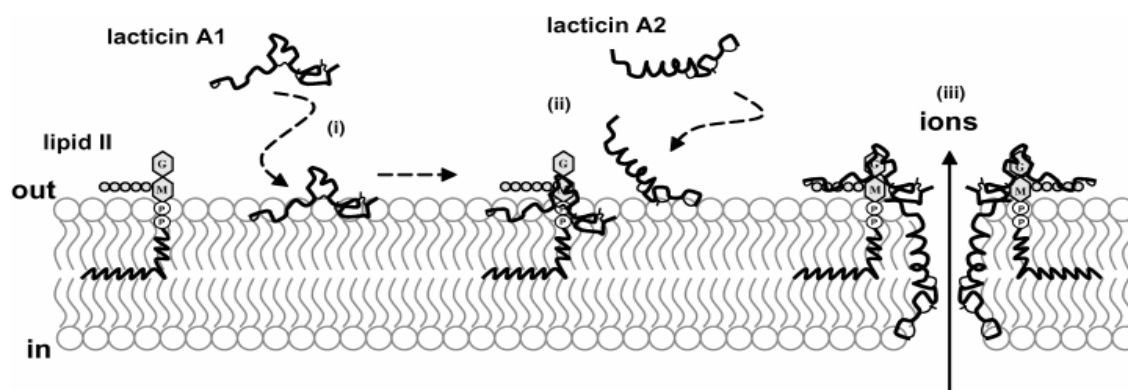of the membrane potential and hydrolysis of internal ATP, the collapse of the pH gradient and cell death.

Fig 3. Proposed model by Weidemann et al. (2006) describing mode of action of lacticin 3147.

Lacticin 3147 has a broad spectrum of antimicrobial activity and inhibits the growth of *Bacillus* sp., *Enterococcus* sp., *Lactobacillus* sp., *Pedicococus pentriceans*, *S. aureus*, *S. thermophilus* and most mastitis-causing streptococci. Food-borne spoilage bacteria, including *L. monocytogenes* and *C. tyrobutyricum*, are sensitive to lacticin 3147 and the peptide could be used to prevent food spoilage and disease (Ryan et al. 1996).

Mastitis treatment

Lacticin 3147 was investigated for use as an antimicrobial agent as it inhibited common mastitis-causing pathogens, including *S. aureus*, *S. dysgalactiae*, *S. uberis* and *S. agalactiae* (Ryan et al. 1998).   The producing organism is GRAS and is active at both low and physiological pH and was heat stable (Ryan et al. 1996; Ryan et al. 1999).

Teat seal formulations such as Orbeseal® (Pfizer Animal Health 2004) are recommended for use during the dry period as a prophylactic measure to reduce the number of new mastitis infections (Berry and Hillerton 2002). The inert property of the teat seal formulation has no antimicrobial effect and therefore relies on good udder hygiene practices for effective treatment. Antibiotics such as cloxacillin have been added to the formulations

(Orbenin® Extra Dry Cow, Pfizer Animal Health) to prevent new infections during this period. However, prolonged exposure to antibiotics at low levels could increase the risk of antibiotic resistance by pathogenic bacteria. Bacteriocins, such as lacticin 3147 could replace antibiotics in these formulations (Ryan et al. 1998; Ryan et al. 1999; Twomey et al. 2000). Studies to date have shown that resistance by mastitis pathogens *S. dysgalactiae* and *S. aureus* to the bacteriocin lacticin 3147 were not significant (Ryan et al. 1998).

In separate studies, the bismuth subnitrate-based teat seal (Osmonds Teat Seal 2, Cross Vetpharm Group Ltd., Dublin, Ireland) combined with lacticin 3147 was evaluated against the mastitis-causing pathogens *S. dysgalactiae* (Ryan et al. 1999) and *S. aureus* (Twomey et al. 2000; Crispie et al. 2005). Irritancy to the teat area and the somatic cell response were evaluated.

The protection given by the teat seal plus lacticin 3147 and the teat seal only were compared after experimental challenge with *S. dysgalactiae*. The results showed significant improvements in the level of protection afforded by the teat seal containing the bacteriocin 3147 (Table 7). Ninety-one percent of quarters treated with the teat seal plus lacticin 3147 remained free of new infections compared with only 33.3 % of quarters treated with the teat seal alone (Ryan et al. 1999).

Tissue tolerance studies were done comparing the SCC in the milk from quarters treated with the teat seal alone, teat seal plus lacticin 3147 and with a commercially available antibiotic infusion containing sodium cloxacillin. The SCC over 5 consecutive days after infusion was 7.22×10^5 and 5.71×10^5 SCC·mL^{-1} for the teat seal and the teat seal plus lacticin 3147 respectively. The highest SCC of 1.01×10^6 SCC·mL^{-1} was for the quarter infused with the antibiotic cloxacillin, while the untreated quarter had a SCC of 6.27×10^5 SCC·mL^{-1}. This data indicated that the lacticin 3147 was tolerated within the udder tissue and no visible sign of irritation or abnormality was reported (Ryan et al. 1999).

Table 7. Clinical mastitis and recovery of *S. dysgalactiae* in non-clinical mastitis in quarters after treatment with the teat seal only and the teat seal plus lacticin 3147 (Ryan et al. 1999).

Treatment	Total no of quarters treated	New clinical infections by *S. dygalactiae*	New non-clinical isolations of *S. dysgalactiae*
Teat seal	33	16 (48.5 %)	6 (18.2 %)
Teat seal plus lacticin 3147	35	3 (8.6 %)	0 (0 %)

Twomey et al. (2000) evaluated the effect of the teat seal plus lacticin 3147 with untreated quarters as controls, against experimental challenge by *S. aureus*. The concentration of the bacteriocin and inoculum of the *S. aureus* challenge was varied to optimise treatment conditions. The presence of the teat seal plus lacticin 3147 using a concentration of 32 768 AU/4g of teat seal, resulted in a significant decrease in the number of teats shedding *S. aureus* (Table 8). The antagonistic effect of the bacteriocin at the same concentration was however reduced when the inoculum of the *S. aureus* challenge introduced into the teats was increased. The concentration of the bacteriocin used was found to be significant factor for the teat seal to be effective in reducing *S. aureus* in the teats.

Table 8. The effect of teat seal plus lacticin 3147 in eliminating *S. aureus* in artificially infected cows. Shedding evaluated after 18h (Towmey et al. 2000).

Inoculum	Lacticin 3147 AU/4g of teat seal	Treatment	Total teats inoculated	Teats shedding *S. aureus*	% Teats successfully treated
1.7×10^3	32 768	Untreated	29	19	34.5
		Teat seal + lacticin 3147	29	4	86.2
6.8×10^3	32 768	Untreated	20	16	20.0
		Teat seal + lacticin 3147	20	11	45.0

The initial evaluation of lactitin 3147 by Ryan et al. (1998) indicated that bacteriocin produced in a synthetic growth medium was not adequately released from the teat seal formulation without the addition of a surfactant (Tween 80). Later research improved the efficacy of the teat seal formulation by producing lacticin 3147 in milk-based (whey) medium which resulted in an increase in activity from ~320 $AU \cdot mL^{-1}$ to ~500 $AU \cdot mL^{-1}$ in the fermentate after 24 hr incubation. The increase in activity of the bacteriocin preparation resulted in a significant release of the peptide in the teat seal formulation without the addition of Tween 80, thereby providing a cost-effective method of producing larger quantities of the bacteriocin (Crispie et al. 2005).

The lacticin 3147 produced in the milk-based (whey) medium reduced the number of *S. aureus* recovered after experimental challenge. The average recovery of *S. aureus* from teats infused with teat seal plus lacticin 3147 was 7.3 x 10^2 $cfu \cdot mL^{-1}$ compared with 1.6 x 10^4 $cfu \cdot mL^{-1}$ for those treated with the teat seal alone. The bacteriocin-teat seal preparation also appeared to eliminate *S. aureus* cells already present in the teat canal prior to the infusion of the product compared to the teat seal alone. No viable *S. aureus* cells were recovered from the teats where the bacteriocin was present in the teat seal, compared to four of the teats where only the teat seal was used (n = 8) (Crispie et al. 2005).

The stability of the product for the dry period of 50-60 days would still need to be assessed adequately as the teat seal-bacteriocin product evaluated by Twomey et al. (2000) and Crispie et al. (2005) was only infused for a period of 18 hours. Ryan et al. (1999) however showed that in an 8-day period, lacticin 3147 retained activity in the teat environment.

To summarise, research has shown that the bacteriocin lacticin 3147 has the potential for use in a teat seal preparation to effectively prevent new infections by streptococci and offer some protection to *S. aureus* infection. The bacteriocin could potentially be produced

on large scale using a milk-based (whey) medium at concentrations that are active against target organisms. The bacteriocin is also active and insoluble at physiological pH and thus remains effective in the teat canal environment.

2.2.4.3 Other bacteriocins that could have potential use in mastitis treatment

2.2.4.3.1   Staphylococcal bacteriocins

Bacteriocins from Gram-positive bacteria have, to a large extent, been limited to applications in the food industry. Potential applications of other bacteriocins in mastitis treatment have been limited to that of lacticin 3147 (Crispie et al. 2005) and nisin (Sears et al. 1992).

Growth inhibition studies of mastitis pathogens by normal bovine teat skin flora (Woodward et al. 1987; De Vliegher, et al. 2003) have been attempted to evaluate the antagonistic or other effect that these less pathogenic bacteria could have on major mastitis-causing pathogens such as *S. aureus*, *E.coli* and streptococci.   Staphylococcal strains associated with mastitis were investigated and it was found that bacteriocins active against mastitis-causing *Streptococcus agalactiae* isolates were primarily produced by *S. epidermidis*, *S. saprophyticus* and *S. arlettae* (dos Santos Nascimento et al. 2005).

2.2.4.3.2   Streptococcal bacteriocins

Many streptococci have been found to produce bacteriocins and the potential applications of these bacteriocins range from those produced by the thermophilic lactic acid bacteria, for their potential application in cheese production to the oral streptococci for use in the treatment of dental carries.

No potential streptococcal bacteriocins have as yet been isolated for use in the treatment of mastitis. However, the natural ecological niche of a particular bacteriocin producer is

often the specific area that is targeted for practical application. The mastitis pathogen *S. uberis* is commonly found in the natural environment of dairy cattle and thus could also be competing with other bacteria in this ecological niche. Wirawan et al. (2006a; 2006b) screened more than 200 *S. uberis* strains from their culture collection to determine whether any of these strains produced bacteriocin-like inhibitory substances. Strain 42 was found to produce two bacteriocins, a natural nisin variant, nisin U and a circular peptide, uberlysin (Wirawan, et al., 2006b) The bacteriocin nisin U had activity spectra against *S. agalactiae*, *S. dysgalactiae* and *E. faecalis* that are considered to be potential mastitis-causing pathogens (Wirawan et al. 2006a). The discovery of this natural nisin variant, which is active against mastitis-causing pathogens, could offer a potential alternative to nisin A, especially in cases where nisin A resistance may occur in pathogenic strains. A combination of antimicrobials, such as a nisin variant with other bacteriocins could potentially be more effective in treatment strategies (Wirawan et al. 2006b).

Other streptococcal bacteriocin producers occur in the oral cavity where the normal flora such as *S. salivarius*, *S. pyogenes* and *S. mutans* are readily found. These produce bacteriocins or uncharacterised bacteriocin-like inhibitory substances (Tagg 2004; Tagg et al. 2006). Normal flora of the nasopharynx also consists of bacteriocin producing strains, including *S. salivarius* strains, and has been investigated for the prevention of streptococcal pharyngitis and otitis media (Walls et al. 2003; Tagg 2004). The type of treatment used is known as bacteriotherapy or bacterial interference, where bacteriocin producing, non-pathogenic strains are introduced into the nasopharynx to protect against recurrent streptococcal infections (Wall et al. 2003). The bacteriocin salvaricin A2 (SalA2), produced by *S. salivarius* K12 has been developed as an oral probiotic (BLIS K12 Throat Guard, BLIS Technologies, New Zealand) to treat streptococcal infections especially by *S. pyogenes* in children (Tagg et al. 2006).

Streptococcal bacteriocins produced by *Streptococcus thermophilus* strains are often investigated for use in yoghurt starter cultures, including thermophilin 81 (Ivanova et al. 1998) and thermophilin 13 (Marciset et al. 1997), while thermophilin 580, produced by *S. thermophilus* 580 has been studied for possible application in cheese production as starter cultures with the added benefit of bacteriocin inhibition of *C. tyrobutyricum* in the cheese ripening process (Mathot et al. 2003).

Larger bacteriocins (>10kDa) also produced by some streptococci are characterised as non-lytic inhibitory agents or as bacteriolytic enzymes. Examples include dysgalactin produced by *S. dygalactiae* subsp. *equisimilis* and streptococcin A-M57 produced by *S. pyogenes* M-57. Stellalysin is an example of a large 29-kDa bacteriocin produced by *S. constellatus* subsp. *constellatus*. The activity spectra of stellalysin includes *S. pyogenes, S. gordonii* and *S. mutans* (Heng et al. 2006).

The mutacins B-Ny266, J-T8 and B-JH1140, produced by *S. mutacin* have been characterised as belonging to the lantibiotic class of bacteriocins. Potential practical applications of mutacins include the treatment of dental carries (Hillman 2001). Mutacin B-Ny266 has been of particular interest due to its wide-spectrum of activity against many pathogenic Gram-positive and Gram-negative bacteria, including staphylococcal and streptococcal strains resistant to antibiotics. It could therefore find application for therapeutic use (Mota-Meira et al. 2000; Mota-Meira et al. 2005).

Rumen streptococci have also been investigated as a source of bacteriocins, with *S. bovis* as the predominant strain isolated (Whitford, et al., 2001). Bovacin 255 produced by *S. gallolyticus* 255, a class II bacteriocin and bovicin HC5 from *S. bovis* HC5 could find application in cattle farming (Whitford et al. 2001; Mantovani et al. 2002). Bacteriocins that inhibit Gram-positive LAB found in rumen can be advantageous as these bacterial species, through fermentation produce large quantities of methane and ammonia waste products.

