# Therapeutic properties of the lantibiotic nisin F

by
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# Declaration

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# Summary

Bacterial resistance against antibiotic treatments is a global concern and resistance to almost every known antibiotic has already been reported. There is thus a significant need for the development of novel antimicrobial drugs.

In addition to probiotic traits, certain bacteria have the ability to produce antimicrobial peptides, referred to as bacteriocins. Lantibiotics, a group of small ribosomally synthesized bacteriocins, recently gained interest for their application in the medical field. Lantibiotics have a very specific structure, including lanthionine rings, that stabilise the peptides. Due to their small size and specific action, these peptides reach specific sites of infection without affecting the composition of the host's natural microbiota. As with any therapeutic agent, antimicrobial peptides are also prone to *in vivo* degradation, binding, clearance via immune action and development of bacterial resistance.

Nisin F, a class Ia lantibiotic produced by *Lactococcus lactis* subsp. *lactis* F10, has already shown activity against the well-known pathogens *Stapylococcus aureus*, *Listeria monocytogenes* and various antibiotic resistant strains. The aim of this study was to assess the antimicrobial activity of nisin F against systemic *S. aureus* infections in mice and possible immune responses elicited by the peptide.

A single administration of nisin F to the peritoneal cavity protected mice from *S. aureus* infection for at least 15 min. After continuous administration, the peptide showed no significant antimicrobial activity against *S. aureus*. The peptide did, however, convey some degree of protection to infected mice by stimulating a pro-inflammatory action through lymphocyte protection. When administered to uninfected mice, nisin F had an immune boosting effect via interleukin (IL)-6 and IL-10 without being detrimental to the host. The *ex vivo* effects of nisin F was compared to nisin A, a natural nisin variant, and Nisaplin®, a commercially purified form of nisin A. None of the three peptides inhibited the functional capacity of leukocytes in terms of 1L-1β en IL-6 production, not even in the presence of an external stimulus (lipopolysaccharides from *Escherichia coli*). Cytotoxicity was detected in response to high dosages of nisin F. Serum inhibited the antimicrobial effect of nisin F and nisin A, but Nisaplin® remained unaffected.

Nisin F was applied against systemic infection for the first time and the immunological effect of the peptide was investigated. Nisin F partially protected mice against *S. aureus* infections through immunomodulatory effects. This study provided valuable knowledge on the *in vivo* application of nisin F. With further optimization of nisin F preparation and application systems, the peptide might be more effective against *in vivo* infections.

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# Opsomming

Bakteriële weerstand teen antibiotika wek wêreldwyd kommer en weerstand teen amper elke bekende antibiotikum is reeds aangemeld. Daar is dus 'n groot behoefte vir die ontwikkeling van nuwe antimikrobiese middels.

Bykomend tot probiotiese eienskappe, het sekere bakterieë die vermoë om antimikrobiese peptiede, bekend as bakteriosiene, te produseer. ‘n Groep klein ribosomaal-gesintetiseerde bakteriosiene, lantibitiotika, is onlangs vir mediese toepassing oorweeg. Lantibiotika beskik oor 'n baie spesifieke struktuur, insluitend lantionien ringstrukture, wat die peptied stabiliseer. Weens hul klein grootte en spesifieke aksie is hierdie peptiede daartoe in staat om spesifieke areas van infeksie te bereik sonder om die gasheer se natuurlike mikrobepopulasie te beïnvloed. Soos met enige terapeutiese middel, is bakteriosiene ook geneig tot *in vivo* afbreking, binding, klaring via die immuunsisteem en ontwikkeling van bakteriële weerstand.

Nisien F, 'n klas Ia lantibiotikum, deur *Lactococcus lactis* subsp. *lactis* F10 geproduseer, het reeds aktiwiteit teen die bekende patogene *Stapylococcus aureus*, *Listeria monocytogenes* en verskeie antibiotika-weerstandige stamme getoon. Die doel van hierdie studie was om die antimikrobiese aktiwiteit van nisien F teen sistemiese *S. aureus* infeksies in muise te bepaal, asook die moontlike immuunreaksies wat die peptied mag veroorsaak.

'n Enkele toediening van nisien F het muise vir ten minste 15 min teen *S. aureus* beskerm. Na deurlopende administrasie het die peptied geen beduidende antimikrobiese aktiwiteit teen *S. aureus* getoon nie. Die peptied het egter 'n mate van beskerming aan geinfekteerde muise verleen deur ‘n pro-inflammatoriese aksie te inisieer deur limfosiet beskerming. Met toediening aan gesonde diere, het nisien F 'n immuunversterkende effek teweeg gebring via interleukin (IL)-6 en IL-10 vlakke, sonder nadelige uitwerking op die gasheer. Die *ex vivo* effek van nisien F is ook vergelyk met nisien A, 'n natuurlike variant van nisien, asook Nisaplin®, 'n kommersieël-gesuiwerde vorm van nisien A. Nie een van die drie peptide het leukosiete se funksionele kapasiteit in terme van 1L-1β en IL-6 produksie inhibeer nie, selfs nie in die teenwoordigheid van ‘n eksterne stimulus (lipopolisakkariede van *Escherichia coli*) nie. Seltoksisiteit is na blootstelling aan hoë dosisse van nisien F waargeneem. Serum het die antimikrobiese effek van beide nisien F en nisien A geïnhibeer, terwyl die werking van Nisaplin® nie beïnvloed is nie.

Nisien F is vir die eerste keer teen sistemiese infeksies ingespan en die immunologiese impak van die peptied is ondersoek. Nisien F het gedeeltelike beskerming aan muise met *S. aureus* infeksies verleen

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deur die immuunsisteem te versterk. Die resultate het ‘n waardevolle bydrae gelewer tot die *in vivo* toediening van nisien F. Met verdere optimisering van nisien F voorbereiding en toedieningsisteme, mag die peptied moontlik meer effektief teen *in vivo* infeksies aangewend word.

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# Preface

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. Two articles have been published and two manuscripts have been prepared for submission to ISI-accredited journals. All other chapters have been written according to the instructions of the Journal of Applied Microbiology.

The literature review is an overview of global and South African occurrence of antimicrobial resistance and description of *Staphylococcus aureus*, one of the most prevalent organisms in this regard. The biosynthesis, mode of action, *in vivo* monitoring and application of bacteriocins, with the focus on nisin, are also discussed.

The manuscript “Development of a murine model with optimal routes for bacterial infection and treatment, as determined with bioluminescent imaging in C57BL/6 mice” has been published in *Probiotics and Antimicrobial Proteins* (2011; **3**, 125-131) and is presented in Chapter 3 as published.

The manuscript “The ability of nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice” has been published in *Letters in Applied Microbiology* (2010; **51**, 645-649) and is presented in Chapter 4 as published.

The manuscript “The effects of continuous *in vivo* administration of nisin F on *Staphylococcus aureus* infection and immune response in mice” has been prepared for submission to *International Journal of Antimicrobial Agents* and is presented in Chapter 5.

The manuscript “Antimicrobial activity of nisin F, nisin A and Nisaplin in the presence of serum and the effect of these lantibiotics on leukocyte functional capacity” has been prepared for submission to *Journal of Peptide Science* and is presented in Chapter 6.

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# Contents

**Page**

**Chapter 1** 1

**Introduction**

**Chapter 2** 6

**Literature Review**

1. Antimicrobial resistance 7
2. *Staphylococcus aureus* 8
 2.1 Prevalence 10
 2.2 Infection 10
 2.3 Virulence 13
 2.4 Treatment 15
 2.4.1 *CA-MRSA* 15
 2.4.2 *HA-MRSA* 16
3. Antimicrobial peptides 16
 3.1 Lactic acid bacteria and bacteriocins 18
 3.2 Nisin 18
 3.2.1 *Biosynthesis* 19
 3.2.2 *Mode of action* 21
 3.2.3 *Resistance* 23
 3.2.4 *Applications* 24
 3.3 Limitations of bacteriocins and possible solutions 25
4. Bioluminescence imaging 27
5. *In vivo* animal infection models 29
6. Drug development 30

**Chapter 3** 57

**Development of a murine model with optimal routes for bacterial infection and treatment, as determined with bioluminescent imaging in C57BL/6 mice**
Published in *Probiotics and Antimicrobial Proteins* **3**, 125-131 (2011)

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**Chapter 4** 71

**The ability of nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice**

Published in *Letters in Applied Microbiology* **51**, 645-649 (2010)

**Chapter 5** 83

**The effects of continuous *in vivo* administration of nisin F on *Staphylococcus aureus* infection and immune response in mice**

Prepared for publication in *International Journal of Antimicrobial Agents*

**Chapter 6** 97

**Antimicrobial activity of nisin F, nisin A and Nisaplin in the presence of serum and the effect of these lantibiotics on leukocyte functional capacity**

Prepared for publication in *Journal of Peptide Science*

**Chapter 7** 114

**General Discussion and Conclusions**

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# Chapter 1

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# Introduction

Antimicrobial resistance is a serious problem, also in Africa (Okeke and Sosa 2003). The infectious disease burden in Africa is high and financial constraints prevent proper distribution and implementation of the latest and more expensive antibiotics. The situation is further worsened by poor sanitation and general hygiene, over-population, food-animal husbandry and the abuse of antibiotics. Although the World Health Organization (WHO) implemented a global strategy in 2001 to control antimicrobial resistance, developing regions such as Africa seems to be worse-off (WHO 2001; Nyasulu et al. 2012).

*Staphylococcus aureus* is an opportunistic pathogen with asymptomatic colonization of epithelial and mucosal surfaces. Apart from being one of the most important hospital-associated pathogens, infection can lead to minor skin diseases and invasive diseases such as bacteraemia, endocarditis and toxic shock syndrome (Lowy 1998; Diekema et al. 2001; Daum 2008). Wounds originating from implants, prosthetic devices or damaged skin create the perfect environment for infection. Before the introduction of antimicrobials, 90% of invasive *S. aureus* infections led to death. Penicillin was initially used as treatment, but certain strains soon developed resistance to the antibiotic (Barber Rozwadowska-Dowzenko 1948), including other popular antibiotics such as methicillin (Jevons 1961) and vancomycin (Moran and Mount 2003). Resistance was also recorded against newly applied antibiotics such as the quinopriston and dalfopristin combination (Luh et al. 2000, Deshpande et al. 2004) and linezolid (Gonzales et al. 2001; Kola et al. 2007). Methicillin-resistant *S. aureus* (MRSA) is often multi-drug resistant and continues to raise concerns with its rapid spreading into health and community settings around the globe (Stefani et al. 2012).

In 2008, approximately 90 000 invasive MRSA infections were recorded in the United States. Of these cases, 15 249 patients died while hospitalized (CDC 2008). According to a study done in 2005, 85% of all invasive MRSA infections were healthcare-associated, with two-thirds reported to have occurred outside the hospital (CDC 2007). Cases recorded were from diverse geographical areas, but were more prominent in people older than 65 years, Blacks and males. In South Africa, MRSA isolates from various hospitals and private laboratories showed high-level multi-drug resistance (Marais et al. 2009). Resistance to the macrolides, tetracycline and the aminoglycosides were observed, while vancomycin, linezolid and quinopristin/dalfopristin seemed appropriate agents for clinical use in South Africa (Marais et al. 2009; Nyasulu et al. 2012). Resistance patterns varied between children and adults, and between public and private systems. With a study conducted in Cape Town, South Africa, two rifampicin-resistant MRSA strains were isolated (Van Rensburg et al. 2012). Global antibiotic resistance against vancomycin (VRSA: Vancomycin-resistant *S. aureus*) and the recently applied antibiotics daptomycin and linezolid (Sakoulas and Moellering 2008) poses a great challenge to and need for the development of alternative treatments for *S. aureus* infections.

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Lactic acid bacteria (LAB) have the ability to produce small antimicrobial peptides (AMPs) called bacteriocins - lately extensively studied for their therapeutic properties. Due to their generally regarded as safe (GRAS) status, small size, specific binding sites, mode of action, and ability to act in nanomolar concentrations, class I bacteriocins are continuing to become more appealing than conventional antibiotics. Nisin F is a class IIa bacteriocin with *in vitro* antimicrobial activity against *S. aureus* (De Kwaadsteniet et al. 2008). Although not successful in *in vivo* antimicrobial treatment, nisin F showed no abnormal side-effects when tested against respiratory *S. aureus* infections in rats and subcutaneous *S. aureus* skin infections in mice (De Kwaadsteniet et al. 2009, 2010). Nisin F successfully cured subcutaneous *S. aureus* infections in mice when incorporated into bone cement (Van Staden et al. 2012).

Bioluminescent imaging (BLI) is a recently explored process that allows researchers to track bacterial and viral infection, gene expression, protein stability and tumour growth in small animals (Contag et al. 1995; Prescher and Contag 2010). The BLI technique is based on the capturing and quantification of visible light produced by luciferase reactions. In this study BLI was implemented to monitor the therapeutic effect of nisin F.