Bacteriocins could be applied as feed additives to alter rumninal fermentation, and as a substitute to conventional antibiotics, such as monesin (Russell and Mantovani 2002).

The first report of a bacteriocin, namely macedocin produced by the thermophilic *S. macedonicus* ACA-DC 198, was characterised by Geogalaki et al. 2002. The bacterium was first isolated from Greek Kasseri cheese from Macedonia in Northern Greece and was subsequently named as *S. macedonicus* (Tsakalidou et al. 1998). Flint et al. (1999) also isolated *S. waius* from biofilms on stainless steel structures exposed to milk, but *S. waius* was subsequently found to be synonymous to *S. macedonicus* isolated by Tsakalidou et al. (1998) and reclassified as such (Manchini et al. 2002). The species forms part of the larger *S. bovis / S. equinus* complex but remains as a separate species, as low level of DNA homology (less than 70 %) exists with other closely related species such as *S. gallolyticus* (International Committee on Systematics of Prokaryotes Subcommitee on the taxonomy of staphylococci and streptococci 2003). More recently, *S. macedonicus* strains isolated from Italian raw milk cheeses were characterised (Lombardi et al. 2004).

Macedocin ACA-DC 198 is a bacteriocin that has been assessed as a food grade bacteriocin for use in cheese manufacturing as a starter culture, because it is able to produce the lantibiotic at pH and temperature conditions that prevail during cheese manufacturing, and it also inhibits the food spoilage bacteria *C. tyrobutyricum* (Van den Berghe et al. 2006). It has a molecular mass of 2,794 Da, as determined by electrospray mass spectrometry. Partial N-terminal amino acid sequence analysis revealed some homology to other streptococcal bacteriocins, SA-F22 and SA-M49, both produced by *S. pyogenes* (Georgalaki et al. 2002). No therapeutic applications have as yet been investigated for macedocin ACA-DC 198 and its activity spectrum has been largely restricted to food spoilage organisms.

2.2.5 Conclusion

The economic implication of mastitis as a recurrent disease in dairy farming warrants further research into developing new technologies in antimicrobial therapy. Bacteriocins can be considered as an alternative and does offer some advantages over conventional antibiotic therapy. Increasing concerns for human health, primarily due to the emergence of antibiotic resistance in pathogenic bacteria, also necessitates the development of alternative anti-infective agents.

Bacteriocins are usually active against specific bacterial strains based on target receptors on the surface of sensitive strains. When diagnosing mastitis, the causative bacteria needs to be clearly identified and a targeted approach for specific pathogens should be considered. Bacteriocins can kill susceptible organisms quickly by cell lysis. This rapid action could ensure that resistance is less likely to develop in pathogens. Antibiotics used are usually broad-spectrum, killing all Gram-positive or Gram-negative bacteria to which it is exposed to, not only those causing infection. Bacteriocins offer the advantage of a target-specific action. If a broader spectrum of activity is required, a combination of two or three bacteriocins could be considered to ensure that more than one pathogen be targeted during treatment.

The lowest minimum inhibitory concentration (MIC) of the bacteriocin should be established, as this would reduce the amount of bacteriocin used in the treatment product. The bacteriocin should also remain active and should persist in the target environment for a given period of time in order to come in contact with potential pathogens.

The method of drug delivery in a treatment strategy for mastitis is important and a teal seal offers many advantages. Firstly it acts as a physical barrier and is prophylactic. By combining an antibacterial agent in a teat seal, the inhibitor is localised in the teat canal, targeting pathogens that may be present near the teat opening and thus prevent bacteria from

colonising the mammary tissue. Topical preparations can also be used and due to the lack of invasiveness are more easily accepted as a form of drug delivery. The persistence and stability of the bacteriocin on the surface of the teat skin is essential but should not cause irritation or an allergic reaction to further inflame the teat area.

Bacteriocin-based products have been successfully tested in the past. Nisin has been used as a teat disinfectant in the commercial product, Wipe-Out® Dairy Wipes (Immucell Corporation) (Cotter et al., 2005a) for use throughout the lactation period, while lacticin 3147 has been evaluated for use as a dry cow therapy in a teat seal formulation (Ryan et al. 1998). Thus the route of administration, considering the teat-canal environment of the cow, as well as the production cycle of the cow are important considerations when determining the type of treatment product produced.

Bacteriocins produced by LAB are considered to be GRAS (generally regarded as safe) and would therefore be more acceptable when compared to antibiotics. Antibiotic therapy during lactation requires a withdrawal period, which results in economic losses due to wastage and loss of production time. Bacteriocin residues in milk are more acceptable as digestive enzymes easily destroy the peptides. Thus, the withholding periods would be significantly reduced if bacteriocin therapy were used instead of antibiotic therapy.

Considering the extensive costs of a disease such as mastitis to the dairy industry, research directed towards viable and safe alternatives should be considered. Bacteriocins can thus be viewed as a real treatment solution to augment other management strategies and reduce the amount of antibiotics used in the treatment of mastitis.

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CHAPTER 3

Bacteriocin ST91KM, produced by *Streptococcus macedonicus* ST91KM, is a narrow-spectrum peptide active against bacteria associated with mastitis in dairy cattle

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Abstract: *Streptococcus macedonicus* ST91KM, isolated from Bulgarian-style yoghurt, produces a bacteriocin (macedocin ST91KM) active against *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis*. Peptide ST91KM is, according to tricine-SDS PAGE, between 2.0 and 2.5 kDa in size. The activity of peptide ST91KM remained unchanged after 2 h of incubation at pH 2.0 to 10.0 and 100 min at 100 °C. The peptide was inactivated after 20 min at 121 °C and when treated with pronase, pepsin and trypsin. Treatment with α-amylase had no effect on activity, suggesting that the mode of action does not depend on glycosylation. Precipitation with 60 % saturated ammonium sulphate, followed by Sep-Pak C18 separation recovered 43 % of peptide ST91KM. Amplification of the genome of strain ST91KM with primers designed from the sequence of the macedocin prescursor gene (*mcdA*) produced two fragments (approximately 375 and 220 bp) instead of one fragment of 150 bp recorded for macedocin produced by *S. macedonicus* ACA-DC 198. Strain ACA-DC 198 was not available. However, the DNA fragment amplified from strain LMG 18488 (ACA-DC 206), genetically closely related to strain ACA-DC 198, revealed 99 % homology to the *mcdA* of *S. macedonicus* ACA-DC 198 (accession number DQ835394). Macedocin ST91KM may thus be a second putative bacteriocin described for *S. macedonicus*.

Key words: Bacteriocin ST91KM, *Streptococcus macedonicus*

Introduction

Mastitis in dairy cattle is caused by *Staphylococcus aureus*, *Streptococcus agalactiae*, methicillin-resistant strains of *S. aureus* (MRSA) and macrolide-resistant, coagulase-negative staphylococci and streptococci (Khan et al. 2000; Loch et al. 2005; Lüthje and Schwarz 2006). Infected animals are usually treated with broad-spectrum antibiotics, e.g. β-lactam penicillins and cephalosporins, tetracyclines, aminoglycosides and macrolides (Ziv 1980; Philpot and Nickerson 1999). Prophylactic dosages of these antibiotics are used in mastitis control programmes during the dry period and during lactation (NMC 2007). Although disputed by some authors (Erskine et al., 2004), this practise could select for strains with resistance to antibiotics (Passantino 2007). Macrolide-resistant strains of *S. aureus* (MRSA) and streptococci have been isolated from bovine milk (Khan et al. 2000; Loch et al. 2005; Lüthje and Schwarz 2006) and could be transferred to humans (Lee et al. 2003).

Bacteriocins (ribosomally synthesized peptides with antibacterial activity) produced by lactic acid bacteria are generally regarded as safe (GRAS). Bacteriocins are divided into three classes based on their structure and mode of action (Klaenhammer 1993). Class I are small peptides (< 5kDa) with unusual amino acids, such as lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine. Class II are small (<10 kDa), heat-stable, non-lanthionine containing peptides. The larger peptides (>30 kDa) are grouped into class III.

Lacticin 3147 and nisin, produced by *Lactococcus lactis* subsp. *lactis*, have been evaluated as possible alternatives to prevent mastitis infections (Sears et al. 1992; Ryan et al. 1998). Nisin-based teat sanitisers and teat dips proved effective in reducing the cell numbers of *S. aureus*, *S. agalactiae* and *Streptococcus uberis* by 62 %, 99 % and 67 %, respectively (Sears et al. 1992).

In this paper, we describe a bacteriocin produced by *Streptococcus macedonicus* with activity against *S. agalactiae, S. dysgalactiae, S. uberis, S. aureus* and *Staphylococcus*

epidermidis. As far as we could determine, only one bacteriocin has been described for *S. macedonicus,* e.g. the lantibiotic macedocin produced by strain ACA-DC 198 (Georgalaki et al. 2002).

Materials and Methods

Isolation of lactic acid bacteria and screening for production of antimicrobial compounds

A sample of Bulgarian goat yoghurt, obtained from a local cheese manufacturer, was serially diluted in sterile physiological water and plated onto MRS Agar (Biolab, Biolab Diagnostics, Midrand, SA). The plates were allowed to dry and overlaid with a second layer (10 mL) sterile MRS Agar (Biolab) to obtain micro-aerophilic conditions. After 24 h of incubation at 30 °C, the plates were overlaid with a thin layer (10 mL) of semi-solid BHI agar (BHI Broth, Merck, Darmstadt, Germany), supplemented with 0.7 % (w/v) agar and inoculated with an indicator strain ($1x10^4$ cfu·mL^{-1}). The indicator strains are listed in Table 1. After 18 h of incubation at 37 °C, colonies with the largest inhibition zones were selected and streaked onto MRS Agar (Biolab) to obtain pure cultures.

Antimicrobial activity was confirmed using the agar-spot method, as described by Van Reenen et al. (1998). Cell-free culture supernatants were exposed to 80 °C for 10 min to inactivate proteolytic enzymes and then adjusted to pH 6.0 to 6.5 with sterile 1 M NaOH. Antimicrobial activity was expressed as arbitrary units (AU)·mL^{-1}, calculated as follows: a^b x 100, where "a" represents the dilution factor 2 and "b" the last dilution that produces an inhibition zone of at least 2 mm in diameter. The strain with the broadest spectrum of antimicrobial activity was selected for further studies.

Identification of the producer strain

Key identification tests (Holt et al. 1994) were used to identify the selected strain (ST91KM) to genus-level. Identification to species-level was by PCR with species-specific primers, according to the method described by Papadelli et al. (2003). *Streptococcus gallolyticus* subsp. *macedonicus* LMG 18488 (ACA-DC 206) was used as reference strain. Amplified fragments were sequenced on an automatic sequencer (ABI Genetic Analyzer 3130XL Sequencer, Applied Biosystems), and compared with DNA sequences in Genbank using BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information http:// www.ncbi.nlm.nih.gov/BLAST/).

Isolation of plasmid DNA

The procedure of Anderson and McKay (1983), adapted for use with the Qiagen® Plasmid Midi Kit, was used. One hundred mL of MRS broth (Biolab) was inoculated with 1.0 % (v/v) of an overnight culture of strain ST91KM (OD_{600nm} = 1.4) and incubated at 30 °C until OD_{600nm} = 2.2. The cells were harvested (3000 x g, 15 min, 4 °C), washed in STE buffer (6.7 % sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8.0) and resuspended in 4 mL STE buffer with lysozyme (10 mg·mL^{-1}). After incubation at 37 °C for 2 h, 4 mL lysis buffer (200 mM NaOH, 1.0 %, w/v, SDS) was added to the cell suspension, mixed gently by inversion and left at 25 ºC for 5 min. Four mL of the neutralization buffer (3 M potassium acetate, pH 5.0) was added, mixed by gentle inversion, and incubated on ice for 15 min. The plasmid-containing supernatant was removed after two centrifugation steps (14 000 x g, 30 min, 4 °C). The Qiagen®-tip was equilibrated with 4 mL equilibration buffer (750 mM NaCl, 50 mM MOPS, pH 7.0, 15 %, v/v, isopropanol, 0.15 %, v/v, Triton® X-100) and the plasmid-containing supernatant allowed to enter the resin by gravity flow. The tip was washed twice with buffer (1.0 M NaCl, 50 mM MOPS pH 7.0, 15 %, v/v, iso-propanol) and eluted from the

resin. The DNA was precipitated with iso-propanol, collected (15 000 x g, 30 min, 4 °C) and washed with 70 % (v/v) ethanol. The pellet was suspended in TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and kept at 4 °C.

Partial purification of the antimicrobial peptide and size determination

Strain ST91KM was cultured in MRS broth (Biolab) for 15 h at 30 °C. The cells were harvested (8 000 x g, 10 min, 4 °C) and the bacteriocin precipitated from the cell-free culture supernatant with 60 % saturated ammonium sulphate (Sambrook et al. 1989). The precipitate was suspended in 25mM ammonium acetate buffer (pH 6.5) to one-tenth of the original volume and loaded onto a SepPak C18 cartridge (Water Millipore, MA, USA). Peptides were eluted with 20 %, 40 %, 60 % and 80 % (v/v) isopropanol, respectively, in 25 mM ammonium acetate buffer (pH 6.5). Fractions of 1 mL were dried under vacuum at 50 °C and stored at -20 °C. Antimicrobial activity was determined as described before. *S. agalactiae* (RPSAG2) and *Lactobacillus sakei* (LMG 13558) served as indicator organisms.

Protein precipitated with ammonium sulphate was separated by tricine-SDS-PAGE according to the method of Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.5 to 45 kDa (Amersham Bioscience, Europe GmbH, Freiburg, Germany) was used. The gels were fixed and one half stained with Coomassie Blue and a second half overlaid with MRS soft agar (0.7 %, w/v, agar), inoculated with *L. sakei* LMG 13558 (1×10^4 cfu·mL^{-1}).