Drug discovery and development take several years and involve a few critical steps. The drug should not only exert *in vitro*, but also *in vivo* activity. Usually *in vivo* pre-clinical studies are carried out on small mammals such as rodents, and when successful, progress to larger mammals and human clinical trials. Results obtained through *ex vivo* studies on a drug, e.g. toxicity to eukaryotic cells or immunomodulatory properties, often add valuable information to knowledge gained from *in vitro* and *in vivo* studies.

The objectives for this study were:

- to optimize *in vivo* infection of *S. aureus* and optimize treatment dosages of nisin F;
- to determine the ability of a single administration of nisin F to control *S. aureus* infection in the peritoneal cavity, as studied in mice;
- to determine the antimicrobial ability of continuous administration of nisin F to control *S. aureus* infection in the peritoneal cavity of mice; and
- to determine the immunological effect and stability of nisin F in *ex vivo* studies.

Thus, the final outcome of the study was to gain more information on the stability of nisin F *in vivo* and to serve as a stepping stone in the process of developing nisin F as an anti-staphylococcal drug.

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# Chapter 2

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# Literature Review

## 1. Antimicrobial resistance

The discovery of penicillin by Fleming in 1928 brought a dramatic revolution in medicine and created the belief that antibiotics will cure all infectious diseases (Fleming 1928). Soon, pathogenic microorganisms started to develop resistance to penicillin and other antibiotics that were subsequently developed. Today, resistance to almost every known antibiotic has been reported. Clinical isolates use different mechanisms to acquire resistance to antibiotics. These may include alterations of the target/binding site of the agent, modification of the agent, or restricting intracellular access of the agent (Konings et al. 2000). Factors influencing antimicrobial resistance (AMR) include inappropriate prescription and use of antibiotics, mobility of humans or animals, antibiotics used in food production and animal husbandry, as well as contaminated water and soil sources.

According to the World Health Organisation (WHO), AMR is still a world-wide crisis (2012). Although a global strategy was implemented in 2001, AMR is increasing morbidity and mortality rates (especially resistance to malaria, tuberculosis, HIV and common bacterial diseases) in healthcare facilities and communities. In 2009, the annual number of deaths in Europe caused by resistant pathogens exceeded 25 000, with MRSA responsible for 5 400 (37%) of the mortalities and more than a million additional hospitalisation days of patients (ECDC/EMEA 2009). Thus, AMR also has a negative economic impact. A patient infected with a resistant strain has to undergo more diagnostic testing, receive treatment with second-line antibiotics, and may even have to be hospitalized for a longer period. This leads to an increased possibility for the resistant strain to spread, or for the patient to acquire secondary infections (Wenzel and Edmond 2000; Laxminarayan 2003). In 1999, a full course of treatment for multidrug-resistant tuberculosis (TB) would have cost a patient R26 354, versus R215 for treatment of infection with a susceptible TB strain (Hensher 1999).

It is essential to assess the level of AMR in a country to put the necessary precautions in place. A few surveillance attempts were carried out by the South African Society of Clinical Microbiology, formerly known as the National Antimicrobial Surveillance Forum (NASF), as well as the Group for Enteric, Respiratory and Meningeal Diseases Surveillance (GERMS-SA), amongst others. Recently, an AMR surveillance program was initiated in South Africa by the Global Antibiotic Resistance Partnership (GARP) of the Centre for Disease Dynamics, Economics and Policy (CDDEP). Although of value, a standard surveillance program is still lacking (Nyasulu et al. 2012). Results from previous surveillance studies revealed that *Klebsiella pneumonia*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most resistant nosocomial pathogens (Apalata et al. 2011; Nyasulu et al. 2012). In a systematic review of nine AMR studies done in South Africa, Nyasulu et al. (2012) summarized antibiotic resistant *S. aureus* strains from clinical origin. On average, 38% of tested *S.*

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*aureus* strains were resistant to erythromycin, while no resistance to vancomycin or linezolid were observed (Fig. 1).

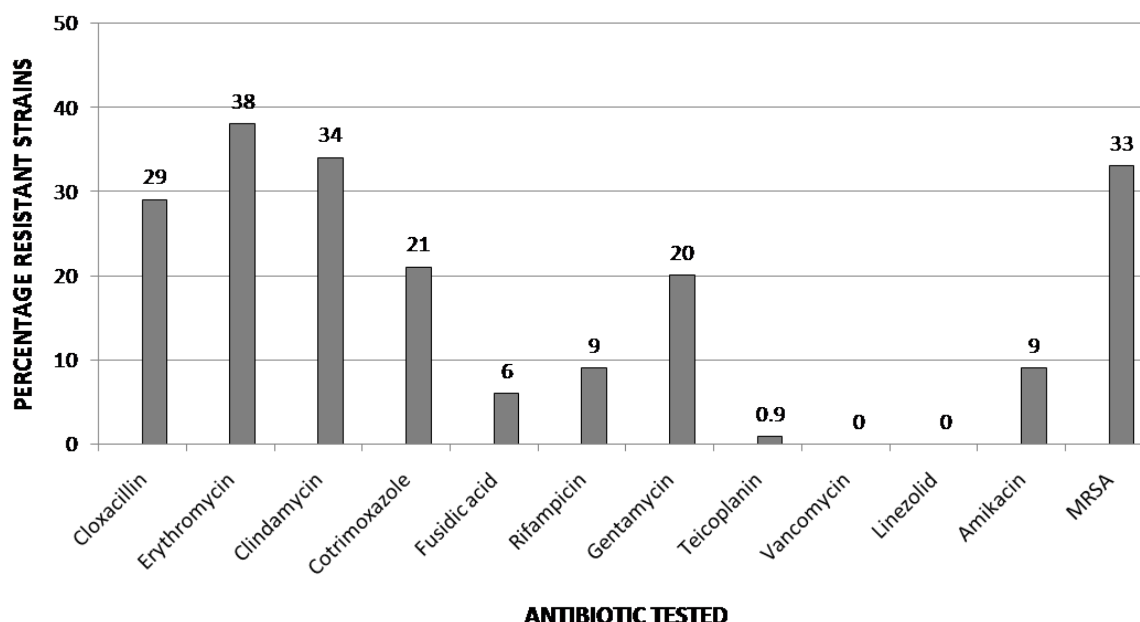


**Figure 1** Antibitiotic resistance patterns amongst strains of *S. aureus* isolated from patients in South African clinics (adapted from Nyasulu et al. 2012).

## 2. *Staphylococcus aureus*

*Staphylococcus aureus* is a member of the Staphylococcaceae family and is characterized by Gram-positive clusters of microcapsulated, nonsporulating, nonmotile cocci (Lowy 1998). The golden yellow colonies ferment mannitol (Gould and Chamberlain 1995) and flourish in saline conditions, as normally found on the skin surface (Thomas 1988). *Staphylococcus aureus* is a facultative anaerobe that grows between 15 and 45˚C, withstand high NaCl concentrations and drying (Matouskova and Janout 2008), and is catalase and coagulase positive.

*Staphylococcus aureus* became resistant to penicillin in the late 1940s to mid-1950s, with hospitals reporting 50% of all strains resistant (Barber and Rozwadowska-Dowzenko 1948; Colebrooke 1955). Strain 80/81, the first penicillin-resistant strain, originated in Australia and was transferred to America and the UK (Williams et al. 1959). The arrival of the semi-synthetic penicillin, methicillin, led to a decrease in cases of 80/81 infection (Matouskova and Janout 2008). Within six months, methicillin-resistant strains that originated were detected in England (Jevons 1961) and were classified as hospital-acquired methicillin resistant *Staphylococcus aureus* (HA-MRSA). These strains are also resistant to the antibiotics cloxacillin, fluocloxacillin and cephalosporins used before methicillin (Gould and Chamberlain 1995). Multidrug-resistant MRSA strains with combined resistance to peniciliin, streptomycin, tetracycline and erythromycin were reported in 1971 (Grundmann et al.

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2006). During the 1970s and early 1980s, the prevalence of multidrug-resistant MRSA strains decreased only to be followed by an increase in the 1980s and 1990s with outbreaks that lasted just more than two years (Tuffnell et al. 1987). Gentamycin resistance was detected in strains at the start of the eighties. A healthcare worker (HCW) transferred the strain from Australia to England (Duckworth et al. 1988). Vancomycin resistance emerged in 1987 (Scwalbe 1987), increased from 1996 (Hiramatsu et al. 1997) and a fully-resistant strain was isolated from a patient in Michigan in 2002 (CDC 2002). *Staphylococcus aureus* strains with intermediate susceptibility to vancomycin (VISA), heterogeneous VISA (hVISA) and linezolid-resistant *S. aureus* (LRSA) strains have been isolated, as well as MRSA isolates with decreased glycopeptide susceptibility (glycopeptide intermediately susceptible *S. aureus*, GISA). Isolates that spread globally were termed epidemic MRSA (EMRSA), opposed to methicillin-susceptible *S. aureus* (MSSA).

Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) has become more prevalent in the last two decades (CDC 1999). Infections with CA-MRSA are classified according to certain criteria. Firstly, the diagnosis should be made outside of a hospital or within 48h after admission to a hospital. Secondly, the patient should not have any medical history of MRSA before or after admission to the hospital and finally, the patient should not have any internal catheters or medical devices (CDC 2005). CA-MRSA infections usually occur in young individuals by means of skin and soft tissue infections (SSTIs) (Table 1).

**Table 1** Main characteristics of HA-MRSA and CA-MRSA strains (Matouskova and Janout 2008)

| Characteristic | HA-MRSA | CA-MRSA |
|---|---|---|
| Clinical | surgical site infections, invasive | skin infections, “bug bites”, rarely invasive, multiple, recurrent |
| Epidemiology | Age, healthcare | young, athletes, drug users, correctional facilities and military |
| Antibiotic resistance | multi-drug resistant | β-lactam resistant |
| Molecular markers | PVL –<br>SCC*mec* I-III | PVL +<br>SCC*mec* IV, V |

Panton-Valentine leukocidin (PVL) is a toxin and virulence factor lethal to neutrophils. It causes tissue necrosis by forming heptameric pores in leukocyte membranes and is associated with CA-MRSA, SSTIs (Lina et al. 1999) and severe necrotizing pneumonia (Labandera-Rey et al. 2007). CA-MRSA strains are β-lactam resistant, PVL+ and possess SSC*mec* types IV or V. HA-MRSA are more prevalent in older individuals in hospital or healthcare facilities, are multidrug-resistant, PVL- and possess SSC*mec* types I-III (Matouskova and Janout 2008). Differentiating between HA-MRSA and CA-MRSA based on epidemiological aspects is becoming more difficult due to multiple definitions, the rapidly changing molecular features of CA-MRSA and the adaptation to transmission within hospitals and healthcare-facilities by certain strains, e.g. US300 (David et al. 2008). In addition,

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infection acquired in the community can be transmitted in the hospital and *vice versa* (Flynn and Scott 2008). HA-MRSA infections can be sub-classified into hospital-onset (positive culture obtained more than 48h after admission) or community-onset (positive culture obtained less than 48h after admission) (Klevens et al. 2007).

## 2.1 Prevalence

Humans are a natural reservoir of *S. aureus* with asymptomatic colonization primarily in the anterior nares of the nose and on the skin (Williams 1963; Peacock et al. 2001). In hospitals, patients are regarded as the main reservoirs with doctors and nurses as carriers (Gould and Chamberlain 1995). In severe CA-MRSA cases, colonization was also detected in areas of the axillae, inguinal and rectum (Decker 2008). Colonization occurs more often in males (Ayliffe et al. 1977), especially those who have homosexual intercourse (Diep et al. 2008). Intravenous drug users, patients who underwent hemodialysis or surgery and individuals with type 1 diabetes, acquired immunodeficiency syndrome (AIDS), or leukocyte malfunction are more prone to colonization and subsequent *S. aureus* infection (Lowy 1998). Furthermore, foreign material used in invasive therapy, such as intravenous catheters, intubation or ventilation also increases the risk of infection (Gould and Chamberlain 1995; Lowy 1998). Older people often receive surgery and orthopaedic implantations, which places them into a high-risk category (Ayliffe et al. 1977). While the control of MRSA in long-term care facilities and nursing homes are increasing, only a few studies have been done on these settings (Navarro et al. 2008). Transmission usually occurs via skin contact with health workers or less frequently from environmental vectors. Athletes are often infected by CA-MRSA from contaminated objects (e.g. shared razors, soap bars, towels), whirlpools and damaged skin (Begier et al. 2004; Kazakova et al. 2005). Contaminated stethoscopes (Smith et al. 1996), pagers (Singh et al. 2002), workstations and bed sites (Hardy et al. 2006) can also spread infection. Airborne transmission occurs only across distances of a few feet (Gould and Chamberlain 1995). Domesticated animals may also spread MRSA to humans when in close contact with them or eating contaminated meat products (Navarro et al. 2008; Matouskova and Janout 2008).

## 2.2 Infection

Human infections occur often, but mostly stay localized at the site of entry due to the host defences. *Staphylococcus aureus* infection can lead to minor SSTIs, abnormalities such as boils, styes and furuncles. More serious diseases caused by *S. aureus* include bacteraemia, sepsis, mastitis, toxic shock syndrome, endocarditis and metastatic infections which spread to the bones (osteomyelitis), joints (arthritis), kidneys, lungs, heart and skeletal muscle (Lowy 1998; Todar). Outbreaks in hospitals are also associated with discharging wounds, urinary tract infections, otitis media, pneumonia (Gould and

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Chamberlain 1995), skin lesions and erythema (Navarro et al. 2008). *Staphylococcus aureus* also causes food poisoning. A summary of diseases caused by *S. aureus* is shown in Fig. 2.

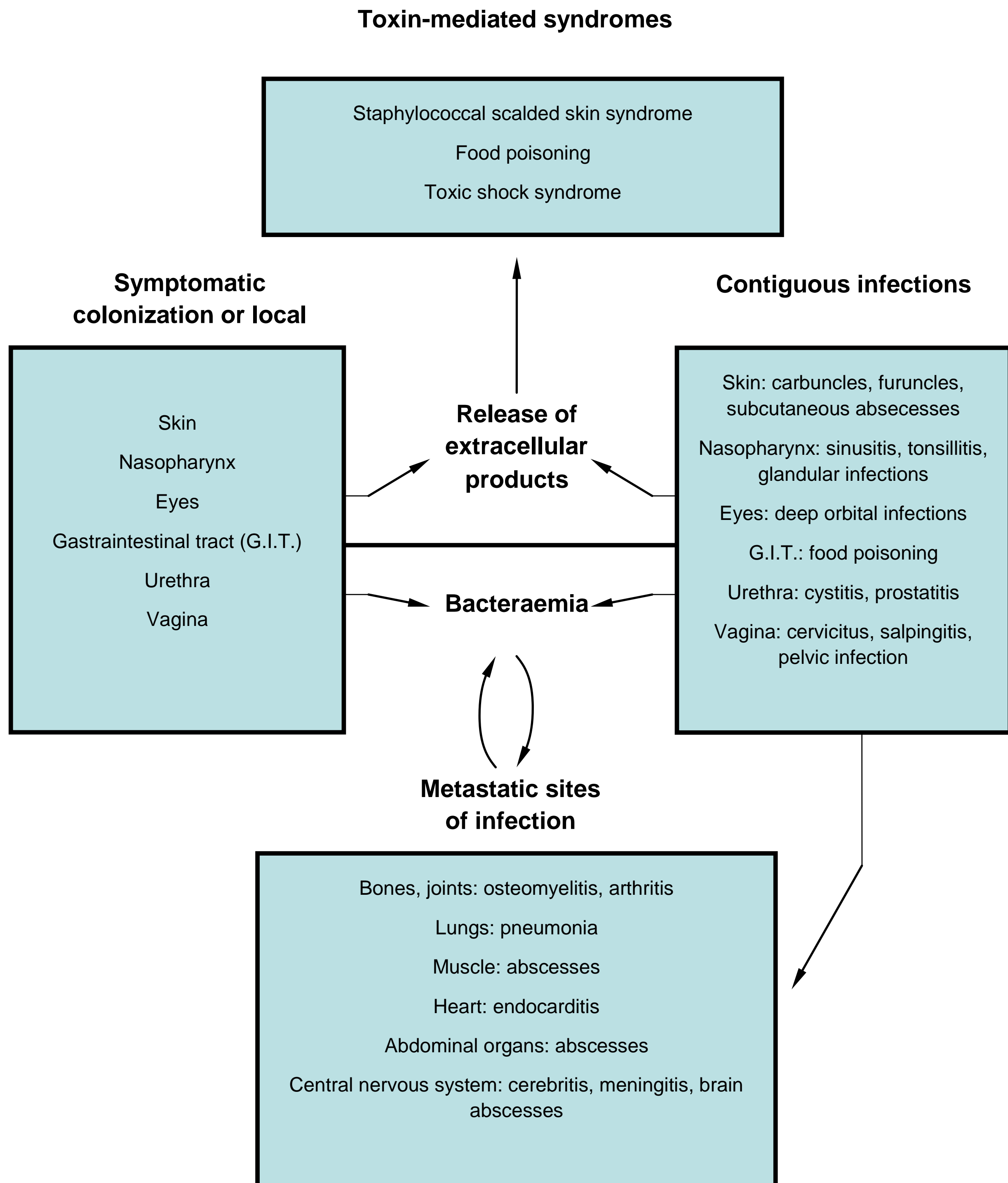


**Figure 2** Diseases caused by *Staphylococcus aureus* (adapted from Arbuthnott et al. 1990).

Exterior signs of infection usually include increased temperatures at the site of infection, formation of pus and necrosis of tissue. Fibrin causes blood clotting around the area, preventing bacteria from spreading. *Staphylococcus aureus* attaches to host proteins such as laminin and fibronectin with the

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help of surface proteins it produces. It also produces a fibrin or fibrinogen binding protein, referred to as a clumping factor (Todar 2009).

The mode of *S. aureus* infection is illustrated in Fig. 3 (Lowy 1998). Circulating staphylococci bind to sites of endovascular damage where platelet–fibrin thrombi (PFT) have formed. Bacterial adhesion can take place through MSCRAMM-mediated mechanisms, through endothelial cells via adhesin–receptor interactions, or via bridging ligands such as fibrinogen. The bacteria produce proteolytic enzymes that facilitate the spread to nearby tissues after phagocytosis by endothelial cells. This is followed by the release of staphylococci into the bloodstream. Endothelial cells express the tissue factor that facilitates the deposition of fibrin and the formation of vegetations. An inflammatory response occurs once the infection has spread to the adjoining sub-epithelial tissues, leading to abscess formation. After phagocytosis, endothelial cells express Fc receptors, adhesion molecules [vascular-cell adhesion molecules (VCAM)] and intercellular adhesion molecules (ICAM), and release interleukin-1, interleukin-6, and interleukin-8. As a result, leukocytes adhere to endothelial cells, with diapedesis to the site of infection.

Changes in the conformation of endothelial cells result in increased vascular permeability, with transudation of plasma proteins. Both tissue-based macrophages and circulating monocytes release interleukin-1, interleukin-6, interleukin-8, and tumor necrosis factor *a* (TNF-*a*) after exposure to staphylococci. Macrophage activation occurs after the release of interferon-*g* by T cells. Cytokines released into the bloodstream from monocytes or macrophages and endothelial cells, contribute to the manifestation of the sepsis syndrome and vasculitis associated with systemic staphylococcal disease. Expression of Fc receptors may contribute to vasculitis occasionally encountered during bacteremia by acting as a binding site for immunoglobulin (Ig) or immune complexes.

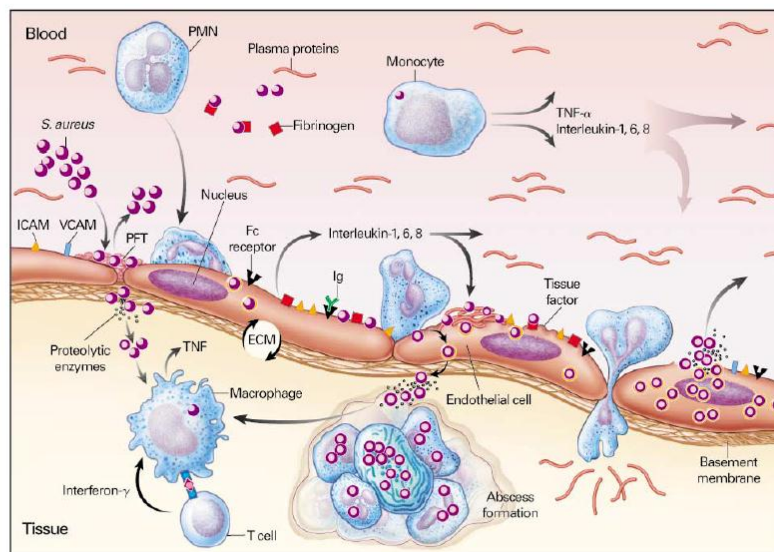


**Figure 3** Mechanism of infection (from Lowy 1998). PMN denotes polymorphonuclear leukocyte.

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## 2.3 Virulence

*Staphylococcus aureus* expresses a wide variety of virulence factors. Colonization of host tissues is aided by surface proteins and the spread of infection by invasins such as leukocidin, kinases, and hyaluronidase. Phagocytic engulfment are prohibited by surface factors including capsules and Protein A, but once inside phagocytes, these bacteria produces carotenoids and catalases to survive. Protein A and coagulase act as immunological disguises. Eukaryotic cell membranes are lysed by toxins such as hemolysins, leukotoxin and leukocidin. *Staphylococcus aureus* also produces exotoxins (SEA-G, TSST and ET), which damages host tissues or provoke symptoms of disease. Inherent and acquired antimicrobial resistance form a very important part of the virulence of *S. aureus*.

The staphylococcal genome consists of a circular chromosome (of approximately 2.8 Mbp), with prophages, plasmids and transposons. Genes encoding antibiotic resistance (Table 2) and virulence are transferred between species and strains through extrachromosomal elements including plasmids (Lowy 1998). Approximately 75% of the *S. aureus* genome is conserved between strains and is referred to as the 'core' genome. The remaining 25%, the 'accessory' genome, consists of mobile genetic elements (MGEs) providing virulence and resistance (El Garch et al. 2009). Little is known about the bacterial and host factors that promote *S. aureus* colonization (Foster 2004). Adaptations to the external environment (Wilson and Salyers 2003) and lateral gene transfer (Boyd and Brüssow 2002; Fitzgerald et al. 2001) between strains are partly responsible for evolution of pathogens.

**Table 2** Genes involved in antibiotic resistance

| Gene | Product | Resistance to | Reference |
|---|---|---|---|
| *bla* | *β*-lactamase | penicillin | Lowy 1998 |
| *fem* | factors essential for methicillin resistance | methicillin | Lowy 1998 |
| *mecA* | PBP2 | penicillin | Matouskova and Janout 2008 |
| *lukS-PV* | | | Matouskova and Janout 2008 |
| *lukF-PV* | | | Matouskova and Janout 2008 |
| *qacA* and *qacB* | | antiseptics | Matouskova and Janout 2008 |
| *vanA* | | vancomycin + teicoplanin | Matouskova and Janout 2008 |
| *vanB* | | vancomycin only | Matouskova and Janout 2008 |
| *vanC* + *vanC*-like phenotype | | | Matouskova and Janout 2008 |

Expression of virulence-associated proteins is controlled by the regulatory operons, *agr* (Kornblum et al. 1990), *sar* (Cheung et al. 1992), *xpr* (Smeltzer et al. 1993) and *sae* (Giraudo et al. 1994). The accessory gene regulator (*agr*) is the most important (Sabersheikh and Saunders 2004) among the above mentioned and varies between group I, II, III and IV (Diep et al. 2006). *Staphylococcus areus agr* specificity groups are classified as follows: strains causing invasive infections such as bacteraemia in group I, endocarditis strains in group II, menstrual toxic shock strains in group III and exfoliatin-

producing strains in group IV (Ji et al. 1997; Jarraud et al. 2000; Ben et al. 2006). *Staphylococcus aureus* has various virulence factors, including surface proteins that facilitate adhesion to damaged tissue, eliminate host immune responses in the blood (Foster 1998) and promote iron uptake (Mazmanian, 2003). Host immune responses are affected by the lysis of white blood cells by the leukotoxin Panton-Valentine Leukocidin (PVL), superantigens (TSST) or prevention of the migration of white blood cells (WBCs) to the infected site by the chemotaxis-inhibiting protein (CHIP) (de Haas et al. 2004). Bacterial proteins with superantigen activity which leads to overstimulation of cytokine release, include staphylococcal enterotoxins A–E, G–R and U (encoded by the genes *sea– see*, *seg–ser* and *seu*), toxic shock syndrome toxin-1 (TSST-1, encoded by *tst*), exfoliative toxins A and B (*eta* and *etb*) and other toxins such as α-, β-, γ- and δ-toxin and the leukotoxins, Panton–Valentine leukocidin (*pvl*) and leukocidin E-D (*lukE-D*) (Arbuthnott et al. 1982; Bhakdi and Tranum-Jensen 1991; Bohach et al. 1990; Prevost et al. 1995). Mertz et al. (2007) observed that the amount of WBCs in a specific area of tissue is directly related to the type of strain infecting the area. Strains able to produce ETA and ETB would more likely infect WBC-poor areas and PVL-positive strains will infect areas with more WBCs (Mertz et al. 2007). Surface proteins are predominantly synthesized during the exponential growth phase and the secreted proteins during the stationary phase (Lowy 1998). Surface proteins are more dominant during colonization whereas secreted proteins feature during persistence (Novick 1993).