Effect of enzymes, surfactants, protease inhibitors, temperature and pH on the bacteriocin

Strain ST91KM was cultured in MRS broth for 24 h at 30 °C. Cells were harvested (8 000 x g, 10 min, 4 °C) and the cell-free culture supernatant heat-treated as described before.

Volumes of 1 mL were treated with 1 mg·mL^{-1} (final concentration) pronase, pepsin and catalase (Boehringer Mannheim, GmbH, Germany), trypsin (BDH Chemicals Ltd, Poole, England) and α-amylase (Sigma Diagnostics, St. Louis, USA), respectively, and incubated for 2 h at 37 °C. Antimicrobial activity was monitored using the agar-spot method, as described before.

In a separate experiment, cell-free culture supernatants were treated with 1 % v/v (final concentration) SDS, Tween 20, Tween 80, Triton X-114, Triton X-100, EDTA and urea, respectively. Cell-free culture supernatant not treated served as control. All samples were incubated at 37 °C for 5 h and tested for antimicrobial activity as described before.

Cell-free culture supernatant (pH adjusted to 6.0 - 6.5 with 1 M NaOH) was incubated at 40, 60, 80 and 100 °C for 10, 30 and 60 min, respectively, and autoclaved (15 min at 121 °C). Residual antimicrobial activity was tested as described before. Cell-free supernatant was adjusted with sterile 1 M NaOH or 1 M HCl to pH values between 2 and 10, with increments of two pH units, kept at 80 °C for 10 min and then incubated for 2 h at 30 °C. The pH of each sample was corrected to 6.0 with sterile 1 M NaOH or 1 M HCl and tested for antimicrobial activity as described before.

Adsorption of the bacteriocin to strain ST91KM

Adsorption of the bacteriocin to strain ST91KM was determined according to the method described by Yang et al. (1992). Strain ST91KM was grown in MRS broth (Biolab) for 15 h at 30 °C, the pH of the culture adjusted to 6.0 with sterile 1 M NaOH and the cells harvested (20 000 x g, 15 min, 4 °C). The cells were resuspended in 10 mL 100 mM NaCl (pH 2.0) and incubated for 1 h at 4 °C on an orbital shaker (200 rpm). Cells were harvested (8 000 x g, 30 min, 4 °C) and the cell-free culture supernatant adjusted to pH 6.0 with sterile 1 M NaOH. Antimicrobial activity was determined as described before.

Amplification of gene mcdA, precursor of macedocin ACA-DC 198

S. macedonicus ACA-DC 198 was not available. Instead, *S. macedonicus* LMG 18488 (ACA-DC 206), a strain genetically closely related to ACA-DC 198, was used. Genomic DNA was isolated from *S. macedonicus* ST91KM and LMG 18488 according to the method of Dellaglio et al. (1973). Primers were designed for amplification of the macedocin prescursor gene *mcdA* according to the sequence listed in Genbank (accession number DQ835934). The primers were mcdAF (5′- ATGGAAAAAGAAACAACAAT, forward) and mcdAR (5′- TTATGAGCAACATGTAGCAA, reverse). Each 20 µL PCR-reaction mixture contained 1 x PCR buffer (with 2 mM Mg^{2+}), 100 µM of each dNTP, 200 µM of each primer, 0.5 U *Taq* DNA polymerase (*Takara Ex Taq*™, Separations, Johannesburg, South Africa) and 1 ng·µL^{-1} genomic DNA. The following conditions for PCR were used: denaturation at 94 °C for 5 min, followed by 30 x 30 s cycles at 94 °C, annealing at 45 °C for 30 s, and extension at 72 °C for 40 s, followed by final extension at 72 °C for 7 min. Amplicons were visualised on a 1.0 % (w/v) agarose gel stained with ethidium bromide. Bands corresponding in size to the *mcdA* precursor gene were excised from the gel and purified using the Qiagen PCR Purification Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa). The fragment was sequenced on an automatic sequencer (ABI Genetic Analyzer 3130XL Sequencer, Applied Biosystems), and compared with DNA sequences in Genbank using BLAST.

Results and Discussion

Heat-treated and pH-neutralised cell-free culture supernatant of strain ST91KM inhibited *S. agalactiae* RPSAG1 and RPSAG2, *Staphylococcus epidermidis* RPSE1, *L. sakei* LMG 13558 and *Micrococcus varians* RPMV1. Partially purified bacteriocin ST91KM, obtained by precipitation from a cell-free culture supernatant with 60 % saturated ammonium

sulphate, inhibited the growth of *S. dysgalactiae* RPSD1, RPSD2, RPSD3 and RPSD4, *S. uberis* RPSU1, RPSU2, EDSU1 and EDSU2, and *S. aureus* RPSA1, RPSA2, EDSA0267 and EDSA0269. Growth of *Lactobacillus* sp., *Enterococcus* sp. and *Listeria innocua* were not inhibited (Table 1). These results correspond to that recorded for lacticin 3147 and nisin, in that the peptide inhibits the growth of *S. agalactiae*, *S. dysgalactiae, S. uberis* and *S. aureus* (Broadbent et al. 1989; Ryan et al. 1998). The spectrum of activity of bacteriocin ST91KM differs from that recorded for macedocin ACA-DC 198 and bacteriocins SA-F22 and SA-M49 produced by *Streptococcus pyogenes* in that it does not inhibit the growth of *Lactobacillus plantarum, Bacillus cereus, Clostridium tyrobutyricum, Clostridium butyricum*, *L. innocua*, *Bifidiobacterium* sp. and *S. thermophilus* (Georgalaki et al. 2002). Thermophilin 110, 580 and 81, produced by *Streptococcus thermophilus,* has a narrow spectrum of activity, but differ from bacteriocin ST91KM by inhibiting *L. innocua* (Ivanova et al. 1998; Mathot et al. 2003; Gilbreth and Somkuti 2005).

Strain ST91KM is a Gram-positive coccus, arranged in pairs, and is catalase and oxidase negative. No growth was recorded in MRS broth (Biolab) supplemented with 6.5 % (w/v) NaCl. Growth was recorded at 45 °C and at pH 9.6. Metabolism is fermentative, with the production of L-lactate but no CO_2 from D-glucose. Acid is produced from lactose, glucose and maltose but not from arabinose, inulin, mannitol, salicin, sorbitol and trehalose. Aesculin is not hydrolysed. Colonies on blood agar are α-haemolytic. Closely related streptococci such as *S. gallolyticus* and *Streptococcus bovis* are distinguished from *S. macedonicus* in that they are able to hydrolyse aesculin. *S. gallolyticus* produces acid from inulin and trehalose, while *S. bovis* has variable reactions for these (Schlegel et al. 2004) *S. thermophilus* is similar to *S. macedonicus* in both habitat, resistance to high temperatures (45 °C) and also does not hydrolyse aesculin, but can be distinguished by a negative reaction for maltose (Tsakalidou et al. 1998)

Amplification of the genomic DNA of strain ST91KM with species-specific primers yielded a single DNA fragment of 350 kb with 99 % similarity to the 16S rDNA of *S. macedonicus* (Genbank accession number Z94012). Strain ST91KM is thus regarded as *S. macedonicus*.

No plasmid DNA could be isolated from strain ST91KM, suggesting that the genes encoding peptide ST91KM are located on the genome. Amplification of the genomic DNA of *S. macedonicus* ST91KM with primers designed from the sequence of *mcdA* produced two amplicons (approximately 375 and 220 bp). Amplification of the genome of *S. macedonicus* LMG 18488 (ACA-DC 206) yielded a single PCR product of approximately 150 bp. Sequencing of the PCR product amplified from strain LMG 18488 revealed 99 % homology to the *mcdA* gene of *S. macedonicus* ACA-DC 198 (accession number DQ835394). As no amplicon of this size was produced for *S. macedonicus* ST91KM, the gene producing the precursor peptide for bacteriocin ST91KM is considered different from the precursor peptide described for macedocin ACA-DC 198. Bacteriocin ST91KM may thus be a second putative bacteriocin described for *S. macedonicus*.

Precipitation with 60 % ammonium sulphate recovered 77 % of macedocin ST91KM and yielded a 82-fold increase in specific activity. Separation by SepPak C18 resulted in a 43 % recovery (Table 2).

According to tricine-SDS-PAGE, macedocin ST91KM is between 2.0 and 2.5 kDa in size (Fig 1.), corresponding to salivaricin A (2.3 kDa), streptococcin SA-FF22 (2.8 kDa) and macedocin ACA-DC 198 (2.8 kDa) (Ross et al. 1993; Jack et al. 1994; Georgalaki et al. 2002);. Thermophilin 110 and 81, produced by *S. thermophilus*, are 4.0 to 5.0 kDa in size (Ivanova et al. 1998; Gilbreth and Somkuti 2005).

Complete inactivation of ST91KM was observed after treatment with pronase, pepsin and trypsin. Treatment of bacteriocin ST91KM with EDTA had no inhibitory effect on

activity, suggesting that EDTA could be used as an additive in growth medium to protect degradation of the bacteriocin by proteases. Activity remained unchanged when treated with α-amylase, suggesting that the mode of action does not depend on glycosidic moieties. Treatment with α-amylase has resulted in the inactivation of other bacteriocins such as thermophilin T, produced by Str*eptococcus thermophilus* ACA-DC 0040 (Aktypis et al. 1998). Treatment with surfactants SDS, Tween 20, Tween 80, triton X-114 and triton X-100 had no effect on the activity of peptide ST91KM, suggesting that the active molecule does not have a three-dimensional conformation that may be denatured by anionic detergents.

No loss in activity was recorded at 100 °C and macedocin ST91KM remained active after incubation at pH values ranging from 2.0 to 10.0. Similar results have been reported for macedocin produced by *S. macedonicus* ACA-DC 198 (Georgalaki et al. 2002) and bovicin 255 produced by *S. gallolyticus* LR0255 (Whitford et al. 2001). However, macedocin AC-ADC 198 and bovicin 255 remained active after 15 min at 121 °C (Whitford et al. 2001; Georgalaki et al. 2002), whereas bacteriocin ST91KM was inactivated. Streptococcin SA-FF22 produced by *S. pyogenes* and mutacin MT6223 produced by *S. sobrinus* remained active at pH 2 to 7, but was inactivated at higher pH values (Tagg et al. 1973; Loyala-Rodrigues et al. 1992;). Thermophilin 81 remained active at a broad pH range, but was rapidly inactivated by heating at 60 °C for 15 min and was not inactivated when treated with trypsin (Ivanova et al. 1998).

No antimicrobial activity was recorded after treatment of bacteriocin ST91KM with 100 mM NaCl at pH 2.0 (data not shown), indicating that the peptide was released into the culture supernatant and did not adhere to the surface of the producer strain.

Conclusion

Macedocin ST91KM differs from other bacteriocins described and has a narrow-spectrum of activity against Gram-positive pathogens that are often associated with mastitis. This, and the fact that the peptide remained stable at physiological pH and over a broad temperature range, renders it ideal for control of mastitis in dairy cows. Further research is necessary to determine if the bacteriocin is a safer option than the disinfectants and antibiotics currently in use.

Acknowledgements

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Table 1. Spectrum of antimicrobial activity recorded for macedocin ST91KM

Target organism	Strain	Growth media and temperature (°C)	Antimicrobial activity (SUP[a])	Antimicrobial activity (PPT[b])
Streptococcus agalactiae	RPSAG1	BHI, 37	+	+
	RPSAG2			
Streptococcus dysgalactiae	RPSD1	BHI, 37	-	+
	RPSD2	BHI, 37	-	+
	RPSD3	BHI, 37	-	+
	RPSD4	BHI, 37	-	+
Streptococcus uberis	RPSU1	BHI, 37	-	+
	RPSU2	BHI, 37	-	+
	EDSU1	BHI, 37	-	+
	EDSU2	BHI, 37	-	+
Staphylococcus aureus	RPSA1	BHI, 37	-	+
	RPSA2	BHI, 37	-	+
	EDSA	BHI, 37	-	-
	EDSA0267	BHI, 37	-	+
	EDSA0269	BHI, 37	-	+
Micrococcus varians	RPMV1	BHI,37	+	+
Staphylococcus epidermidis	RPSE1	BHI, 37	+	+
Escherichia coli	RPEC1	BH1, 37	-	-
	RPEC2	BH1, 37	-	-
Bacillus cereus	RPBC1	BHI, 37	-	-
Listeria innocua	F	BHI, 37	-	-
Lactobacillus plantarum	423	MRS, 30	-	-
	ST26MS	MRS, 30	-	-
Lactobacillkus casei	LHS	MRS, 30	-	-
Lactobacillus sakei	LMG13558	MRS, 30	+	+
Enterococcus faecalis	E92	MRS, 30	-	-
Enterococcus muntii	ST4SA	MRS, 30	-	-

[a] SUP, cell-free culture supernatant.
[b] PPT, partially purified bacteriocin obtained after precipitation with 60 % ammonium sulphate and dialysis
MRS, De Man, Rogosa, Sharpe Broth
BHI, Brain Heart Infusion Broth
+, sensitive to bacteriocin ST91KM
-, resistant to bacteriocin ST91KM

Table 2. Activity of macedocin ST91KM at different stages of purification

Purification stage	Volume (mL)	Total activity (AU)	Protein (mg·mL^{-1})	Specific activity (AU·mg^{-1} protein)	Recovery (%)	Purification factor
Cell-free culture supernatant	500	4.0 x 10^5	0.29	2.8 x 10^3	100	1
Ammonium sulphate precipitation	48	3.1 x 10^5	2.86	2.2 x 10^3	77	82
SepPak C18 separation, followed by isopropanol extraction	13.5	1.7 x 10^5	1.57	8.2 x 10^3	43	296
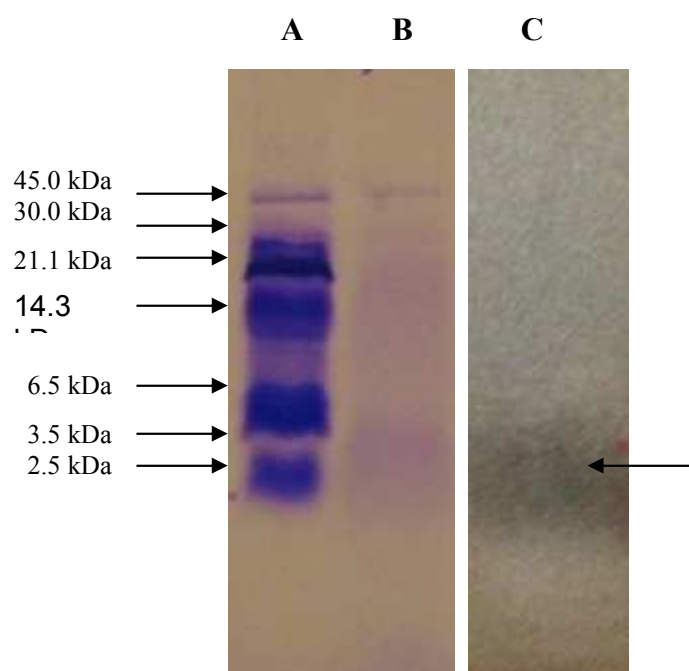
Fig. 1. Separation of peptide ST91KM by Tricine-SDS-PAGE. A: Molecular weight marker (Amersham Bioscience), B: Protein band visible after staining the gel with Coomassie Blue, C: Inhibition zone, indicated by the arrow, obtained after overlaying the SDS gel with *L. sakei* LMG 13558 (1×10^4 cfu·mL^{-1}).