*Staphylococcus aureus* produces various toxins that target membranes of mammalian cells and cause tissue damage (Bohach et al. 1999). Superantigen toxins are responsible for diseases such as septic shock (Bohach et al. 1999). Enzymes such as hyaluronidase, lipase, staphylokinase, phospholipase, DNase, phosphatase and protease are secreted to destroy tissue (Arbuthnott et al. 1990; Lowy 1998). Other enzymes include coagulase and haemolysin that catalyse the conversion of soluble to insoluble fibrin. This in turn leads to blood clotting that surrounds the bacteria and protect them from host immune responses (Gould and Chamberlain 1995). Some virulence factors are encoded by genes situated on mobile genetic elements (e.g. TSST-1), pathogenicity islands, lysogenic bacteriophages (e.g. PVL) (Novick 2003) or genes integrated in the chromosome (de Haas et al. 2004). The horizontally transferred *mecA* gene is situated on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) and confers resistance to methicillin and other β-lactam antibiotics. Other resistance genes include *bla* (*β*-lactamase) and *fem* (factors essential for methicillin resistance). The SCC*mec* element integrates into the *S. aureus* chromosome at the *att*B*scc* site (Ito et al. 1999). This integration is facilitated by the invertase/resolvase family encoded by the *ccr* gene complex consisting of *ccrA-C* (Ito et al. 2003). The *mecA* gene encodes the 78-kDa penicillin-binding protein PBP2a or PBP. Five SCC*mec* (types I–V) have been characterized. Type I emerged in 1961 in the UK, type II in 1982 from Japan, type III in 1985 from New Zealand, type IV in 1990s and type V after 2000 from Australia (Hiramatsu et al. 2001; Ma et al. 2002; Ito et al. 2004). Several variants of these SCC*mec* types have been described. Three tentative types have recently been discovered - type VI (Oliveira et al. 2006),

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type VII (Berglund et al. 2008) and type VIII (Zhang et al. 2009). The level of antibiotic resistance depends on the expression of related genes, the degree of expression and the presence of additional virulence genes (Peacock et al. 2002).

The most common staphylococcal proteins anchored in the cell wall are proteins with affinity to fibrinogen (i.e. clumping factors A and B, encoded by the *clfA* and *clfB* genes, respectively), fibronectin (*fnbA*), collagen (*cna*), sialoprotein (*bbp*), elastin (*ebpS*) and adhesins with unknown function (*sdrC* and *sdrE*) (Speziale et al. 1986; Jonsson et al. 1991; Park et al. 1996; McDevitt et al. 1997; Josefsson et al. 1998; Nı´ et al. 1998; Tung et al. 2000). Phenol-soluble modulin peptides, a new class of staphylococcal peptides, is associated with virulence of CA-MRSA skin infections. These peptides attack the main host defence response against *S. aureus* through lysis of neutrophils (Wang and Barret 2007).

Virulence factors can act in combination, e.g. exfoliative and pyrogenic toxin superantigen production leads to staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS) and food poisoning (Sauer et al. 2008). Some genes tend to associate with others, e.g. *seg* and *sei* (Jarraud et al. 1999; Becker et al. 2003; El-Huneidi et al. 2006; Sauer et al. 2008) as well as *sej* and *sed* (Becker et al. 2003; Mendoza and Martin 2005). Certain genes are also associated with specific diseases, e.g. PVL production with skin diseases such as furuncles, carbuncles and necrotizing pneumonia (Lina et al. 1999), TSST-1 with septic shock and TSS (Uchiyama et al. 1989) and SSSS-causing isolates often express exfoliative toxins, ETA and ETB (Opal et al. 1988). The *IL4* _524 C/C host genotype is associated with an increased probability of nasal *S. aureus* colonization, irrespective of the strain's genotype. Persistent nasal colonization is associated with both the *IL4* _524 C/C genotype and certain *S. aureus* AFLP strain markers, signifying the complexity of host-microbe interaction (Emonts et al. 2008).

## 2.4 Treatment

### *2.4.1 CA-MRSA*

CA-MRSA strains are spreading fast into newly established residential areas and are becoming more drug resistant, probably due to migration and travelling of individuals (Bartels et al. 2007). Aggressive control in these areas is practically impossible, but systematic topical decontamination of carriers remains an option (Navarro et al. 2008). Screening, contact tracing and decolonization of CA-MRSA carriers were also implimented in Denmark (Navarro et al. 2008). Vaccines have shown improved phagocytosis *in vitro* (Lowy 1998) and are currently in development for treatment of large communities (Wang and Barret 2007). However, according to the World Health Organization (WHO) prevention of *S. aureus* infections by vaccination remains challenging, especially regarding possible antigen development and ethical concerns among vaccinated patients. Vaccines developed against *S.*

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*aureus* include StaphVAX, a polysaccharide conjugate vaccine (Fattom et al. 2004) and PentaStaph™, a staphylococcal polysaccharide conjugate and toxoid vaccine (NABI Biopharmaceuticals). Prevention of CA-MRSA in hospitals is challenged by the constant emergence of new EMRSA strains (Otter and French 2006), rapid molecular changes (Kennedy et al. 2008) and difficulty in distinguishing between CA-MRSA and HA-MRSA (Maree et al. 2007). Since CA-MRSA is becoming more prevalent in hospital settings, HA-MRSA control techniques can also be implemented (Navarro et al. 2008).

### *2.4.2 HA-MRSA*

Some hospitals implemented the technique of screening all patients upon admission, but delayed test results (4-5 days) compromised effective treatment. The majority of patients were infected in the hospital as a result of post-operative contamination or prolonged stay. The personal hygiene of HCWs continues to play an important role in preventing HA-MRSA infections. Alcoholic hand rubs has proven to be very effective (Johnson et al. 2005), although *S. aureus* has developed resistance to quaternary ammonium-type antiseptic compounds (Rouch et al. 1990; Bjorland et al. 2001). HCWs that frequently come into contact with colonized or infected patients should be regularly screened for the presence of MRSA (Siegel et al. 2007). Decolonization of patients by whole body bathing or eradication of the gastro-intestinal tract and wounds is another way of preventing MRSA infection in hospitals (Wendt et al. 2007). Systemic decolonization with mupirocin is also an option, but can lead to resistance (Vasquez et al. 2000). Infected patients can be cohorted in a single room where only certain HCWs treat these patients (Sexton et al. 2006).

Decontamination of the hospital environment is also very important. Approximately 64-74% of all hospital surfaces including bedding, mattresses, door handles, grab bars and taps are contaminated. Equipment includes keyboards, pagers, stethoscopes, membranes of blood pressure gauge sleeves and tourniquets used in blood sampling (Oomaki et al. 2006; Dancer 2008). Antibiotics used in treatment are the semi-synthetic penicillin, oxacillin or nafcillin, vancomycin, daptomycin, quinopristin-dalfopristin, rifampicin and ciprofloxacillin (Lowy 1998), teichoplanin (Gould and Chamberlain 1995) and various oxzolidinones. Linezoid and synercid are also relatively new drugs used (Foster 2004). Antibiotic resistance to the new drugs quinopristin-dalfopristin and tigecycine has already been reported (Peeters and Sarria 2005; Skiest 2006). Medical practitioners often use combinations of antimicrobials in an attempt to limit antimicrobial resistance.

## 3. Antimicrobial peptides

Antimicrobial peptides (AMPs) are produced by a variety of organisms including humans, insects, plants and bacteria. In addition to the host's innate and acquired immune responses, AMPs are often incorporated in response to pathogenic invasion (Reddy et al. 2004). Most AMPs are small (<10 kDa),

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cationic, hydrophobic proteins with antibacterial, antifungal or antiviral activity (Bals 2000). Some AMPs have also shown anti-tumour (Bals 2000) and spermicidal (Reddy et al. 1996; Aranha et al. 2004) activity. Certain AMPs administered in low concentrations also proved to diminish well-established biofilms (bacterial populations protected by extracellular macromolecules) on surgically implanted medical devices (Upton et al. 2012). Biofilms on medical devices are often targets for secondary infections in the hosts due to their resistance to high levels of conventional antibiotics.

The fact that AMPs are mostly active against specific groups of bacteria, indicate that they are less detrimental to the host's natural flora. Their activity in a wide range of temperature and pH, specific mode of action and low toxicity to eukaryotic cells, render them favourable candidates for novel therapeutic agents. In general, AMPs can benefit the host in one of four ways: 1) to treat infection when administered alone, 2) to act synergistically when administered in combination with conventional antibiotics or antivirals, 3) to act as immunostimulators of the innate immune system, and 4) to neutralize endotoxins produced by pathogens (Gordon and Romanowski 2005).

A few AMPs have been found to enhance the penetration of conventional antibiotics through the bacterial membrane of multidrug-resistant *Pseudomonas aeruginosa* when administered in combination with the antibiotic. They include magainin II and cecropin A administered with rifampicin (Cirioni et al. 2008), tachyplesin III with imipenem (Cirioni et al. 2007), and P5 with isepamicin (Jeong et al. 2010). The synergistic effect allows lower dosages of the antibiotics (Barriere 1992), as well as reduced resistance development in certain cases (Wu et al. 1999; Steenbergen et al. 2009). A number of AMPs have been evaluated in clinical trials, but with limited success and with FDA-approval for topical application only (Gordon and Romanowski 2005; Park et al. 2011). They include omiganan/ MBI-226 for catheter-related local and bloodstream infection (Isaacson 2003; Rubinchik et al. 2009; Yeung et al. 2011), pexiganan/ MSI-78 for diabetic foot ulcers (Hancock and Sahl 2006), plectasin against drug-resistant bacteria (Hancock and Sahl 2006; Zaiou 2007), opebacan for endotoxemia in hematopoetic stem cell transplants (Zhang and Falla 2006; Yeung et al. 2011), iseganan/ IB-367 for oral mucositis (Hancock and Sahl 2006), MBI 594AN and XMP.629 for topical treatment of acne, P113 P113D mouthrinse for oral candidiasis in HIV patients, and neuprex/ rBPI21 for systemic treatment of pediatric meningococcemia (Gordon and Romanowski 2005). Phase I clinical trials were recently completed on oral administration of the class II lantibiotic NVB302 for *Clostridium difficile* infections in humans (www.evaluatepharma.com, June, 2012). In 2012 two pharmaceutical companies (Oragenics and Intrexon) initiated a "lantibiotics program" in which they will focus on the development of lantibiotics.

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### 3.1 Lactic acid bacteria and bacteriocins

Lactic acid bacteria (LAB) are one of the most extensively studied and best characterized groups of bacteria and were even referred to as “the bugs of the new millennium” at the turn of the century (Konings et al. 2000). They are Gram-positive, anaerobic bacteria with lactic acid as one of their main fermentation products and play an important part in the fermentation of milk products, vegetables, meat and wine because of their generally regarded as safe (GRAS) status approved by the United States Food and Drug Administration (FDA). LAB has the ability to produce bacteriocins, which are AMPs with activity against strains closely related to the producing species (Daw and Ikiner 1996).

Since the discovery of bacteriocins in 1925 (Gratia 1925), they have been classified and reclassified numerous times (Daw and Ikiner 1996). Fredericq (1957) proposed a classification system based on the specificity of absorption and further sub classified them according to immunity (Fredericq 1957). Reeves implemented a system consisting of 16 classes of bacteriocins based on its producer species. Ten years later, Bradley (1967) designed a criterion for bacteriocin classification based on natural division, with two groups: low and high molecular weight forms. Klaenhammer (1993) classified bacteriocins into four classes based on structure, mechanism of action, genetics and biochemical properties. Cotter et al (2005) changed it to three groups: Lantibiotics (class I), heat-stable non-lantibiotics (class II) and the bacteriolysins (class III).

Rea et al. (2011) suggested sub classification of class 1 (a-c) bacteriocins, while class II consists of four subdivisions (a-d). Class 1a bacteriocins include, amongst others, nisin, subtilin, epidermin and Pep5, while the lantibiotics mersacidin, lacticin (3147 and 481), and nukacin ISK-1 belong to class Ib. Lantibiotics are bacteriocins of 19-38 amino acids and contain certain unusual amino acids and various circular structures (lanthionine rings). The latter aids this group of proteins in stability in terms of heat, pH, oxidation and proteolysis (Sahl et al. 1995; Bierbaum et al. 1996). Examples of class II bacteriocins include pediocin PA-1 and AcH (IIa), lactococcin G and Q (IIb), enterocin AS-48 and gassericin A (IIc) and lacticin Q and lactococcin A (IId) (Nishie et al. 2012). Class II bacteriocins are also heat stable.

### 3.2 Nisin

Nisin, a class Ia bacteriocin, is generally regarded as safe (GRAS) and due to its relatively low toxicity to humans (Fowler 1973), it has been used as a food preservative for decades (Van Heusden et al. 2002). Since nisin will be used in this study, it will be discussed as a model bacteriocin. Nisin A (Mattick and Hirsch 1947), F (De Kwaadsteniet et al. 2008), U, U2 (Wirawan et al. 2006), Q (Zendo 2003) and Z (Mulders et al. 1991) are small lantibiotics ribosomally synthesised by certain *L. lactis* species, with the exception of nisin U that is produced by *Streptococcus uberis* (Wirawan et al. 2006). These natural variants of nisin usually differ with only a few amino acids and have antimicrobial

activity against various Gram-positive species, including *Staphylococcus, Listeria, Bacillus* and *Clostridium* spp. (Breukink and De Kruijff 1999). Nisin have also shown activity against multidrug-resistant strains such as MRSA and VRE (Kruszewska et al. 2004; Piper et al. 2010). Nisin consists of 34 amino acid molecules and contains five lanthionine or β-methyllanthionine rings (Sun et al. 2009), as illustrated in Fig. 4. Unusual amino acids include dehdryalanine (Dha), dehydrobutyrine (Dhb), lanthionine (Ala-S-Ala) and 3-methyllanthionine (Abu-S-Ala).

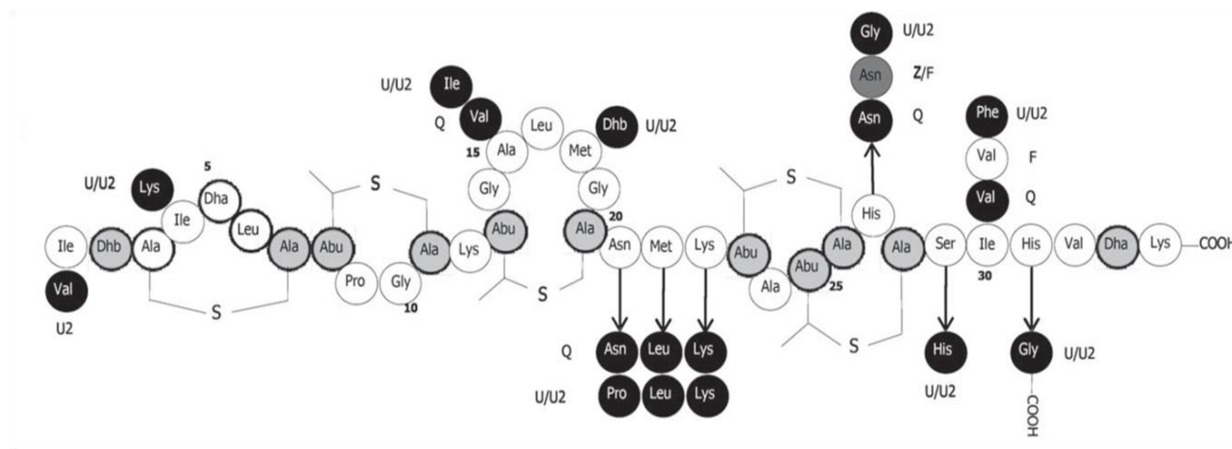


**Figure 4** Comparison of structures of nisin A (illustrated as basis), Z, Q, F, U and U2 after post-translational modification (adapted from Piper et al. 2010).

### *3.2.1 Biosynthesis*

Nisin is produced on the ribosome of its producer cells. The initial precursor peptide consists of a leader peptide at its N-terminal and a pro-region (eventually to become the mature peptide) at its C-terminal (Cotter et al. 2005; Willey and Van der Donk 2007; Bierbaum et al. 2009; Ross and Vederas 2011). While inside the bacterial cell, the protein is kept inactive by the leader peptide, which is recognized by modification enzymes and subsequently cleaved off (Fig. 5). Class I lantibiotics are modified by the enzymes LanB and LanC – NisB and NisC in the case of nisin. NisB is responsible for the dehydration of serine (Ser) and threonine (Thr) residues to form Dha and Dhb, respectively, as illustrated in Fig. 6 (Patton and Van der Donk et al. 2005). NisC mediates cyclization of the dehydrated residues with intramolecular cysteine (Cys) to form lanthionine (Lan) or methyllanthionine (MeLan) rings. Subclass II lantibiotics on the other hand, rely on LanM for modification (both dehydration and cyclization).

During the biosynthesis of nisin, the peptide interacts with various additional cellular compounds (Konings et al. 2000). After the precursor peptide has been modified, the leader peptide is cleaved of by a serine-type protease, LanP. The protease domain of the ATP-binding cassette protein, LanT, can also facilitate cleavage. The mature peptide is then secreted out of the cell by LanT. NisR

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constitutively regulates transcription while the signal transducer histidine kinase, NisK, is involved in sensing nisin, allowing it to act as an extracellular regulator of its own biosynthesis (Kuipers et al. 1995; Van Kraaij et al. 1999).

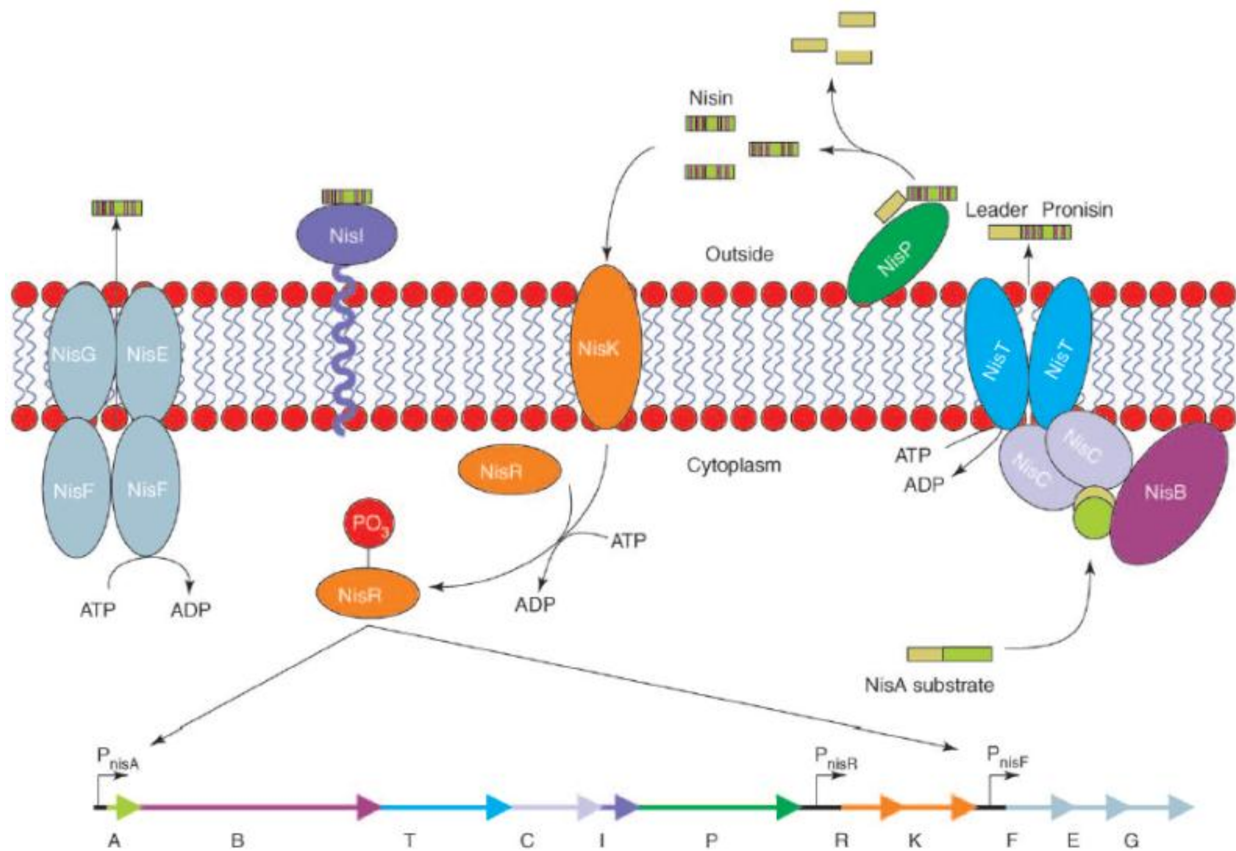


**Figure 5** Molecular components involved in the biosynthesis, regulation and immunity of nisin A (from Patton and Van der Donk 2005).

NisRK acts as a two-component regulatory system. Similar autoregulation is also observed for epidermin (Peschel et al. 1993), subtilin (Kleerebezem et al. 1999; Kleerebezem 2004), mutacin II (Qi et al. 1999), lacticin 3147 (McAuliffe et al. 2001), SapB (Keijser et al. 2002; Nguyen et al. 2002; O'Connor and Nodwell 2005), mersacidin (Schmitz et al. 2006) and bovicin HJ50 (Ni et al. 2011). Nisin resistance genes *nisI* and *NisFEG* equip nisin-producing strains with immunity from its own bacteriocins (Engelke et al. 1994; Siegers and Entian 1995). While nisI acts as a specific immunity protein, NisFEG acts as an ABC transporter system (Draper et al. 2008). The expression of immunity genes have to be regulated very precisely to ensure activity the moment peptides are produced.

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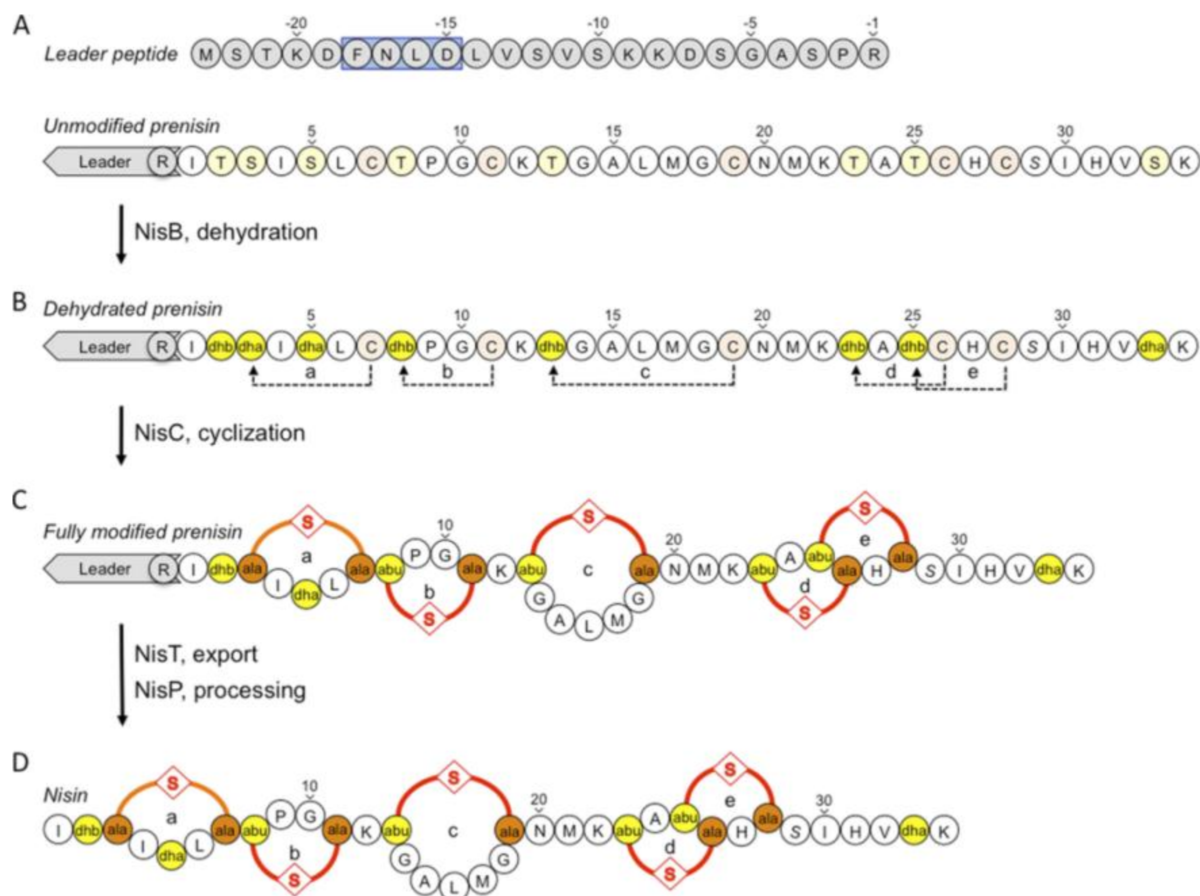


**Figure 6** Posttranslational modification of amino acids of nisin A (from Mavaro et al. 2011).

### *3.2.2 Mode of action*

Nisin exerts its antimicrobial activity in multiple unique ways. The main mechanism is binding to the cell wall precursor lipid II as illustrated in Fig. 7 (Breukink et al. 1999; Wiedeman et al. 2001; Wiley et al. 2007). Bound lipid II prevents cell wall biosynthesis in Gram-positive bacteria. The overall positive charge of nisin facilitates its mobilization to the negatively charged bacterial cell wall. The amide groups of the A and B lanthionine rings on the amino (N)-terminal of nisin binds to the pyrophosphate structures of the docking molecule lipid II, N-acetylmuramic acid (MurNAc) (Wiedemann et al. 2001; Hasper et al. 2004; Hsu et al. 2004;). A similar binding to lipid II without the formation of pores was observed in the lantibiotics subtilin, mutacin (Hasper et al. 2006), epidermin and gallidermin (Bonelli et al. 2006), while the antibiotics vancomycin and ramoplanin bind to the terminal D-Ala D-Ala residues (Patton and Van der Donk 2005; Wiley et al. 2007) and the class Ib lantibtiotic, mersacidin to the N-acetylglucosamine (GlcNAc) saccharide of lipid II. Transmembrane pores are formed after the bending of nisin at the flexible hinge region between the C and D rings and insertion of the carboxy (C)-terminal end into the phospholipid bilayer (Hsu et al. 2004; Van Heudsen et al. 2002).