CHAPTER 4

Parameters affecting the adsorption of the bacteriocin macedocin ST91KM to sensitive cells

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Abstract: Macedocin ST91KM, produced by *Streptococcus macedonicus* ST91KM, inhibits the growth of *Lactobacillus sakei*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Micrococcus varians*. The peptide adsorbed equally well (66 %) to *L. sakei* LMG13558 and insensitive cells, e.g. *Enterococcus faecalis* BFE 1071 and FAIR E92, and *Streptococcus caprinus* ATCC 700066. Optimal adsorption of macedocin ST91KM was recorded at 37 °C and 45 °C and at pH of 8 - 10. Addition of solvents decreased adsorption by 50 %, suggesting that the receptors to which the bacteriocin binds have lipid moieties. The addition of $MgCl_2$, KI and Na_2CO_3 completely prevented adsorption of macedocin ST91KM to the target cells, possibly due to competitive ion adsorption on the bacterial cell surface. The peptide has a bacteriocidal mode of action, resulting in lysis and the release of DNA and β-galactosidase. Atomic force microscopy of sensitive cells treated with macedocin ST91KM have shown deformation of the cell structure and developing of irregular surface areas.

Key words: Macedocin ST91KM, adsorption, *Streptococcus macedonicus*

Introduction

Lactic acid bacteria produce a variety of substances, including bacteriocins, with antibacterial activity. Bacteriocins are ribosomally synthesized peptides, usually active against bacteria of the same or closely related species (Jack et al. 1995). They are divided into three classes based on their structure and mode of action (Klaenhammer 1993). Class I are small peptides (< 5k Da) with unusual amino acids, such as lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine. Class II are small (<10 kDa), heat-stable, non-lanthionine containing peptides. The larger peptides (>30 kDa) are grouped into class III.

Streptococcus macedonicus ST91KM, isolated from Bulgarian goat yoghurt, produces the bacteriocin macedocin ST91KM. The peptide has a narrow spectrum of antibacterial activity and inhibits the growth of *Lactobacillus sakei*, *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis*. *S. agalactiae*, *S. dysgalactiae*, *S. uberis* and *S. aureus* are associated with mastitis in dairy cows (Philpot and Nickerson 1999). Macedocin ST91KM has been characterised by Pieterse et al. (2007). The peptide is approximately 2.0 − 2.5 kDa in size and remained stable after incubation for 2 h at pH 2.0 to 10.0. No decrease in activity was recorded after treatment at 100 °C for 100 min, but the peptide was inactivated at 121 °C for 20 min. No change in activity was recorded after treatment with SDS, urea, Tween 20, Tween 80 and EDTA. Treatment with pronase, pepsin and trypsin inactivated the bacteriocin. Activity was not reduced after treatment with α-amylase, suggesting that the peptide is not glycosylated.

A limited number of bacteriocins with antimicrobial activity against pathogens causing mastitis have been studied, i.e. lacticin 3147 and nisin, both produced by *Lactococcus lactis* subsp. *lactis* (Sears et al. 1992; Ryan et al. 1998). More recently, strains of *S. aureus* isolated

from cows infected with mastitis have been screened for production of bacteriocins that could be used to inhibit strains of *S. agalactiae* associated with mastitis (Dos Santos Nascimento et al. 2005).

As far as we could determine, only one bacteriocin has been described for *S. macedonicus*, i.e. the lantibiotic macedocin produced by *S. macedonicus* ACA-DC 198 (Georgalaki et al. 2002). In this study, the parameters affecting the adsorption and mode of action of macedocin ST91KM to target organisms was evaluated in order to use the bacteriocin in the most effective way.

Materials and Methods

Growth conditions and preparation of macedocin ST91KM

S. macedonicus ST91KM was grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) at 30 °C. The target strains used in the study were grown either in MRS broth or BHI broth (Merck, Darmstadt, Germany), at temperatures indicated in the respective culture collection catalogues. *S. agalactiae* RPSAG2, isolated from a clinical mastitis case was obtained from the Western Cape Provincial Veterinary Laboratory (Stellenbosch, South Africa) and was cultured in BHI broth at 37 °C. All strains were stored at −80 °C in MRS or BHI broth with 40 % (v/v) glycerol.

Macedocin ST91KM was prepared as follows: Strain ST91KM was inoculated (2 %, v/v) into 100 mL MRS and incubated for 17 h at 30 °C. The cells were harvested (1000 x *g*, 10 min, 4 °C), the pH of the cell-free culture supernatant adjusted to 6.0 with 1M NaOH, and then heated for 10 min at 80 °C to inactivate proteolytic enzymes. The supernatant was filter-sterilised (0.20 µm pore size membrane filter, Minisart®, Sartorius, Aubagne, France) and

the activity of macedocin ST91KM (800 AU·mL^{-1}) determined by using the agar-spot method (Van Reenen et al. 1998). Active samples of macedocin ST91KM were stored at 4 ºC.

Antimicrobial activity assays

The agar-spot method (Van Reenen et al. 1998) was used to determine the activity of macedocin ST91KM against target strains listed in Table 1. Overnight cultures of the target strains (OD$_{600nm}$ = 0.1 – 0.2) were inoculated (0.1 % v/v) into 10 mL MRS or BHI soft agar (0.7 %, w/v, agar), poured into sterile petri dishes and allowed to solidify. A dilution series of macedocin ST91KM was prepared and 10 µL spotted onto the surface of the solid media. Antimicrobial activity was expressed as arbitrary units AU·mL^{-1}, calculated as follows: a^b x 100, where "a" represents the dilution factor 2 and "b" the last dilution that produces an inhibition zone of at least 2 mm in diameter. Activity was expressed per mL by multiplication with 100.

Effect of macedocin ST91KM on cell growth and cell membrane permeability

Twenty mL macedocin ST91KM was added to 50 mL *S. agalactiae* RPSAG2 (OD$_{600nm}$ = 0.17). The final concentration of macedocin ST91KM was approximately 229 AU·mL^{-1}. Optical density readings were recorded every hour for 10 h.

In a separate experiment, an overnight culture of *L. sakei* LMG13558 (OD$_{600nm}$ = 2.2) was harvested (8 000 x *g*, 15 min, 25 °C), the cells washed twice with 2.0 mL 5 mM phosphate buffer (pH 6.5), and then incubated in the presence of macedocin ST91KM (0.1:10 ratio). After 1 h at 37 °C, the cells were harvested (10 000 x *g*, 15 min, 4 °C) and the supernatant filtered through a 0.20 µm pore size filter membrane (Ministart®, Sartorius). The presence of nuclear material was recorded by absorbance readings at 260 nm (SmartSpec™ Plus Spectrophotometer, Bio-Rad Laboratories, Hecules, CA USA). The

presence of K$^+$ in the filtrate was determined by atomic absorption spectrometry (Avanta Σ, GBC Scientific Equipment, Victoria, Australia). Controls were cells prepared the same way, but not treated with macedocin ST91KM.

In another experiment, extracellular levels of β-galactosidase activity were determined according to the methods of Nagy et al. (2001) and Hsu et al. (2005). Cells of *L. sakei* LMG13558 were harvested (10 000 x *g*, 15 min, 25 °C) after 11 h incubation at 30 °C, washed twice with 0.03 M sodium phosphate buffer (pH 6.8) and then re-suspended in 2.0 mL of the same buffer. The cell suspension was treated with 2 mL macedocin ST91KM (800 AU·mL^{-1}) for 5 min at 25 °C, followed by the addition of 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min incubation at 37 °C for 10 min, 2.0 mL 0.1 M sodium carbonate was added to stop the reaction of the β-galactosidase. The cells were harvested (10 000 x *g*, 15 min, 25 °C) and absorbance readings of the cell-free culture supernatant recorded at 420 nm, using a spectrophotometer (SmartSpec™ Plus Spectrophotometer). Cells of *L. sakei* LMG 13558 treated with sterile water instead of macedocin ST91KM served as control. All experiments were performed in duplicate.

Adsorption of macedocin ST91KM to target cells

Adsorption to target cells (Table 1) was tested according to Yildrim et al. (2002). The bacterial strains were grown overnight in MRS or BHI broth at 30 °C or 37 °C to OD$_{600nm}$ = 0.1 – 0.2 and the cells harvested (10 000 x *g*, 15 min, 4 °C). Cells were washed twice in 5 mM phosphate buffer (pH 6.5) and re-suspended in the same buffer to the original volume. Each cell suspension (0.7 mL) was mixed with an equal volume of macedocin ST91KM and incubated at 37 °C for 1 h. The cells were harvested (10 000 x *g* for 15 min) and the activity of unbound macedocinST91KM in the cell-free supernatant determined using the agar-spot

method as described before. Adsorption of macedocin ST91KM to the target cells was calculated according to the following formulae: % Adsorption = 100 – [(macedocin ST91KM activity after treatment/original macedocin ST91KM activity) x 100]. The experiment was performed in duplicate.

Effect of pH and temperature on adsorption of macedocin ST91KM to *L. sakei*

Cells from an overnight culture of *L. sakei* LMG13558 (OD_{600nm} = 2.2) were harvested and re-suspended in 5 mM phosphate buffer of pH 2.0 to 10.0. Macedocin ST91KM was added to the cell suspension and incubated at 4, 10, 25, 30, 37, 45 and 60 °C for 1 h. The cells were harvested (10 000 x *g*, 15 min, 25 °C) and the pH of the supernatants adjusted to 6.0 with sterile 1 M NaOH. Macedocin ST91KM activity was determined as described before. All experiments were performed in duplicate.

Effect of surfactants, inorganic salts and organic compounds on the adsorption of macedocin ST91KM to *L. sakei*

Cell suspensions of *L. sakei* LMG 13558 were prepared as described before and were treated with 1 % (w/v) NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, $(NH_4)_3C_6H_5O_7$, CH_3COONa, Na_2CO_3, EDTA ($C_{10}H_{16}O_8N_2$), SDS and 1 % (v/v) Triton X-100, Triton X-114, β-mercaptoethanol, and 80 % (v/v) ethanol, methanol and chloroform, respectively. The pH of each suspension was adjusted to 6.5 with 1 M NaOH or 1 M HCl. Macedocin ST91KM was added to the treated cells, as before, and incubated at 37 °C for 1 h. The cells were harvested (10 000 x *g*, 15 min, 25 °C) and the activity of the cell-free supernatant determined as before. The experiment was performed in duplicate.

Effect of macedocin ST91KM on cell morphology

Strain ST91KM was cultured in MRS broth for 15 h at 30 °C. The cells were harvested (8 000 x g, 10 min, 4 °C) and the bacteriocin precipitated from the cell-free culture supernatant with 60 % saturated ammonium sulphate (Sambrook et al. 1989). The precipitate was suspended in 25 mM ammonium acetate buffer (pH 6.5) to one-tenth of the original volume and loaded onto a SepPak C18 cartridge (Water Millipore, MA, USA). Peptides were eluted with 20 %, 40 %, 60 % and 80 % (v/v) isopropanol, respectively, in 25 mM ammonium acetate buffer (pH 6.5). Fractions of 1 mL were dried under vacuum at 50 °C and stored at -20 °C until required.

Cells from an overnight culture (OD_{600nm} = 0.16) of $S.$ $agalactiae$ RPSAG2 were harvested (8 000 x g, 10 min, 4 °C) and washed five times with sterile distilled water. The dried bacteriocin fractions of macedocin ST91KM were reconstituted and concentrated with milli Q water, filter-sterilised (0.20 µm pore size membrane filter, Minisart®, Sartorius Aubagne, France) and incubated with the prepared cells overnight at 8 °C (approximately 2 000 $AU \cdot mL^{-1}$). The cells were then washed five times with sterile distilled water and re-suspended in 1 mL sterile distilled water. Untreated cells of $S.$ $agalactiae$ RPSAG2 served as control. Images of cell surface areas of treated and untreated cells were visualised by atomic force microscopy (Multimode AFM from Veeco, Santa Barbara USA). The cell suspension was applied onto freshly cleaved mica surface and allowed to dry for 5 min before subjected to AFM. All images were obtained in air and with tapping mode. A silicone non-contact cantilever from Nansensors (Neuchatel, Switzerland) with a resonance frequency of 160 kHz and a spring constant of approximately 50 N/m was used. Height and size information was acquired by using imaging software from Veeco.

Results and Discussion

Effect of macedocin ST91KM on cell growth and membrane permeability of target cells

Addition of macedocin ST91KM (229 AU·mL^{-1}) to *S. agalactiae* RPSAG2 decreased the cell numbers from 10^6 cfu·mL^{-1} (OD$_{600nm}$ = 0.16) to 10^5 cfu·mL^{-1} (OD$_{600nm}$ = 0.10) after 6 h (Fig. 1). Optical density readings of the control culture (no bacteriocin added) increased to OD$_{600nm}$ = 0.27 for *S. agalactiae* RPSAG2 over the same period.