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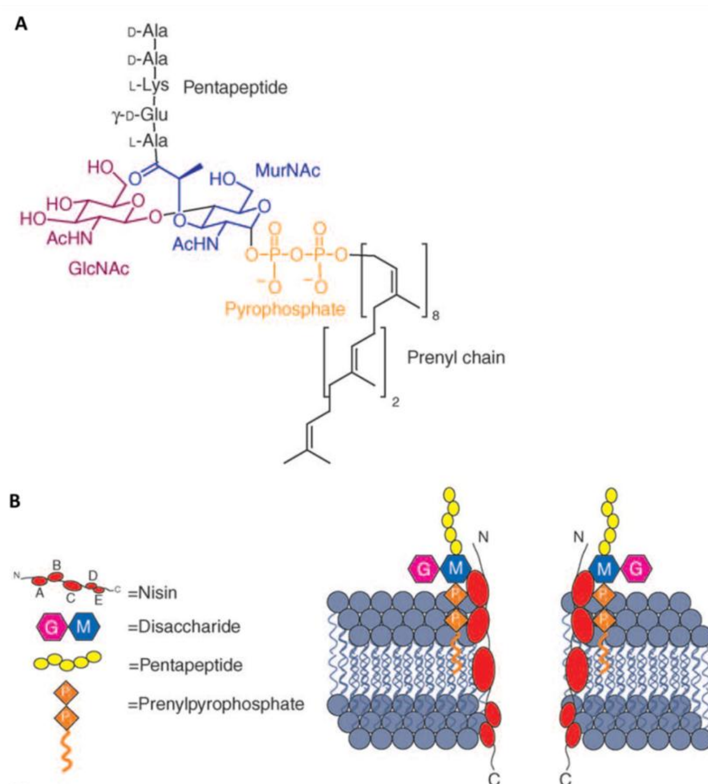


**Figure 7** The chemical structure of lipid II (A) and the binding mechanism of nisin to lipid II (B) (adapted from Patton and Van der Donk 2005).

The pores generally consist of eight nisin and four lipid II molecules (Hasper et al. 2004) and have a diameter of 2-2.5 nm (Wiedemann et al. 2004). Pores cause an efflux of potassium ions, amino acids and ATP ions, which is detrimental to the bacterial cell (Van Heudsen 2002). The formation of pores can take place in one of three proposed models: the “wedge”, “barrel-stave” or “carpet” models (Sahl 1991; Driessen et al. 1995; Moll et al. 1999; Hechard and Sahl 2002). The lantibiotic peptides use the wedge model (Moll et al. 1999).

Nisin also has the ability to disrupt the functional localization of lipid II (Hasper et al. 2006). While lipid II is normally present at sites of emerging cell wall biosynthesis, treatment with nisin results in patches of dysfunctional lipid II across the bacterial membrane, away from the site of biosynthesis.

Furthermore, nisin (and subtilin) has the ability to inhibit spore germination in some *Bacillus* and *Clostridium* species (Thomas et al. 2002; Montville et al. 2006; Gut et al. 2008). The mode of action seems to be connected to the residue in position 5 of the lantibiotic. Replacement of dehydroalanine normally present at position 5, showed enhanced inhibition of spore outgrowth (Rink et al. 2007).

Nisin and Pep5 were also shown to induce autolysis in Staphylococcal strains. In this case, the mode of action involves the binding of nisin to lipoteichoic and teichoic acids of the cell and displacing and

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activating N-acetyl-alanine aminidase and N-acetylglucosaminidase supposed to interact with lipoteichoic and teichoic acids (Bierbaum and Sahl 1985).

### *3.2.3 Resistance*

The combination of the mode of action of nisin (pore formation, inhibition of cell wall biosynthesis, cell autolysis and the inhibition of spore outgrowth) gives it a unique advantage over other antibacterial compounds. Although these features decrease bacterial resistance development to nisin, it is not impossible.

Resistance to nisin was reported as early as the 1960's, with the proteolytic enzymes collectively termed nisinase (Jarvis 1967). The enzyme was later identified as a dehydroalanine (Dha) reductase, with the ability to reduce carboxyl Dha to Ala to inactivate nisin (Jarvis and Farr 1971). Resistant strains of *Streptococcus* spp. (Alifax and Chevalier 1962; Galesloot 1956; Lipinska and Strzalkowska 1959*), B. subtilus, B. licheniformis, B. cereus* (Jarvis 1967), *S. aureus* (Carlson and Bauer 1957; Jones 1974), *L. monocytogenes* (Harris et al. 1991; Ming and Daeschel 1993; Mazzotta and Montville 1997) and *C. botulinum* (Mazzotta et al. 1997) developed. Particular concern was raised by a study where nisin-resistant *Streptococcus bovis* mutants showed 1000 times greater resistance to ampicillin, in comparison to their nisin-susceptable predecessors (Mantovani and Russel 2001). This phenomenon is termed cross-resistance. Another case of acquired multidrug-resistance was observed when *Salmonella enterica* was exposed to the AMP, microcin (Carlson et al. 2001).

Although much less than for conventional antibiotics, a number of genes have been assigned to bacterial resistance to AMPs and bacteriocins. Resistance can be acquired in response to repeated exposure to increasing concentrations of the peptide. In this case, the microorganism alters expression of its genes involved in the general composition of the cytoplasmic membrane biosynthesis - from there the term physiological adaption (Peschel et al. 1999; Thedieck et al. 2006; Sun et al. 2009). ABC transporters (Kramer et al. 2006; Draper et al. 2008; Hansen et al.; 2009) or regulators and two-component signal transductions systems (Cotter et al. 1999; Kallipolitis et al. 2003; Mascher et al. 2004; Gravesen et al. 2004; Guinane 2006) are other forms of resistance. Genes encoding ABC transporters involved in nisin resistance include *ysaBC* in *L. lactis* (Kramer et al. 2006), *yvcRS* in *B. subtillis* (Hansen et al. 2009), *sp0913* and *sp0912* in *S. pneumonia* (Sass et al. 2008). The transporter encoded by *vraDE* provides *S. aureus* with resistance to the class II lantibiotic mersacidin. Kramer et al. (2006) suggested a putative nisin resistance pathway in *L. lactis* with four mechanisms of action: (A) - thickening of the bacterial wall to prevent nisin from binding to lipid II (*pbp2A*), becoming packed more densely (*galE* and *pbp2A*) or less negatively charged (*dltD*); (B) expressing genes encoded by the *arc* operon to increase pH outside of the cell wall and possibly degrade nisin; (C) increasing fluidity of the membrane by decreasing expression genes of the *fab* operon responsible for

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the saturation of membrane phospholipid fatty acids, which possibly prevents nisin from inserting into the membrane and (D) ABC transporters pumping nisin out of the membrane and preventing binding to lipid II.

Most research on nisin resistance has been done on *L. monocytogenes* and the genes involved are listed in Table 3. The nisin resistance protein (NSR) was discovered by Froseth and McKay (1991), with additional studies revealing its ability to cleave nisin between MeLan (position 28) and Ser (position 29, Sun et al. 2009). An important factor in the bacterial stress response is the alternative sigma factor SigB, which was found to reduce the sensitivity of *L. monocytogenes* to nisin and lacticin 3147 (Begley et al. 2006). The signal-transduction system LisRK and the VirRS system, which controls the expression of *dltA* and *mrpF*, also plays a role together with the *pbp*, *anrB*, *telA*, and *gad* genes. VirRS controls the expression of the *dlt*-operon, involved in alanylation of the lipoteichoic acids in the cell envelope, and *mprF*, conferring resistance to cationic antimicrobial peptides (CAMPs) (Abachin et al. 2002; Mandin et al. 2005; Thedieck et al. 2006). The ability of *L. monocytogenes* to sense antimicrobial agents, ethanol and lysozyme are conferred by CesRK. CesRK also regulates orf2420, responsible for virulence in mice. LisRK aids the pathogen to sense and enhance virulence against nisin and other harmful molecules such as ethanol, acid and hydrogen peroxide stress (Kallipolitis et al. 2003).

**Table 3** Genes rendering resistance of *Listeria monocytogenes* to nisin

| Gene | Function | Reference |
|---|---|---|
| *nsr* | Nisin resistance protein | Froseth and McKay 1991; Sun et al. 2009 |
| *pbp* | Penicillin-binding protein | Gravesen et al. 2001, 2004 |
| *lisRK* | LisRK signal transduction system | Cotter et al. 1999, 2002 |
| *dltA* & *mprF* | Two-component regulatory system VirRS | Mandin et al. 2005 |
| *SigB* | Sigma factor B | Begley et al 2006 |
| *anrB* | Permease component of ABC transporter | Collins et al. 2010a |
| *telA* | Telurite resistance | Collins et al. 2010b |
| *gad* | Glutamate decarboxylase | Begley et al. 2010 |
| *lmo1021* & *lmo1022* | LiaSR two-component signal transduction system | Collins et al. 2012 |
| *CesR, CesK & , orf2420* | CesRK two-component signal transduction system | Kallipolitis et al. 2003 |

### *3.2.3 Applications*

Nisin has been applied in the food industry for more than 50 years. Due to its FDA-approved status and specific mode of action, it reduces the chances of the development of bacterial resistance. Due to its activity against plaque and gingivitis-causing bacteria, nisin has been incorporated in mouthwashes (van Kraaij et al. 1999). A number of studies applied nisin in the treatment of mastitis, a bacterial infection of the mammary glands. Mastitis is known to cause huge economic losses in the dairy

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industry (Soltys and Quinn 1999; Sordelli et al. 2000; Twomey et al. 2000; Riffon et al. 2001; Bradley 2002). Intramammary infusions of nisin effectively treated streptococcal and staphylococcal mastitis-related infections (Taylor et al. 1949). In another study *in vivo*, Broadbent et al. (1989) showed that nisin inhibited the growth of several Gram-positive, mastitis-causing pathogens. Sears et al. (1992) acquired 66% inhibition of *S. aureus*, 95% of *Streptococcus agalactiae*, and 100% for *Streptococcus uberis* after combining nisin A and lysostaphin in intramammary infusions. Nisin A was also applied in teat wipes (Broadbent et al. 1989; Ross et al. 1999; Cotter et al. 2005).

Contaminated biomedical implant devices are the cause of serious surgical (Campoccia et al. 2005). Nisin, adsorbed to silanized surfaces, successfully prevented the growth of *L. monocytogenes* (Bower et al. 1995). Intravenous catheters and tracheotomy tubes coated with nisin were implanted in sheep and ponies, respectively (Bower et al. 2002). The endotracheal tubes prevented colonization of *S. aureus*, *S. epidermidis*, and *S. faecalis*. The authors, however, concluded that the antimicrobial activity of nisin may be short lived *in vivo*, as the peptide controlled bacterial infection for only 5 h in sheep. However, the tracheotomy tubes retained activity for seven days (Bower et al. 2002).