The cell numbers of the control strain of *S. agalactiae* RPSAG2 increased to 1.24 x 10^7 cfu·mL^{-1} (OD$_{600nm}$ = 1.21) after 10 h of incubation. In contrast, the optical density readings of a culture of *S. agalactiae* RPSAG2 where the bacteriocin was added did not increase significantly over the 10 h incubation period (OD$_{600nm}$ = 0.11). This indicates that the bacteriocin had a bacteriocidal effect against actively growing cells of *S. agalactiae* RPSAG2.

A bacteriocidal mode of action has been demonstrated for lantibiotics lacticin 3147 (McAuliffe et al. 1998) and macedocin ACA-DC 198 (Georgalaki et al. 2002). The addition of 300 AU·mL^{-1} of lacticin 3147 to cells of *Lactococcus lactis* subsp. c*remoris* HP, resulted in a 30 % decline in viable cell numbers within 15 min. An increase in potency of lacticin 3147 to 1200 AU·mL^{-1} resulted in complete repression of growth (McAuliffe et al. 1998). A similar mode of action was demonstrated for macedocin ACA-DC 198 against *L. sakei* LMG 13558. Treatment of the cells with macedocin ACA-DC 198 (400 AU·mL^{-1}) lowered the cell numbers from 10^8 to 10^7 cfu·mL^{-1} after 1 h. Treatment of the target cells with higher levels of macedocin ACA-DC 198 (800 AU·mL^{-1}) did not result in a more rapid reduction of viable cell numbers (Georgalaki et al. 2002). The mode of action of macedocin ST91KM is thus comparable to lacticin 3147 and macedocin ACA-DC 198. The maximum number of viable cells were killed within the first hour after the addition of macedocin ST91KM and remained

constant for the remainder of the incubation period. The effect of a higher concentration of macedocin ST91KM was not tested.

The optical density readings measured at 260nm and 420nm gave an indication of the presence of nuclear material and β-galactosidase caused by cell leakage (Table 2). The cell-free supernatant of strain ST91KM had readings of 0.581 (OD_{260nm}) and 0.631 (OD_{420nm}), for nuclear material and β-galactosidase leakage, respectively and can be attributed to some cell lysis occurring during stationary growth of strain ST91KM. The optical density readings recorded for untreated and treated cells of *L. sakei* (LMG 13558) indicated an increase in the levels of nuclear material and β-glactosidase in the cell-free culture supernatant after the supernatant containing macedocin ST91KM has been added to the target cells. Potassium levels were only slightly higher at 534 mg·K^+L^{-1}, compared to untreated cells (509 mg·K^+L^{-1}). This was attributed to leakage from the cells of *L. sakei* (LMG 13558). Similar results have been reported for buchnerin LB, a bacteriocin produced by *Lactobacillus buchneri* (Yildirin et al. 2002). However, a much weaker efflux of K^+ was reported for macedocin ST91KM.

Adsorption of macedocin ST91KM to target cells

MacedocinST91KM adsorbed to both sensitive and non-sensitive cells (Table 1). Sixty-six percent of the peptide adsorbed to *L. sakei* LMG13558. However, 66 % of the peptide also adsorbed to non-sensitive strains of *Enterococcus faecalis* (BFE 1071 and FAIRE 92) and *Streptococcus caprinus* (ATCC 700066). No, or very little adsorption (up to 33 %) of macedocin ST91KM has been recorded for the other resistant strains (Table 1). Yildirim et al. (2002) reported a high percentage adsorption of buchericin LB to resistant strains of *L. lactis* (94 %), *Pediococcus cerevisiae* (100 %) and *S. aureus* (80 %). Manca de Nadra et al. (1998), on the other hand, reported weak adsorption of pediocin N5p to resistant

strains (13 to 20 %) and higher adsorption to sensitive strains (30 – 100 %). This indicated that the bactericidal action of pediocin N5p is dependent on specific receptors on sensitive strains. The mode of action of macedocin ST91KM did not only depend on adsorption, as sensitive and non-sensitive strains revealed similar levels of adsorption of the peptide.

Effect of pH, temperature, surfactants and salts on the adsorption of macedocin ST91KM to *L. sakei*

Treatment of cells with macedocin ST91KM at 4 – 60 °C and at pH 2.0 – 10.0 did lead to a significant increase in adsorption (Table 3). Adsorption of the peptide to target cells decreased from 66 to 33 % below 30 °C and was completely inhibited at 4 °C. Optimal adsorption at different pH levels was recorded at pH 8.0 to 10.0.

The addition of inorganic and organic salts reduced adsorption of the bacteriocin. $MgCl_2$, KI and Na_2CO_3 salts completely prevented adsorption of the peptide to target cells (Tabel 3). Similar results have been reported for plantaricin C19 (Atrih et al. 2001). Adsorption of plantaricin C19 was steadily reduced at increasing salt concentrations, possibly due to competitive ion adsorption on the bacterial cell surface.

The surfactants SDS, Triton X-100 and Triton X-114 did not affect adsorption of macedocin ST91KM to target cells, while the solvents, β-mercapto-ethanol, 80 % ethanol and methanol reduced adsorption to 33 %, suggesting that the binding sites on target cells may have lipid moieties. Chloroform had no effect on adsorption.

Effect of macedocin ST91KM on cell morphology

Cells of *S. agalactiae* RPSAG2 treated with partially purified macedocin ST91KM (approximately 2 000 AU·mL^{-1}) were deformed and had irregular surfaces compared to cells that have not been treated. This suggested that presence of macedocin ST91KM may result

in the formation of pores in the membrane of the target cells. This is in line with the mode of action of other bacteriocins, such as lacticin 3147 (McAuliffe et al. 1998).

Conclusion

The action of macedocin ST91KM resulted in efflux of cellular components in sensitive strains. The peptide adsorbed to both sensitive and non-sensitive cells, indicating that activity is not species-specific but rather dependent on specific cell-surface receptors. Binding sites for the peptide could be lipid in nature, as the addition of solvents reduced adsorption. Salts prevented the adsorption of macedocin ST91KM to target cells, possibly due to competitive ion adsorption on the cell surface. Macedocin ST91KM could potentially be used as an antimicrobial agent against pathogens associated with mastitis due the rapid bacteriocidal mode of action against the mastitis pathogen *S. agalactiae* RPSAG2. Optimal adsorption of macedocin ST91KM was recorded at physiological pH and temperature, suggesting that the peptide could be included in a teat seal. Macedocin ST91KM is also heat stable remaining active at 100 °C (Pieterse et al. 2007). This may be an important consideration to ensure that macedocin ST91KM would remain active after storage at a variety of storage condition in a final teat seal product.

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Table 1. Activity spectrum of macedocin ST91KM and adsorption to target cells.

Target organism	macedocin ST91KM	
	Sensitivity to macedocin ST91KM[a]	Adsorption (%)
Enterococcus faecalis BFE 1071 and FAIRE 92	-	66
Enterococcus mundtii ST4SA	-	0
Enterococcus sp. HKLHS	-	33
Lactobacillus curvatus DF38	-	33
Lactobacillus plantarum LMG 13556	-	33
Lactobacillus salvarius 241	-	33
Lactobacillus sakei LMG 13558	+	66
Lactococcus lactis subsp. *lactis* HV219	-	0
Listeria innocua LMG 13568	-	33
Streptococcus caprinus ATCC 700066	-	66

[a] - = not inhibited, and + = inhibited by macedocin ST91KM

Table 2. Effect of macedocin ST91KM on the permeability of *L. sakei* LMG13558 cells.

	nucleotides (O. D. $_{260nm}$)	β-galactosidase (O. D. $_{420nm}$)	Potassium ions (mg/l)
Treated cells	2.979	1.265	534
Untreated cells	0.624	0.116	509
Macedocin ST91KM, no cells	0.581	0.631	8

Table 3. Effect of pH, temperature, surfactants and salts on the adsorption of macedocin
ST91KM to *L. sakei* LMG 13558.

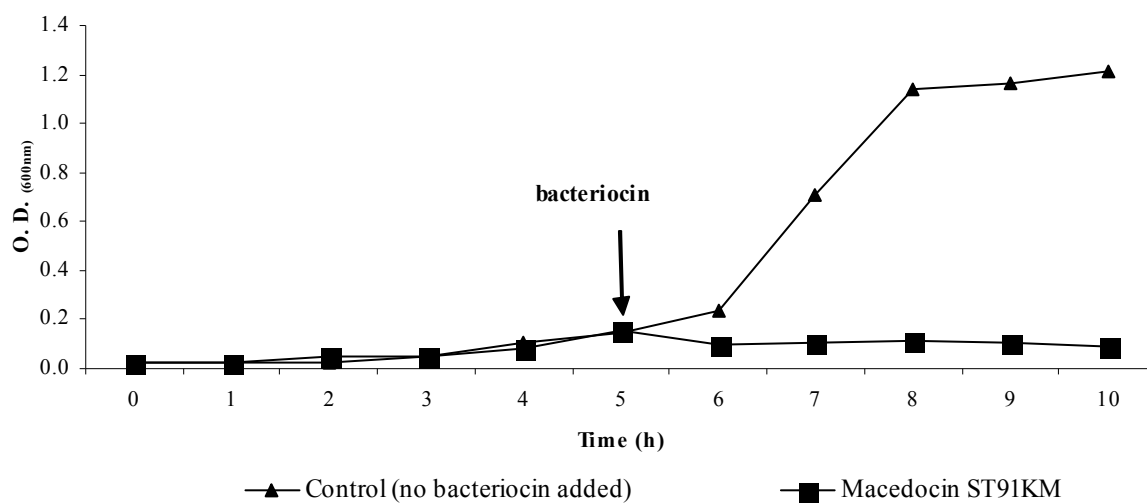|	Adsorption (%)
Control (not treated)	66
Effect of pH	
2, 4 and 6	33
8 and 10	66
Effect of temperature (°C)	
4	0
10, 25, and 30	33
37 and 45	66
60	33
Treatment with 1 %	
Na-acetate	66
Na_2CO_3	0
EDTA(-Na)	33
SDS, Triton X-100 and Triton X-114	66
B-mercapto-ethanol, 80 % ethanol and methanol	33
Chloroform	66
NaCl, K_2HPO_4, KH_2PO_4, KCl and NH_4-citrate	33
$MgCl_2$	0
KI	0
Tris	33

Fig. 1. The effect of macedocin ST91KM on *S. agalactiae* RPSAG2. The arrow indicates the point at which macedocin ST91KM was added.
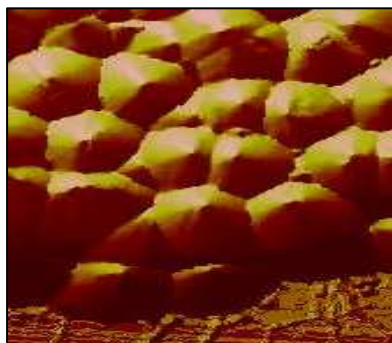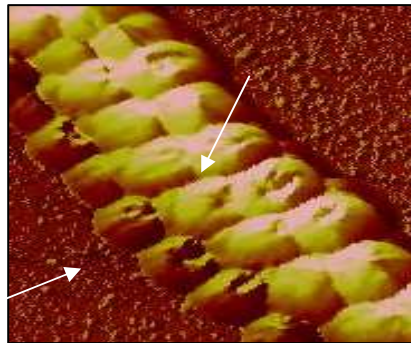
(A) Untreated cells   (B) Cells treated with macedocin ST91KM

Fig. 2. Changes in cell morphology of *S. agalactiae* RPSAG2 treated with macedocin ST91KM. A: before treatment, B: after treatment. The arrow indicates irregularity on the cell surface.

CHAPTER 5

Antimicrobial susceptibility of mastitis pathogens to macedocin ST91KM and antibiotics and possible application of ST91KM in a teat seal preparation against *Streptococcus agalactiae* infection

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Abstract: Mastitis is considered to be the most economically costly disease affecting the dairy industry. Regular dosage of animals with antibiotics, including use of prophylactic concentrations, may select for resistant strains. The purpose of this study was to evaluate the antimicrobial resistance of mastitis pathogens to antibiotics commonly used in treatment remedies and to introduce the possible use of an alternate antimicrobial agent. The bacteriocin macedocin ST91KM, produced by *Streptococcus macedonicus* ST91KM, is bacteriocidal to *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Staphylococcus aureus*. Antimicrobial susceptibility patterns were evaluated against seventeen mastitis pathogens. All isolates tested were resistant to methicillin and oxacillin, but had minimum inhibitory concentrations (MICs) falling in the intermediate and susceptible range against erythromycin. *S. agalactiae* and *S. epidermidis* had the highest sensitivity to macedocin ST91KM. A teat seal preparation containing macedocin ST91KM effectively released the peptide and inhibited the growth of *S. agalactiae*. Macedocin ST91KM could form the basis for an alternative dry cow therapy to prevent mastitis infections in dairy cows as it is effective against pathogens that display resistance to conventional antibiotic therapy.

Introduction

Mastitis is the most important disease affecting production in the dairy industry. Losses can be attributed to rejected milk, reduced production, reduced milk quality, culling, treatment costs and veterinary expenses (Petrovski et al. 2006). Mastitis is primarily caused by the presence and infection of pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli* (Philpot and Nickerson 1999).

Antibiotic therapy remains an important component of treatment strategies during lactation and dry periods (Crist et al. 2007). The presence of antibiotic residues in milk and other animal food products is likely if the required withdrawal periods are not adhered to. In addition, it has been suggested that antibiotic therapy is a selective force in the development of antibiotic resistance in pathogenic bacteria. Resistance to various antibiotics by coagulase-negative staphylococci, *S. aureus* and streptococci have been reported (Gentilini et al. 2002; Guérin-Faublée et al. 2002; Rajala-Schultz et al. 2004; Moroni et al. 2006). Concern has also been raised that contaminated food products could be a possible source of human infections if homologous antibiotic resistance genes are transferred to human pathogens (Khan, et al. 2000; Lee 2003, Loch et al. 2005; Lüthje and Schwarz 2006). However, trends in antibacterial resistance patterns during a seven-year period (1994 – 2000) in the USA investigated by Erskine et al. (2002) showed that the proportion of isolates that were susceptible to antibiotics actually increased. Despite these conflicting reports, the use of antibiotics for prophylactic use and treatment of bacterial infections should be managed responsibly.