Nisin was also applied in the treatment of respiratory tract infections. Nisin inhibited *S. pneumonia* associated with otitis media *in vivo* (Goldstein et al. 1998). Additionally, Nisin F inhibited *S. aureus* in the nasal cavities of immunosuppressed rats (De Kwaadsteniet et al. 2009). When applied together with polymyxin E and clarithromycin, nisin inhibited the growth of *P. aeruginosa* (Giacometti et al. 1999). A number of studies found nisin effective against diarrhoea-causing *Clostridium* species i.e. *C. botulinum* (Delves-Broughton et al. 1996) *C. tyrobutyricum* (De Carvalho et al. 2007) and *C. difficile* (Bartoloni et al. 2004). Another possible application is the treatment of peptic ulcers, since nisin successfully inhibited *Helicobacter pylori in vitro* (Delves-Broughton et al. 1996; Kim et al. 2003). Commercially produced nisin is in phase I clinical trials for treatment of gastric conditions and oral mucositis, while both nisin A and Z are in preclinical trials against vancomycin-resistant enterococci (VRE). Lastly, nisin has excellent spermicidal activity. It was successfully applied as a contraceptive in rats, with restored fertility after trials (Aranha et al. 2004). This finding could lead to an improvement on current contraceptive products, since many of them contain Nonoxynol-9 (N-9), a compound harmful to epithelium cells.

### 3.3 Limitations of bacteriocins and possible solutions

As previously mentioned, most clinical trials involving AMPs are limited to topical applications and not systemic application. In addition, resistance to almost every known antimicrobial compound has been reported and for the lucky few, it is probably only a matter of time. Since AMPs mostly interact with bacterial membranes to exert its activity, resistance is mostly acquired through alterations in the bacterial membrane (Ming and Daeschel 1993; Mazzotta et al. 1997; Crandall and Montville 1998).

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Another major disadvantage of AMPs is their limited *in vivo* stability due to proteolytic degradation inside the intestines, tissues serum and kidneys of the host (Joerger 2003; McGregor 2008). The terminal parts of the peptides are prone to enzymatic degradation by aminopeptidases and carboxypeptidases, whilst various endopeptidases can target specific cleaving sites within the peptide (Rink et al. 2010). Trypsin will cleave cationic amino acids like lysine and arginine (Svenson et al. 2008; Park et al. 2009), while chymotrypsin cleaves phenylalanine, tryptophan and tyrosine, and elastase cleaves alanine, glycine, and valine (Gray and Cooper 1971). AMPs like defensins (Kagan et al. 1994) and bactencin (Rademacher et al. 1993) produce toxins and are prime targets for *in vivo* proteolytic inactivation as a mechanism to decrease the concentration and potency of the bacteriocin. Probably due to this fact, successful application of nisin is mostly limited to topical applications.

Since the ring-formations in lantibiotics enhance their stability, the introduction of D-amino acids (Besalle et al. 1990; Hong et al. 1999) and cyclization (Li and Roller 2002) are possible options to enhance the stability of bacteriocins. Rink et al (2010) combined the two techniques to create peptides with enhanced *ex vivo* stability. Other techniques includes conjugation of fatty acids (Avrahami and Shai 2002), substitution by peptoids (Chongsiriwatana et al. 2008; Wang et al. 2009) and incorporation of beta-peptides (Porter et al. 2002), fluorinated amino acids (Meng and Kumar 2007) and acylation (Radzishevsky et al. 2007). Peptide engineering on nisin include incorporation of its unique biosynthethic genes onto unrelated peptides with improved *in vitro* and *in vivo* activity and stability (Kuipers et al. 2006; Kluskens et al. 2009; Kuipers et al. 2009; Majchrzykiewicz et al. 2010; Moll et al. 2010). Recently, Field and co-workers created nisin S29 derivatives with antibacterial activity against Gram-positive as well as Gram-negative pathogens (Field et al. 2012).

Another route to overcome peptide degradation is the development of drug delivery systems. One such example is to protect the protein inside a polymer complex that will systematically release the active protein as the polymer matrix dissolves. This application was successfully implemented in food preservation, but very few systems are available for medical application (Balasubramanian et al. 2009). When encapsulated in poly (L-lactide) (PLA) nanoparticles, released nisin inhibited the *in vitro* growth of *Lactobacillus delbrueckeii* subsp. *bulgaricus* (Salmaso et al. 2004). In another study, the bacteriocin plantaricin 423, released from electrospun nanofibers consisting of polyethylene oxide (PEO) successfully inhibited growth of *Lactobacillus sakei* and *Enterococcus faecium* (Heunis et al. 2010). Similar fibers containing nisaplin (nisin A) successfully treated *S. aureus*-infected burn wounds in mice when applied as an antibacterial dressing (Heunis 2012). Nisin F incorporated in bone cement (similar to implants used for hip replacements) successfully prevented *S. aureus* growth when inserted into infected subcutaneous pockets as a prophylactic treatment in mice (Van Staden et al. 2012).

Due to the small size of AMPs, they are easily adsorbed by the kidneys during circulation through the host (Jeong et al. 2010). Strategies to increase stability *in sera* include the lengthening of peptides

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through the attachment of polyethylene glycol (PEG), referred to as PEGylation (Veronese and Mero 2008; Pasut and Veronese 2009; Jevsevar 2010). Although also decreasing cytotoxicity of peptides, PEGylation unfortunately has a negative effect on in vitro activity (Imura 2007a, b). This obstacle was overcome in a study using shorter PEGylation (Zhang et al. 2008).

The large-scale production of active, purified bacteriocins is very expensive (Joerger 2003). To cross this financial obstacle, Van t'Hof et al. (2001) suggested transgenic plants as an option. In the food industry, nisin can be applied in combination with traditional food preservation mechanisms such as acidification and increased salt concentration (Harris et al. 1991; Mazzotta and Montville 1997), hydrostatic pressure, heat treatment, refrigeration, (Roberts and Hoover 1996; De Martinis et al.1997) or additives (Taylor et al. 1985). The synergistic effect increases the antimicrobial effect of nisin whilst decreasing the formation of nisin-resistant strains. In a study on nisin-resistant strains, Mazzotta et al. (2000) showed that the combination of nisin and food preservatives did not result in cross-resistance and had no effect on the susceptibility of bacterial strains to preservatives.

## 4. Bioluminescent imaging

Biomedical research has become very reliant on imaging techniques in recent years. Instrumentation for imaging small animals include x-ray computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET, or microPET), single photon emission computed tomography (SPECT, or microSPECT), ultrasonography, and optical imaging, including fluorescence and bioluminescence (Zinn 2008). Optical imaging is less expensive and time consuming than other techniques mentioned. Bioluminescence (or chemiluminescence) imaging (BLI) is based on the detection of imaging contrast formed by light emission from a reporter protein called luciferase. Luciferase catalyzes the conversion of D-luciferin to oxyluciferin. BLI has been used to effectively monitor transgene expression, tumor growth and metastasis, development of infection, transplantations, toxicology, viral infections and gene therapy (Benaro et al. 1997; Contag et al. 1997; Edinger et al. 1999; Koransky et al. 2001). Organisms, cells or genes are genetically modified to express the luciferase enzyme in the presence of oxygen, magnesium (Mg), adenosine triphosphate (ATP) and the substrate D-luciferin if needed. D-luciferin can also be administered to animals by intravenous (i.v.) or intraperitoneal (i.p.) injection, osmotic pumps (Gross et al. 2007) or in drinking water (Gross et al. 2007; Hiler et al. 2006).

Various luciferases are used in optical imaging, but the most popular is luciferase from the North American firefly (*Photinus pyralis*; FLuc). Other useful luciferases have been cloned from the sea pansy (*Rinilla*; RLuc), jellyfish (*Aequorea*), corals (*Tenilla*), the click beetle (*Pyrophorus plagiophtalamus)* and various bacteria (*Vibrio fischeri, V. harveyi*) (Hastings 1996). Luciferases vary in substrate specificity, wavelength of light emission and optimal parameters (Zhao et al. 2005), but

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more than one luciferase can be imaged simultaneously. Bacterial species can also bioluminesce, but in this case employ long-chain aldehydes and flavin mononucleotides in combination with heterodimeric luciferases. Luciferases and all proteins required for substrate biosynthesis are encoded by a single lux operon (Contag et al. 1995). The *luxCDABE* operon originally suitable for Gram-negative organisms only, was genetically modified to *luxABCDE* for use in Gram-positive organisms. The fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction is encoded by *luxCDE*. The *luxAB*-genes encode the luciferase enzyme.

A few factors determine the sensitivity of detection: the level of luciferase expression, the depth of the labelled cells inside the animal, and the sensitivity of the detection system (Wilson and Hastings 1998). BLI can detect bioluminescence and fluorescence with a specialized charged coupled device (CCD) camera. The camera has the ability to convert emitted photons into electrons. Images are generated by encoding the intensity of the photons to electrical charge patterns. Background noise is eliminated by cooling the camera down to -90˚C and taking images in a light-tight box. The system is controlled by a computer where the operator can also acquire and analyse images. Heat (usually 37˚C) is also provided to maintain body temperature. Image acquisition can take from as long as 1 second to 10 minutes and the imaging software ensure that no pixels are saturated during imaging.

A colour scale represents the light intensities of each image and allows the researcher to measure and compare the rate of light emitted from different areas (regions of interest) on a specific image. The colour scales of two or more images can be standardized to the same minimum (violet) and maximum (red) values to allow comparison among images. Such a bioluminescent image is overlaid on a black and white image of the animal. The software can be manipulated to measure light (relative or absolute) emitted from certain region(s) of interest (ROI) on an image or numerous images. Light emission can be relatively quantified as total flux (photons/second), the radiance in each pixel summed or integrated over the ROI area ($cm^2 x 4\Pi$) or absolute as average radiance (photons/second/$cm^2$/steridian), photons per second of light that radiate from the mouse in a unit area (1 $cm^2$) and unit angle (1 steridian).

BLI has various advantages over normal animal models. The spatial and temporal course of diseases can be detected from the same animal without killing it. The animal serves as its own negative control, which further decreases the amount of experimental animals and biological variation. These animal models can lead to more trustworthy and accurate predictions on how humans will react to involved agents (Sadikot and Blackwell 2005). BLI is also relatively inexpensive and accessable to any scientist, as specialised operators are not required (Luker and Luker 2010). Also, BLI has a signal-to-noise ratio with only little background light from chlorophyll pigments present in ingested food. The technique is also less time-consuming, allowing a researcher to monitor more animals in a specific subgroup. Unexpected sites of infection can also be detected by BLI, which would otherwise have been overseen with the use of conventional techniques where only specific body parts or organs of

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animals are examined post mortem. Recently advanced systems have been developed to allow researchers to obtain 3-dimentional images using x-ray technologies (Cronin et al. 2012).

Like every other technique, BLI also has its own limitations. The most important one is the attenuation of the signal through absorption and scattering of light by hemoglobin, melanin and other pigmented particles present in mammalian tissues (Contag et al. 1995). It is thus important to consider the latter when monitoring superficial and deep infections simultaneously. Also, two-dimensional images obtained through BLI, complicate specific allocation of infection inside the animal. Another drawback of BLI is the requirement of oxygen and ATP, which limits *in vivo* applications to aerobic conditions (for instance, not intragastric). Foucault et al. (2010) engineered an *E. coli* strain which enabled bioluminescent studies in the GIT of mice.

The field of optical imaging and its use in animal studies are widely studied and continually evolving (Leevy et al. 2007; Luker et al. 2008; Dothager et al. 2009; Luker and Luker 2010; Prescher et al. 2010; Andreu et al. 2011; Close et al. 2011; Waidmann et al. 2011).

## 5. *In vivo* infection models

Mice are ideal animals to study human infectious diseases, due to the high similarity in immune systems (Flajnik 2002). BLI has been included in various *in vivo* studies monitoring gene expression, protein stability, bioactive small molecules, tracking cells and therapeutic delivery (Prescher and Contag 2010). Studies of particular interest (systemic administration and nisin F) will be discussed (Table 4). Firstly, the bacteriocin mutacin B-Ny266 was intraperitoneally (i.p.) injected into mice infected with $3.1 \times 10^7$cfu *S. aureus* Smith immediately prior to treatment (Mota-Meira et al. 2005). The bacteriocin protected animals from mortality, as seen in the control groups. Secondly, microbisporicin effectively cured septicemic mice i.p. infected with $1 \times 10^6$ cfu of *S. aureus* Smith 819 ATCC 19636 (Castiglione et al. 2008). Thirdly, Nisin F showed no adverse histological effects or weight gain when applied as treatment to $4 \times 10^5$ cfu of *S. aureus* K administered to the nostrils of immunocompromised animals (De Kwaadsteniet et al. 2009). In a related study, C57BL/6 mice were subcutaneously (s.c.) infected with $4 \times 10^6$ cfu of the bioluminescent *S. aureus* strain xen 36 (De Kwaadsteniet et al. 2010). Infections were treated with 256 Au of nisin F, also s.c. administered. No significant differences in bioluminescence could be recorded. Recently, Piper et al. (2012) showed that lacticin 3147 prevented the systemic spread of BALB/C mice i.p. infected with 1 x $10^6$ cfu *S. aureus* Xen29, a bioluminescent strain.