Alternative antimicrobial therapy, in the form of antimicrobial peptides could be used to prevent and treat mastitis. Bacteriocins are ribosomally synthesized peptides, usually active against bacteria of the same or closely related species (Jack et al. 1995). They are divided

into three classes based on their structure and mode of action (Klaenhammer 1993). Class I are small peptides (<5 kDa) with unusual amino acids, such as lanthionine, α-methyllanthionine, dehydroalanine and dehydrobutyrine. Class II are small (<10 kDa), heat-stable, non-lanthionine containing peptides. The larger peptides (>30 kDa) are grouped into class III. The lantibiotic lacticin 3147 produced by the lactic acid bacteria *Lactococcus lactis* subsp. *lactis* DPC3147 has been evaluated in combination with a teat seal for dry cow therapy (Ryan et al. 1998; Ryan et al. 1999; Twomey et al. 2000). A teat sealant is composed of an inert salt (bismuth subnitrate) in a paraffin base, which forms a paste. It is infused into the teat of each quarter using a sterile syringe to form an impermeable plug as it lines the teat canal and results in a physical barrier against invading microorganisms during the dry period (Berry et al. 2002).

Preliminary findings indicate that lacticin 3147 in a teat seal was effective in preventing new infections after exposure to deliberate infection by *S. dysgalactiae* (Ryan et al. 1999). Further studies by Crispie et al. (2005) where lanticin 3147 was produced in crude demineralised whey protein liquor, was also successful in reducing the numbers of *S. aureus* recovered from the teats deliberately infected before infusion with the teat seal plus bacteriocin. Tissue tolerance studies, however, showed that mild irritation was caused by the teat seal plus bacteriocin.

Macedocin ST91KM inhibited pathogenic strains of *S. aureus*, *S. agalactiae*, *S. uberis* and *S. dysgalactiae*. Macedocin is, according to tricine-SDS PAGE, between 2.0 and 2.5 kDa in size, heat-stable up to 100 °C, and remains active between pH of 2.0 to 10.0. Optimum adsorption to bacterial cells occurs at 37 °C and at a pH of 8.0 to 10.0. A specific target-mediated mode of action is likely for macedocin through the binding of membrane-bound cell wall receptors that are lipid in nature. Macedocin has a bacteriocidal effect resulting in cell lysis of target cells.

In this study, the minimum inhibitory concentration (MIC) of β-lactam penicillins, erythromycin and macedocin ST91KM is investigated to determine if the peptide could be used in the prevention and treatment of mastitis pathogens that have become resistant to antibiotics. The possible administration of the bacteriocin in a teat seal preparation was studied *in vitro* against *S. agalactiae*.

Materials and methods

Bacterial strains, media and growth conditions

The bacteriocin producing strain *S. macedonicus* ST91KM was cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) at 30 °C. Mastitis pathogens used in this study were obtained from the Western Cape Provincial Veterinary Laboratory (Stellenbosch, South Africa). These included *S. agalactiae* RPSAG1 and RPSAG2, *S. uberis* RPSU1, RPSU2, EDSU1 and EDSU2, *S. dysgalactiae* RPSD1, RPSD2, RPSD3 and RPSD4, *S. aureus* RPSA1, RPSA2, EDSA 0269, EDSA and EDSA0267, *S. epidermidis* RESE1, and *Escherichia coli* RPEC1 and RPEC2. Mastitis pathogens were cultured in BHI broth (Merck, Darmstadt, Germany) at 37 °C. Stock cultures of all strains were maintained in 50 % glycerol and stored at -80 °C.

Antimicrobial agents

Peptide ST91KM was prepared from a 15-h-old MRS broth culture of *S. macedonicus* ST91KM incubated at 30 °C. The cells were harvested (8 000 x *g*, 10 min, 4 °C) and the bacteriocin precipitated from the cell-free culture supernatant with 60 % saturated ammonium sulphate (Sambrook *et al*. 1989). The precipitate was suspended in 25 mM ammonium acetate buffer (pH 6.5) to one-tenth of the original volume and loaded onto a SepPak C18

cartridge (Water Millipore, MA, USA). Peptide ST91KM was eluted with 40 % (v/v) isopropanol, in 25 mM ammonium acetate buffer (pH 6.5). Fractions of 1 mL were dried under vacuum at 50 °C, reconstituted to final volume of 1 mL with ammonium acetate buffer (pH 6.5) and stored at -20 °C until required for antimicrobial activity tests.

The antibiotics used in this study were selected based on their approval and frequent use in the treatment of mastitis in South Africa. The antimicrobial agents selected included β-lactam penicillins: Penicillin G (PEN) (USB, Amersham Life Science), ampicillin (AMP) (Roche Diagnostics, Mannhein, Germany)), oxacillin (OX) (Sigma-Aldrich, St. Louis, USA), methicillin (MET) (Sigma-Aldrich, St. Louis, USA) and a macrolide antibiotic, erythromycin (EM) (Sigma-Aldrich, St. Louis, USA). The antimicrobial agents were diluted in sterile deionised water to prepare stock solutions, each containing 1280 $\mu g \cdot mL^{-1}$ of the active antibiotic. The final concentration range used for antimicrobial activity tests for each antibiotic was 32.0, 16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.12 $\mu g \cdot mL^{-1}$.

Antimicrobial activity tests

Minimum inhibitory concentration (MIC) tests were performed using the microdilution broth method, with U-bottomed 96-well microtitre plates, recommended by the Clinical and Laboratory Standards Institute (NCCLS 2002). Serial two-fold dilutions of each antimicrobial agent were prepared in cation-adjusted Mueller-Hinton broth (CAMHB) (Oxoid) and 50 µL. pipetted into the wells of sterile microtitre plates. A positive control well contained 50 µL CAMHB, without an antimicrobial agent. Inoculum for each test organism was prepared as follows: Three to five colonies were selected from a 24-h-old agar plate culture and suspended in 2 mL sterile 0.9 % saline. The turbidity was adjusted to 0.5 according to the MacFarland standard. Ten µL of this suspension was transferred to 10 mL CAMHB to obtain a bacterial suspension of approximately 5 x 10^5 $CFU \cdot mL^{-1}$. From this

suspension, 50 μL was pipetted into each well. The microtitre plates were sealed and incubated for 20 h at 37 °C. The MIC was defined as the lowest concentration of the antimicrobial agent at which the bacterial growth was completed inhibited as detected by the unaided eye. MIC breakpoints, listed in the manual of the Clinical Laboratory Standards Institute (NCCLS, 2004), were used to determine resistance and susceptibility for each antibiotic tested. The exact concentration of peptide ST91KM in the bacteriocin preparations was unknown, therefore the antimicrobial activity of the peptide was expressed as arbitrary units per millilitre ($AU \cdot mL^{-1}$), calculated as follows: a^b x 20, where "a" is equal to 2, and "b" the lowest dilution of the antimicrobial agent that prevented visible growth of the microorganisms tested. Activity was expressed per millilitre by multiplication with 20. The protein concentration, expressed as $mg \cdot mL^{-1}$, in the bacteriocin preparations was assayed using the Bradford method (Bradford 1976). The final minimum inhibitory concentration of macedocin ST91KM was expressed as $AU \cdot mg^{-1}$ protein.

Teat seal formulation with macedocin ST91KM

Concentrated bacteriocin was prepared from a 15-h-old MRS broth culture of *S. macedonicus* ST91KM incubated at 30 °C. The cells were harvested (8 000 x *g*, 10 min, 4 °C) and the bacteriocin precipitated from the cell-free culture supernatant with 60 % saturated ammonium sulphate (Sambrook et al. 1989). The precipitate was suspended in 25mM ammonium acetate buffer (pH 6.5) to one-tenth of the original volume. The teat seal consisted of 65 % (w/w) bismuth subnitrate in a paraffin base, similar in composition to that of commercially available intramammary teat sealers such as Orbeseal (Pfizer Animal Health). A second formulation containing 1 % (w/w) Tween 80 in the teat seal paste was also prepared. Macedocin ST91KM (100 μL) was added to 1 g of the teat seal paste with or

without 1 % (w/w) Tween 80 and mixed to form an emulsion. The teat seal formulations were kept in 5 mL sterile syringes at 4 °C.

In vitro effect of macedocin ST91KM combined with the teat seal against *S. agalactiae*

The bacteriocin activity of macedocin ST91KM was determined using the agar well diffusion method. An overnight culture of *S. agalactiae* RPSAG2 was diluted by inoculating 10 µL in 10 mL sterile physiological saline. One millilitre was inoculated into 9 mL semi-solid (0.7 %, w/v) BHI agar (BHI Broth, Merck, Darmstadt, Germany) and wells of 4.6 mm made into the agar. Teat seal preparations combined with bacteriocin ST91KM, with and without 1 % (w/w) Tween 80, were carefully dispensed into the wells to ensure that the seal was in contact with the wall of the wells. The agar plates were incubated at 37 °C overnight for 16 h and the zones of inhibition measured.

Results and Discussion

Antimicrobial susceptibility

Susceptibility of the pathogens to penicillin G (PEN) (USB, Amersham Life Science), ampicillin (AMP) (Roche Diagnostics, Mannhein, Germany)), oxacillin (OX) (Sigma-Aldrich, St. Louis, USA), methicillin (MET) (Sigma-Aldrich, St. Louis, USA) and erythromycin (EM) (Sigma-Aldrich, St. Louis, USA is shown in Table 1. Data were interpreted based on veterinary-specific criteria, except for oxacillin against streptococci, which was interpreted based on data obtained from trials on humans (NCCLS 2004). All isolates were resistant to methicillin and oxacillin, but susceptible to ampicillin. Streptococci showed intermediate resistance to penicillin G. This is in agreement with findings reported by Rossitto et al. (2002) for isolates of *S. dysgalactiae* and *S. uberis*, where 1.3 % and 50.4 %

of isolates showed intermediate resistance to penicillin, respectively. However, Guérin-Faublée et al. (2002) reported that only *S. uberis* (14 % of isolates tested) had an intermediate MIC for penicillin G. Other strains of *S. agalactiae* and *S. dysgalactiae* were susceptible to penicillin G (Guérin-Faublée et al. 2002). Three of the ten streptococci tested had intermediate MICs against erythromycin. Interestingly, these strains (*S. agalactiae* RPSAG1, *S. dysgalactiae* RPSD4 and *S. uberis* EDSU2) also displayed the highest MICs against penicillin G.

Strains of *S. aureus* and coagulase-negative staphylococci (CNS), *Staphylococcus epidermidis,* were resistant to penicillin G. This is in agreement with findings reported by Gentilini et al. (2002) and Moroni et al. (2006). The staphylococci were all susceptible to erythromycin. Oxacillin is recommended as the antimicrobial agent to evaluate susceptibility to methicillin and cloxacillin (NCCLS 2002). The MIC data thus suggests that all the isolates tested are also resistant to cloxacillin, even though the antibiotic was not tested. Most antimicrobial products currently used to treat and prevent intramammary infections in cows contain β-lactam penicillins, i.e. penicillin, cloxacillin and ampicillin (IVS Desk Reference 2005/2006). According to the antimicrobial susceptibility patterns of the isolates tested, ampicillin would be the drug of choice to treat both Gram-negative and Gram-positive bacterial infections. However, the Clinical and Laboratory Standards Institute state that methicillin (oxacillin)-resistant staphylococci should be considered resistant to all β-lactams (including ampicillin), regardless of *in vitro* results (NCCLS 2002). One CNS, *S. epidermidis* was susceptible to ampicillin. CNS strains are often referred to as minor pathogens (Quinn et al. 1994). However, in a study by Rajal-Scultz et al. (2004), 77 % of all isolates associated with bovine mastitis were CNS and 77.3 % of these strains were resistant to ampicillin.

The only Gram-negative mastitis pathogens tested was *E. coli*. Both isolates were resistant to oxacillin, methicillin and erythromycin. This is not unusual as Gram-negative strains are not usually susceptible to these antibiotics (NCCLS 2002). The strains of *E. coli* tested were susceptible to ampicillin. Some strains of *E. coli* that have been isolated from mastitis infections are resistant to ampicillin (Kaspar 2006; Srinivasan et al. 2007).

Antimicrobial susceptibility to the macedocin ST91KM is shown in Table 2. Two preparations of macedocin ST91KM were used: ST91KM precipitate (ST91KMppt) and ST91KM concentrate (ST91KMcon). The minimum concentration of macedocin ST91KM required to completely inhibit visible growth of the sensitive strains was 74.8 AU·mg^{-1} protein for ST91KMppt and 216 AU·mg^{-1} protein for ST91KMcon. The purification and concentration of macedocin ST91KM in ST91KMcon resulted in an average increase of 63.5 % in the AU·mg^{-1} protein. Only Gram-positive isolates (93.3 %) were sensitive to the bacteriocin. Of the four *S. aureus* tested, three were sensitive to the bacteriocin. The activity of macedocin ST91KM, expressed as AU·mL^{-1}, gives an indication of the difference in the sensitivity of the different bacterial isolates tested. *S. agalactiae* RPSAG2 and *S. epidermidis* RPSE1 were the most sensitive, as growth was completely inhibited after a 1: 64 (1280 AU·mL^{-1}) and 1:128 (2560 AU·mL^{-1}) dilution, respectively. Most other sensitive strains were inhibited at a dilution of 1:32 (640 AU·mL^{-1}). Similar results were obtained with the ST91KM concentrate when tested against *S. agalactiae* RPSAG2 and *S. epidermidis* RPSE1. The ST91KM concentrate inhibited the growth of these pathogens at dilutions of 1:512 (10240 AU·mL^{-1}) and 1:256 (5120 AU·mL^{-1}), respectively.

The bactericidal action of some bacteriocins such as lantibiotics is to form pores in the membrane of sensitive bacterial cells in a specific target-mediated manner, binding to membrane-bound cell wall receptors (Jack et al. 1995). It is thus possible that the insensitive strain of *S. aureus* does not have the specific cell wall receptors required for binding to

macedocin ST91KM. Variations in the sensitivity of different strains of *S. uberis* could be attributed to slight differences in the initial inoculum size. *S. uberis* RPSU1 and EDSU1 required a 1:32 (640 AU·mL^{-1}) dilution to inhibit cell growth compared to *S. uberis* RPSU2 and EDSU2, which required higher levels of macedocin ST91KMppt at a 1:8 (160 AU·mL^{-1}).

Bacteriocins that have been considered as antimicrobial agents against mastitis pathogens include the lantibiotics lacticticin 3147 and nisin (Sears et al. 1992; Ryan et al. 1998). Lacticin 3147 and nisin are both broad-spectrum lantibiotics, exhibiting antibacterial activity against Gram-positive mastitis pathogens. Steptococci are more sensitive to these lantibiotics than *Staphylococcus aureus* as was the case for macedocin (Broadbent et al. 1998; Ryan et al. 1998). The potency of lacticin 3147 against *S. dysgalactiae* reported by Ryan et al. (1998) would appear to be significantly higher at 10240 AU·mL^{-1} than that of the macedocin ST91KM concentrate at 2560 AU·mL^{-1}. Further purification of macedocin ST91KM would be necessary to improve potency.

Given that most of the mastitis pathogens tested showed resistance to antibiotics commonly used in dry cow and lactation therapeutics (β-lactam penicillins), this macedocin could be a possible candidate for an alternate antimicrobial agent. Methicillin-resistance in *Staphylococcus aureus* (MRSA) is a widespread problem (Scott 2005) and the antimicrobial susceptibility data would suggest that the *S. aureus* pathogens isolated from bovine milk samples are methicillin-resistant. The risk of contaminated food products containing MRSA entering the food chain could increase the risk of the transfer of resistance genes to human *S. aureus* isolates (Lee 2003). The development of bacteriocins as antimicrobial agents against pathogens, especially those resistant to therapeutic antibiotics, should be considered. A comparative study evaluating the effectiveness of the bacteriocins nisin and mutacin B-Ny266 and the antibiotics oxacillin and vancomycin have shown that these bacteriocins are at least as effective against oxacillin-resistant *S. aureus* strains (Mota-Meira et al. 2000).

In vitro* effect of macedocin ST91KM combined with the teat seal against *S. agalactiae

Dry cow therapy is recommended practice for all cows at the end of lactation. Dry cow therapy would involve the administration of antibiotics in a prophylactic manner to reduce the likelihood of new infections developing during this period. Inert teat sealants have been used in combination with antibiotics such as cloxacillin (Bradley et al. 2005), the lacticin 3147 (Ryan et al, 1998) and alone (Berry et al. 2002) for dry cow therapy.

Given that macedocin ST91KM was effective at inhibiting various mastitis pathogens, it was incorporated into a teat seal formulation (65 % w/w, bismuth subnitrate in paraffin base) to determine if macedocin ST91KM was effectively released to inhibit target organisms. Clear zones of inhibition against the test organism *S. agalactiae* (RPSAG2) were observed in the teat seal formulation containing macedocin ST91KM. The activity of the bacteriocin added to the teat seal formulation was 160 AU·mL^{-1} macedocin ST91KM ppt. per gram. No inhibitory effect was seen in wells that contained the teat seal only. Ryan et al. (1998) performed a similar evaluation incorporating lacticin 3147 into a teat seal, but found that unless Tween 80 (2 % w/w) was added; no inhibitory effect was seen, indicating that this bacteriocin was hydrophobic. Macedocin ST91KM was however released from the teat seal preparation without Tween 80 (1 % w/w) showing an inhibition zone of 14 mm, slightly smaller than the inhibition zone without the addition of Tween 80 (Fig. 1).

Conclusion

Macedocin ST91KM is a narrow-spectrum bacteriocin that should be considered as a viable alternative antimicrobial agent against mastitis pathogens as it effective in inhibiting these pathogens *in vitro*. Antibiotic susceptibility evaluations also indicate that it is effective against bacteria that show resistance to β-lactam antibiotics. A reduction in the use of similar antibiotics in treating mastitis, especially for preventative prophylactic dry cow therapy will

reduce the selective pressure for the development of resistant strains. This bacteriocin is easily released from teat seal preparations and could therefore be used to provide an effective physical barrier against infection whilst inhibiting Gram-positive strains that could be present in the teat canal or potentially invade the teat canal. *In vivo* studies would however be necessary to fully evaluate the effectiveness of this bacteriocin.

Acknowledgements

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Table 1 Antimicrobial agents and the MIC breakpoints (NCCLS, 2004)

MIC breakpoint (μg·mL^{-1})

Antibiotic	**Organism**	**S[b]**	**I[b]**	**R[b]**
Ampicillin	Staphylococci	≤ 0.25	-	≥ 0.5
	Streptococci	≤ 0.25	0.5 – 4.0	≥ 8.0
	Enterobacteriaceae	≤ 8.0	16.0	≥ 32.0
Oxacillin	*S. aureus*	≤ 0.12	-	≥ 0.25
	Staphylococcus spp.	≤ 0.25	-	≥ 0.5
	Streptococci[a]	≤ 2.0	-	≥ 4.0
Penicillin G	Staphylococci	≤ 0.12	-	≥ 0.25
	Streptococci	≤ 0.12	0.25 – 2.0	≥ 4
Erythromycin	*Staphylococcus* spp.	≤ 0.50	1.0 – 4.0	≥ 8.0
	Streptococci	≤ 0.25	0.50	≥ 1.0

[a] Interpretative criteria based on human data
[b] S, sensitive; I, intermediate; R, resistant

Table 2 Minimum inhibitory concentration (MIC, expressed as $\mu g \cdot mL^{-1}$) of selected antibiotics and activity of macedocin ST91KM (expressed as $AU \cdot mg^{-1}$ protein) against mastitis-causing pathogens.

| | | MIC ($\mu g \cdot mL^{-1}$) of antibiotics[a] | | | | | | | | | | Macedocin ST91KM Activity | | | | | |
| | | | | | | | | | | | | ST91KMppt.[c] | | | ST91KMcon[d] | | |
Organism	Strain	AMP		OXA		PEN		MET[b]		EM		$AU \cdot mL^{-1}$	$mg \cdot mL^{-1}$ Protein	$AU \cdot mg^{-1}$ Protein	$AU \cdot mL^{-1}$	$mg \cdot mL^{-1}$ Protein	$AU \cdot mg^{-1}$ Protein
S. agalactiae	RPSAG1	0.25	S	4.00	R	0.50	I	8.00	R	0.50	I	640	0.12	76.8	2560	0.08	204.8
S. agalactiae	RPSAG2	0.25	S	4.00	R	0.25	I	16.00	R	0.25	S	1280	0.06	76.8	5120	0.04	204.8
S. dysgalactiae	RPSD1	0.25	S	8.00	R	0.25	I	8.00	R	0.25	S	640	0.12	76.8	2560	0.08	204.8
S. dysgalactiae	RPSD2	0.25	S	4.00	R	0.25	I	16.00	R	0.25	S	640	0.12	76.8	2560	0.08	204.8
S. dysgalactiae	RPSD3	0.25	S	4.00	R	0.25	I	8.00	R	0.25	S	640	0.12	76.8	2560	0.08	204.8
S. dysgalactiae	RPSD4	0.25	S	4.00	R	0.50	I	16.00	R	0.50	I	640	0.12	76.8	2560	0.08	204.8
S. uberis	RPSU1	0.12	S	8.00	R	0.25	I	16.00	R	0.25	S	640	0.12	76.8	10240	0.02	204.8
S. uberis	RPSU2	0.25	S	8.00	R	0.25	I	16.00	R	0.25	S	160	0.47	75.2	640	0.34	217.6
S. uberis	EDSU1	0.12	S	8.00	R	0.25	I	32.00	R	0.25	S	640	0.12	76.8	5120	0.04	204.8
S. uberis	EDSU2	0.25	S	8.00	R	1.00	I	16.00	R	0.50	I	160	0.47	75.2	1280	0.17	217.6
S. aureus	RPSA1	0.12	S	8.00	R	0.50	R	16.00	R	0.50	S	640	0.12	76.8	1280	0.17	217.6
S. aureus	RPSA2	0.25	S	8.00	R	0.50	R	16.00	R	0.50	S	640	0.12	76.8	1280	0.17	217.6
S. aureus	EDSA	0.25	S	4.00	R	0.50	R	16.00	R	0.50	S	0	3.74	0	0	10.80	0
S. aureus	EDSA0267	0.25	S	8.00	R	8.00	R	16.00	R	0.25	S	640	0.12	76.8	1280	0.17	217.6
S. aureus	EDSA0269	0.12	S	8.00	R	0.50	R	16.00	R	0.50	S	640	0.12	76.8	1280	0.17	217.6
S. epidermidis	RPSE1	0.25	S	16.00	R	0.50	R	16.00	R	0.25	S	2560	0.03	76.8	10240	0.02	204.8
E. coli	RPEC1	1.00	S	>32	(R)	32.00	(R)	>32.00	(R)	>32	(R)	0	3.74	0	0	10.80	0
E. coli	RPEC2	2.00	S	>32	(R)	32.00	(R)	>32.00	(R)	>32	(R)	0	3.74	0	0	10.80	0

[a]ampicillin (AMP), oxacillin (OX), penicillin G (PEN), methicillin (MET), erythromycin (EM);
sensitive (S), intermediate (I), resistant (R);
[b] MET interpretative data based on OX
No veterinary-specific interpretative criteria available for *E.coli* for OX, PEN, MET and EM, breakpoint interpretation is thus given in parentheses.
[c] macedocin ST91KM precipitated in 60 % saturated ammonium sulphate,
[d] macedocin ST91KM precipitated in 60 % saturated ammonium sulphate, eluted with 40 % (v/v) isopropanol, in 25 mM ammonium acetate buffer (pH 6.5). Fractions of 1 mL were dried under vacuum at 50 °C, reconstituted to final volume of 1 mL with ammonium acetate buffer (pH 6.5)

	(a)	(b)	(c)	(d)
Zone appearance				
Zone diameter (mm)	0	15	0	14

Fig. 1. Comparison of macedocin ST91KM activity against *S. agalactiae* (RPSAG2) (a) teat seal only, (b) Teat seal combined with macedocin ST91KM, (c) teat seal plus 1 % (w/w) Tween 80, (d) teat seal combined with macedocin ST91KM, plus 1 % (w/w) Tween 80.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Mastitis is the most important disease in the dairy industry worldwide. It accounts for significant losses due to reduced yield, treatment costs and loss of income if milk is discarded as a result of reduced quality, bacterial contamination or antibiotic residues. The industry is facing many challenges in attempting to manage this disease. Increasing demands for milk require that farming operations are more intensive which in turn places increasing pressure on dairy farms to produce high quality product that meets legislative requirements (Petrovski 2006). The trend is towards a more holistic approach to farming, which demands a stress free environment for cows and a reduction in the use of antibiotics for organic farming enterprises, especially in Europe (Ekaman and Østerås 2003; Østerås and Solverod 2005). Widespread use of antibiotics especially for prophylactic treatment favours selective pressure for the development of antibiotic resistance (Passantino et al. 2007). There is concern that resistant strains could enter the food chain via contaminated food products (Lee 2003). This may make treatment of human pathogens more challenging and would favour the development of alternative antimicrobial agents (Shyock 2004).

Vaccination is considered to be the ultimate solution, especially against *Staphylococcus aureus* and coliform mastitis, as antibiotic treatment has limited success (Wilson et al. 1999). DNA expression vectors as vaccines show the most promise in stimulating the immune system to clear bacterial pathogens during infection (Talbot and Lacasse 2005). The current challenges facing the industry in terms of reducing antibiotic use, however, require a feasible solution that could be reached in the short-term. Bacteriocins could possibly be that solution.

Bacteriocins produced by lactic acid bacteria are generally regarded as safe (GRAS). Some have food-grade status namely, nisin (Jack et al. 1995) and are considered to be

harmless to humans as they are easily destroyed by digestive enzymes. These antimicrobial peptides are ribosomally synthesised. They differ from most antibiotics in that they usually have a narrow-spectrum of activity against closely related species (Jack et al. 1995). They often have a bactericidal mode of action, preventing cell wall synthesis and forming pores in the cell surface of sensitive strains. This results in an efflux of cytoplasmic compounds that are required to maintain ion gradients, trans-membrane potential and the pH gradient across the membrane. Biosynthetic pathways such as ATP synthesis driven by proton motive force cease and cell death occurs (Cotter et al. 2005).

The first aim of this study was to isolate and identify a bacteriocin-producing strain, active against mastitis pathogens. *Streptococcus macedonicus* ST91KM, isolated from Bulgarian goat yoghurt, produces the bacteriocin macedocin ST91KM. As far as we can determine this is only the second putative macedocin to be characterised. Macedocin ACA-DC 198, isolated from Greek Kasseri cheese, was characterised by Georgalaki et al. (2002) for use as a food-grade bacteriocin. Macedocin ST91KM was considered to be unique and dissimilar to macedocin ACA-DC 198 as the macedocin ACA-DC 198 precurser gene, *mcdA* could not be detected on the genomic DNA of the producer strain *S. macedonicus* ST91KM by PCR. Macedocin ST91KM has a narrow spectrum of antibacterial activity against Gram-positive strains. The cell-free supernatant containing macedocin ST91KM was active against mastitis pathogens *S. agalactiae* and *S. epidermidis* as well as other bacteria, *Lactobacillus sakei* and *Micrococus varians*. Other mastitis pathogens were also shown to be sensitive to macedocin ST91KM after precipitation by 60 % ammonium sulphate, including environmental streptococci *S. uberis* and *S. dysgalactiae* and *S. aureus*. These pathogens are considered to be the predominant causes of mastitis by Gram-positive bacteria (Philpot and Nickerson 1999).

Macedocin ST91KM is estimated to be between 2.0 and 2.5 kDA in size, as determined by tricine-SDS-PAGE. The bacteriocin could be considered as a viable treatment option in the udder tissue environment as it is active over broad pH range (2.0 – 10.0) and is heat stable up to 100 °C. Parameters affecting adsorption of macedocin ST91KM to bacterial cells indicated that adsorption was not species-specific and was similar in sensitive and non-sensitive cells. The optimum adsorption occurred at a pH range of 8.0 -10.0 and at 37 °C. A pH of 6.6 increasing to 8.0 when an infection is present and a temperature of at least 37 °C would be expected at the site of infection in the udder and could influence the effectiveness of antimicrobials (Ziv 1980). Macedocin ST91KM, however would appear to be active and able to adsorb to pathogens in *in vitro* experiments at these conditions. Rapid cell death of *S. agalactiae* and the release of cytoplasmic components, indicate a bactericidal mode of action.

Due to widespread antibiotic resistance in pathogenic strains, the antibiotic resistance patterns of selected mastitis pathogens was determined as well as the effect of the bacteriocin ST91KM on sensitive strains. All streptococci and staphylococci screened were resistant to oxacillin and methicillin. Streptococci had intermediate resistance to penicillin G and *S. aureus* and *S. epidermidis* were resistant to penicillin G. These results are not uncommon and coincide with recent trends in antimicrobial susceptibility studies (Gentillini et al. 2002; Moroni et al. 2006; Rositto et al. 2002). Only eighteen mastitis isolates were evaluated but an indication of degree of resistance that could be expected was given. Macedocin ST91KM was also evaluated under similar test conditions. *S. agalactiae* and *S. epidermidis* were the most the sensitive of the Gram-positive isolates. The arbitrary units per mL required to completely inhibit these strains was 1280 AU·mL^{-1} and 2560 AU·mL^{-1}, respectively. The bactericidal activity of macedocin ST91KM was not the same for all strains within a species. Notably, two of four strains of *S. uberis* were less sensitive and one of four strains of *S. aureus* showed resistance to macedocin ST91KM at the highest concentration of bacteriocin

preparation used. It is possible that a resistance mechanism existed in this strain; however, it is perhaps likely that the potency of the bacteriocin in the bacteriocin preparation was perhaps not high enough to result in a bactericidal effect.

In *vitro* studies evaluating the possible administration of the bacteriocin for dry cow therapy in a teat seal preparation were carried out. The bacteriocin was effectively released from the teat seal preparation without the aid of any additional surfactant. The advantages of administering the bacteriocin in combination with a teat seal are two-fold. Firstly, the teat seal would be used during the dry period to prevent and reduce any existing bacterial infections by acting as a physical barrier at the entrance to the teat canal. Secondly, the bacteriocin would be localised at a specific area and inhibit the growth of any bacteria that could potentially be present in the teat canal. Localising the activity of the bacteriocin to this area could mean that less product would need to be used.

Further research can be focused on purifying the bacteriocin in order to determine the amino acid sequence and possibly the gene sequence. *In vivo* studies should also be carried out to fully explore the potential of this bacterioicin as a viable alternative for the treatment of bovine mastitis.

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APPENDIX

Growth of *Streptococcus macedonicus* ST91KM and production of macedocin ST91KM

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Abstract: A new macedocin, produced by *Streptococcus macedonicus* ST91KM isolated from Bulgarian-style yoghurt, is active against a narrow-spectrum of Gram-positive bacteria, including mastitis pathogens *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis.* Macedocin ST91KM is produced in standard MRS broth, BHI, M17 and skim milk. The optimum period of incubation for bacterioicin production occurs at mid-log phase between 12 and 15 h at 30 °C in MRS broth. The initial pH in the growth medium of 6.5 should be used for optimum bacteriocin production. The highest bacteriocin activity against *S. agalactiae* was recorded as 800 AU·mL^{-1}, corresponding to a culture pH of 4.5. Bacteriocin activity was detected after 24 h incubation, but was reduced to 400 AU·mL^{-1}.

Introduction

Lactic acid bacteria produce inhibitory substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocins. Bacteriocins are defined as ribosomally synthesized peptides, usually active against bacteria of the same or closely related species (Jack et al. 1995). The first report of a macedocin, ACA-DC 198 produced by the thermophilic *S. macedonicus* ACA-DC 198 was characterised by Georgalaki et al. (2002). More recently, *S. macedonicus* strains isolated from Italian raw milk cheeses were characterised (Lombardi et al. 2004). Examples of other thermophilic streptococcal bacteriocins include those produced by *Streptococcus thermophilus* strains. These have been investigated for use in yoghurt starter cultures and include thermophilin 81 (Ivanova et al. 1998) and thermophilin 13 (Marciset et al. 1997), while thermophilin 580, produced by *S. thermophilus* 580 has been studied for possible application in cheese production as starter cultures with the added benefit of bacteriocin inhibition of *Clostridium tyrobutyricum* in the cheese ripening process (Mathot et al. 2003).

Streptococcus macedonicus ST91KM, isolated from Bulgarian-style yoghurt, produces a bacteriocin with a very narrow spectrum of activity against Gram-positive bacteria including mastitis causing pathogens *Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis*. Peptide ST91KM is, according to tricine-SDS PAGE, between 2.0 and 2.5 kDa in size. The bacteriocin is heat-stable over a broad pH range (2.0 – 10.0) and could possibly find application in the prophylactic treatment of mastitis in dairy cows). The aim of this experiment was to monitor the production of the bacteriocin by the producer strain *S. macedonicus* ST91KM in MRS broth and to determine if production is affected by pH.

Materials and methods

Bacterial strains

The bacteriocin producer *S. macedonicus* ST91KM was grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, SA) at 30 °C. The indicator strain *Streptococcus agalactiae* (RPSAG2) was obtained from the Western Cape Provincial Veterinary Laboratory (Stellenbosch, South Africa) and *Lactobacillus sakei* (LMG13558) from our own culture collection (Department of Microbiology, University of Stellenbosch). *S. agalactiae* (RPSAG2) was cultured in BHI media (BHI Broth, Merck, Darmstadt, Germany) at 37 °C, while *L. sakei* (LMG13558) was cultured in MRS broth at 30 °C.

Macedocin ST91KM production in MRS broth

S. macedonicus ST91KM was cultured in MRS broth at 30 °C for 24 h. Optical density readings (OD_{600nm}), viable cell numbers ($cfu \cdot mL^{-1}$) and culture pH was recorded. Bacteriocin activity was determined at 3 h intervals during growth, using the agar-spot method described by Van Reenen et al. (1998). Cell-free culture supernatants were exposed to 80 °C for 10 min to inactivate proteolytic enzymes and then adjusted to between 6.0 and 6.5 with sterile 1 M NaOH. Antimicrobial activity was expressed as arbitrary units $AU \cdot mL^{-1}$, calculated as follows: a^b x 100, where "a" represents the dilution factor and "b" the last dilution that produces an inhibition zone of at least 2 mm in diameter. *S. agalactiae* (RPSAG2) was used as the indicator strain.

Effect of growth media and initial growth pH on macedocin ST91KM production

Strain ST91KM was grown in a 10 mL MRS broth for 15 h at 30 °C, the cells harvested (8000 x *g*, 10 min, 4 °C), and the pellet resuspended in 10 mL sterile physiological water to

OD_{600nm} = 1.4. The following growth media were inoculated with 1 % (v/v) of the cell suspension: MRS broth (Biolab), BHI (Biolab), M17 (Merck, Darmstadt, Germany), Skim milk and Soy milk (commercial brands). After growth, the cells were harvested (8000 x *g*, 10 min, 4 °C), the pH of the cell-free culture supernatant adjusted to 6.0 with 1 M NaOH and heat-treated at 80 °C for 10 min. The activity of macedocin ST91KM was determined using the agar-spot method (Van Reenen et al. 1998). *L. sakei* LMG13558 served as target strain.

In a separate experiment, the pH of MRS broth was adjusted to 4.5, 5.0, 6.0 and 6.5 using 6 M HCl or 6 M NaOH to test the effect of the initial pH on the production of macedocin ST91KM. MRS broth was inoculated with a 1 % (v/v) of a 15-h-old culture (OD_{600nm} = 1.4 - 1.5) and incubated at 30 °C for 20 h. Changes in the pH were recorded and bacteriocin activity expressed in $AU \cdot mL^{-1}$ as described before.

Results and Discussion

Macedocin ST91KM production in MRS broth

Bacteriocin activity was observed in MRS broth (Biolab) during mid-log phase, after 9 h of incubation at 30 °C. The highest yield of bacteriocin ST91KM in MRS broth was recorded at 800 $AU \cdot mL^{-1}$ (4.9 x 10^9 $cfu \cdot mL^{-1}$) after 12 h of growth (Fig. 1). This corresponded to a pH of 4.5. Bacteriocin activity was maintained after 15 h of growth, but decreased to 400 $AU \cdot mL^{-1}$ after 24 h. This is indicative of primary metabolite kinetics. The production kinetics of macedocin ACA-DC 198 was similar in that bacteriocin production also occurred in mid log phase, but differed in that bacteriocin levels were still detected after 24 h (Georgalaki et al. 2002). Thermophilin 81 and thermophilin 580 follow similar production kinetics (Ivanova et al. 1998; Mathot et al. 2003).

CHAPTER 2 Effect of growth media and initial medium pH on macedocin ST91KM production

Strain ST91KM cultured in MRS broth produced macedocin ST91KM at 800 AU·mL^{-1} (Table 1). Growth in other media yielded levels ranging from 200 AU·mL^{-1} (BHI broth) to 1600 AU·mL^{-1} (M17 broth) (Table 1). Addition of skim milk (20 – 100 g L^{-1}) reduced activity to 400 AU·mL^{-1}. An initial medium pH of 4.5 yielded low levels of macedocin ST91KM (200 AU·mL^{-1}). The activity increased as the initial pH increased, with a maximum activity (800 AU·mL^{-1}) recorded in medium with an initial pH of 6.5. Production of macedocin ST91KM differed from that recorded for macedocin ACA-DC 198 produced by *S. macedonicus* ACA-DC 198 in that antimicrobial activity was only occasionally detected in MRS broth. A maximum bacteriocin production of 400 AU·mL^{-1} was recorded in skim milk supplemented with nitrogen sources. No macedocin ACA-DC 198 production occurred in M17 broth or BHI (Georgalaki et al. 2002).

Conclusion

Macedocin ST91KM is produced in standard MRS broth, BHI broth, M17 broth and skim milk. Optimal levels of bacterioicin production were recorded between 12 and 15 h when cells were grown at 30 ºC in MRS broth. MRS broth adjusted to pH of 6.5 should be used for optimum bacteriocin production. Macedocin ST91KM differ from macedocin AC-DC 198 in that production was recorded in all the growth media tested. Macedocin ACA-DC 198 production was only detected in skim milk.

Acknowledgements

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Table 1. Effect of MRS, BHI, M17, soy milk and skim milk on production of macedocin ST91KM.

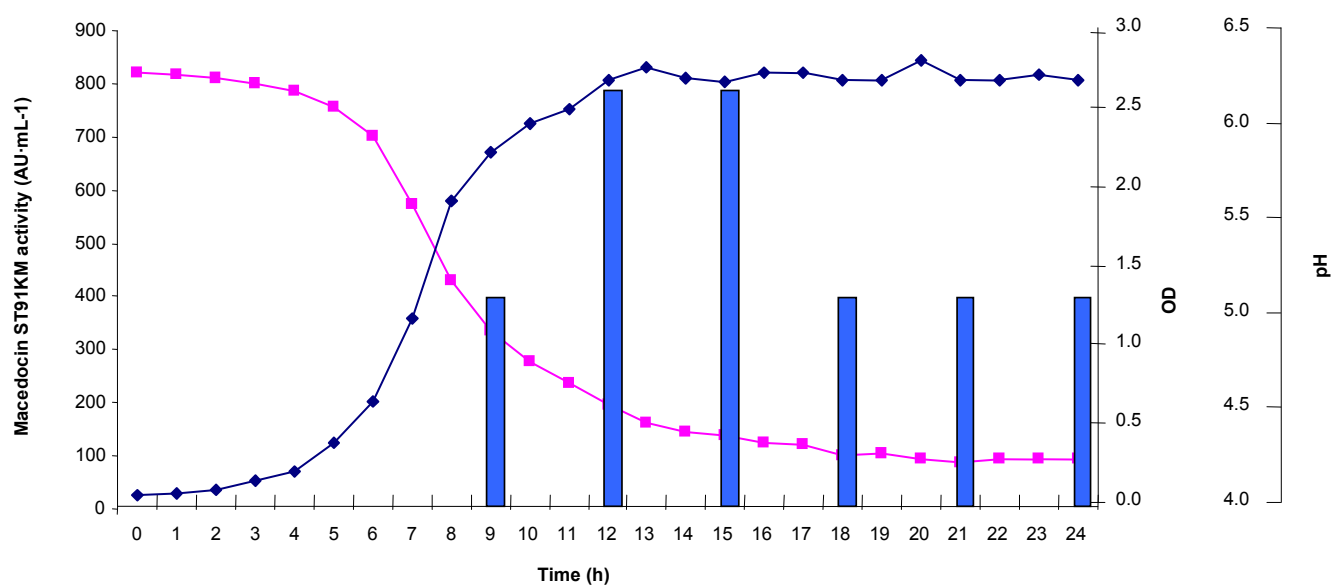| Growth medium	Macedocin ST91KM activity AU ml^{-1}
MRS	800
BHI	200
M17	1600
Soy milk 100 g l^{-1}	200
Skim milk 20 - 100 g l^{-1}	400

Fig 1. Growth of *S. macedonicus* ST91KM (♦), bacteriocin production (bars) and pH (■) in MRS broth (Biolab) at 30 °C.