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**Table 4** *In vivo* bacterial infection models

| Aim of study | Reference |
|---|---|
| Investigating the effectiveness of antibiotics in treating infections in the thigh muscles of mice | Francis et al. 2000 |
| Monitoring the effectiveness of antibiotics on biofilms on catheters planted in mice | Kadurugamuwa et al. 2003, 2004, 2005; Yu et al. 2005 |
| Monitoring staphylococcal foreign-body and deep-thigh-wound infections in mice | Kuklin et al. 2003 |
| Treatment of MRSA infections with the lanbtibiotic mersacidin in mouse rhinitis model | Kruszewska et al 2004 |
| Investigating the effectiveness of antibiotics in a rat endocarditis model | Xiong et al. 2005 |
| *In vivo* activity of mutacin B-Ny266 with i.p. injection in mice | Mota-Meira et al. 2005 |
| Antibiotic treatment to a *Pseudomonas aeruginosa* urinary bladder infection | Kadurugamuwa et al. 2005 |
| Open wound *Escherichia coli* infections in rats | Jawhara and Mordon 2006 |
| Investigating the antimicrobial activity of daptomycin against *S. aureus* peritonitis infections | Mortin et al. 2007 |
| Organ-specific bioluminescent monitoring of *Listeria monocytogenes* infection in mice after i.v. injection | Riedel et al. 2007 |
| Fetoplacental listeriosis and the pathogen's ability to cross the placental barrier in mice | Disson et al. 2008 |
| Monitoring implant-associated osteomyelitis infections | Li et al. 2008 |
| Monitoring the spread of a staphylococcal infection on implanted biomaterial | Engelsman et al. 2008 |
| Treatment of subcutaneous *S. aureus* infections in mice with nisin F | De Kwaadsteniet et al. 2009 |
| Respiratory tract S. aureus infection in rats treated with nisin F | De Kwaadsteniet et al. 2010 |
| Prophylactic treatment of subcutaneous *S. aureus* infections in mice with nisin F incorporated into bone cement | Van Staden et al. 2012 |
| Treatment of *S. aureus* infected burn wounds with nisin A-nanofiber dressings | Heunis 2012 |
| Monitoring systemic spread of *S. aureus* treated with the lantibiotic lacticin 3147 and the antibiotic vancomycin in a mouse peritonitis model | Piper et al. 2012 |

## 6. Drug development

The development of a drug for commercial use can take up to 13 years and a few critical aspects needs to be taken into consideration. In the case of antimicrobials, the drug needs to show *in vitro* antimicrobial activity. Additional *in vitro* and *ex vivo* tests can lead researchers to conclusions as to how the drug might react in an *in vivo* situation, but due to the extreme complexity of the situation once inside a living host, it brings about its own set of uncertainties.

Any compound entering the body exerts a certain immunomodulatory effect, either immunostimulatory or immunosuppressive. The innate immune system acts as the first line of defence and recognizes and responds to the first signs of infection (Hoffmann et al. 1999). In addition to pathogens formerly dealt with, the innate immune system can also initiate responses to new pathogens due to the recognition of highly conserved motifs present in pathogens, pathogen-associated molecular patterns (PAMPs, Bannerman et al. 2004). PAMPs include lipopolysaccharide (LPS), peptidoglycan (PGN) and lipoteichoic acid (LTA), which form part of the bacterial cell wall. One of the primary functions of the innate immune system is the upregulation of cytokine production (Dinarello 1996; Koj

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1996; Suffredini et al. 1999). Cytokines, in turn, act as messengers to various types of immunological cells to facilitate their movement to the site of infection.

Similar to pathogens causing infection or inflammation, treatments foreign to the host can also cause an antigenic effect. The treatment might, for instance, be toxic to eukaryotic cells or seen as a threat and be removed from the system or degraded. Thus it is important to assess the immunomodulatory effect any drug might have on a host and take it in consideration during dosage determination. Information about the immunomodulatory properties of bacteriocins is limited. Bedge et al. (2011) found a commercially purified mixture of nisin A and Z to be toxic to human lymphochytes and neutrophils from whole blood. After administration of Nisaplin® (a commercial form of nisin A) to mice for 30 and 75 days, an increase in CD4 and CD8 T-lymphocytes and a decrease in B-lymphocytes were observed (De Pablo et al. 1999). After 100 days of administration, the effects were, however, normalised again. The authors also found enhanced phagocytic activity of peritoneal cells after long-term administration of nisaplin. In another study, nisin showed immunostimulatory effects on head kidney macrophages in fish (Villamil et al. 2003). When administered to rats as a vaginal microbicide, RP-HPLC purified nisin proved to be non-toxic to host cells (Gupta et al. 2008). Nisin (Suárez et al. 1996), pediocin (Martínez et al. 1997) and peptide AS-48 (Maqueda et al. 1993) also showed an immunogenic effect in antibody studies.

*In vivo* results in the presence of plasma and other complexities often differ greatly from *in vitro* results (Bracci et al. 2003; Pini et al. 2005). Because AMPs (including some conventional antibiotics) are foreign to the host, the peptides often get targeted by the immune system. Protein binding (PB) is a phenomenon which can be studied in many ways and should be included in the preclinical testing of any new drug. Drugs with protein binding higher than 70% need to be assessed very accurately in terms of PB and findings should be taken into consideration in dosage design (Craig and Kunin 1976). Firstly, since only its unbound fraction is free to diffuse to the site of infection (Ogren and Cars 1985; Cars 1990; Kovar et al. 1997; Scaglione et al. 1998) and secondly because suboptimal dosages could lead to antimicrobial resistance (Schmidt et al. 2007). Generally, an increase of MIC was observed in serum for small molecule antibiotics (Chambers et al. 1984; Jones and Barry 1987; Lam et al. 1988) and peptide antibiotics such as LY333328 (Zhanel et al. 1998), daptomycin (Lee et al. 1991), and vancomycin (Dykhuizen et al. 1995). Only one lantibiotic, MU1140 has been tested in serum (Ghobrial et al. 2010), where the authors found contradicting results, where the MIC of the lantibiotic against *S. aureus* decreased in the presence of 50% serum. However, when tested against *S. pneumoniae*, the MIC increased in increasing serum concentrations. When analyzing PB, it is important to use a purified form of the drug in order to determine dosages accurately.

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Currently only a few antibiotics are available to treat infections caused by Gram-positive microorganisms (Upton et al. 2012). Big pharmaceutical companies have not made significant investments over the last few decades due to the time and financial constraints related to the drug development process. The global increase in antibiotic resistance and untreatable infections are not only widely discussed, but also leads to downstream complications. With further increased resistance, complications in surgical procedures and restricted cancer chemotherapy could be at the order of the day. To prevent this from happening, a combination of actions will need to be taken, including responsible use of antibiotics by both professional and public members, as well as the development of novel drugs.

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# Chapter 3

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# Development of a murine model with optimal routes for bacterial infection and treatment, as determined with bioluminescent imaging in C57BL/6 mice

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**Abstract** Mice intragastrically infected with *Listeria monocytogenes* EGDe and *Staphylococcus aureus* Xen 36 showed no visible signs of infection over 48 h. However, high numbers (6.2 x $10^5$ cfu/mg feces) of *S. aureus* Xen 36 were detected 4 h, and 3.3 x $10^5$ cfu/mg feces of *L. monocytogenes* EGDe 8 h, after administration. Mice intraperitoneally infected with *S. aureus* Xen 36 (1 x $10^7$ cfu) developed infection immediately after administration and for at least the following 48 h. Injection with higher cell numbers of *S. aureus* Xen 36 (2 x $10^8$ cfu) resulted in more intense bioluminescence (infection) of the peritoneal cavity. Injection of *S. aureus* Xen 36 in the tail and penile veins resulted in localized tissue infection for the first 120 h. Injection of *S. aureus* Xen 36 into the thigh produced a faint bioluminescent signal for 15 min. Nisin F injected into the peritoneal cavity at the same area of infection led to an immediate statistically significant decrease in infection (from 2 x $10^6$ p/s/cm$^2$/sr to 3 x $10^5$ p/s/cm$^2$/sr), which was maintained for 2 h. Similar results were recorded when nisin F was injected subcutaneously. Intraperitoneal administration is an optimal administration route for bacterial infection and treatment with antimicrobial peptides.

**Keywords** Bioluminescence imaging, Infection Model, *Staphylococcus aureus*

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## Introduction

*Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) are the most prevalent organisms in skin infections and are considered a serious problem, especially in hospitals [10, 21]. MRSA was first reported in the 1960's after the introduction of methicillin in the treatment of *S. aureus* infections [11, 12]. Since then, new enteropathogenic strains of MRSA, capable of rapid molecular changes, have been described [14, 16]. Symptoms vary from minor skin and soft tissue infections (SSTIs) to more serious diseases such as bacteraemia, toxic shock syndrome, endocarditis and pneumonia [13].

*Staphylococcus aureus* infections are treated with oxacillin, nafcillin, quinopristin-dalfopristin, rifampicin, ciprofloxacillin, teichoplanin, cefazolin, mupirocin and cephalothin A, but with limited success [9, 13]. In many cases, vancomycin is used as last resort, but also with limited success [20]. De Kwaadsteniet et al. [7] and Brand et al. [2] reported on nisin F, a class Ia lantibiotic [6], with *in vivo* antimicrobial activity against *S. aureus* in animal models. In these studies, bioluminescent imaging (BLI) was used to record changes in levels of *S. aureus* infection *in vivo.*

In this study, *in vivo* migration of *S. aureus* Xen 36, a strain originally isolated from a bacteremia patient and deposited as *S. aureus* ATCC 49525, was studied in different body parts of mice. Strain Xen 36 is bioluminescent in that it contains a plasmid with the *luxABCDE* operon of *Photorhabdus luminescence* encoding the luciferase enzymes and substrates required for bioluminescence [8]. Infections were treated with nisin F.

## Materials and Methods

Approval for *in vivo* experiments was obtained from the Ethics Committee of the University of Stellenbosch (ethics reference number: 2008B02003A2). Male C57BL/6 mice weighing between 20 and 30 g were used in all experiments. The animals were housed under controlled environmental conditions and fed a standard rodent diet.

### Bacterial Strains

Bioluminescent bacteria with the *luxABCDE* operon of *Photorhabdus luminescence* were purchased from Bioware$^{TM}$ Microorganisms (Caliper Life Sciences, Hopkinton, MA, USA). The strains were cultured at 37°C in specific growth media supplemented with antibiotics (Table 1), as prescribed by the supplier. Cells were grown to exponential phase, harvested and washed twice with sterile saline (15300*g*, 10 min, 4°C). A dilution series was prepared and the optical density of each cell suspension recorded at $A_{600nm}$. *Listeria monocytogenes* EGDe, containing the pMK4 *luxABCDE* plasmid [8], was cultured at 37°C in Brain Heart Infusion (BHI), supplemented with 75 µg/ml chloramphenicol. *Lactococcus lactis* subsp. *lactis* F10, the producer of nisin F, was cultured at 30°C in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa).

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**Table 1** Bioluminescent strains used in this study

| Strain | Origin | Growth medium |
|---|---|---|
| *Pseudomonas aeruginosa* Xen 5 | Septicemia isolate, ATCC19660 | LB, 60 µg/ml tetracyclin |
| *Escherichia coli* Xen 14 | Clinical isolate, WS2572 | LB, 30 µg/ml kanamycin |
| *Salmonella typhimurium* Xen 26 | Clinical isolate, SL1344 | LB, 30 µg/ml kanamycin |
| *Staphylococcus aureus* Xen 29 | Pleural fluid isolate NCTC8532 | BHI, 200 µg/ml kanamycin |
| *Staphylococcus aureus* Xen 30 | Clinical MRSA isolate, I6 | BHI, 200 µg/ml kanamycin |
| *Staphylococcus aureus* Xen 31 | Clinical MRSA isolate, ATCC 33591 | BHI, 200 µg/ml kanamycin |
| *Staphylococcus aureus* Xen 36 | Clinical isolate from bacteremia patient, ATCC49252 | BHI, 200 µg/ml kanamycin |
| *Klebsiella pneumonia* Xen 39 | Clinical isolate, 93A 5370 | LB, 30 µg/ml kanamycin |
| *Listeria monocytogenes* EGDe | | BHI, 75 µg/ml chloramphenicol |

LB= Luria Bertani (Biolab)

BHI= Brain-Heart Infusion (Biolab)

Nisin F Preparation and Activity Tests

*Lactococcus lactis* subsp. *lactis* F10 was cultured anaerobic for 18 h at 37°C in MRS broth. The cells were harvested (11300*g*, 20 min, 4°C) and nisin F was precipitated from the cell-free supernatant with 80% ammonium sulphate according to Sambrook et al. [18]. The precipitate was harvested (22100*g*, 60 min, 4°C) and dialysed against sterile distilled water, using a 1000 molecular weight cut-off membrane (Spectra/Por® Dialysis Membrane, Spectrum Laboratories, Inc. Rancho Dominguez, CA, USA). The desalted preparation was concentrated by freeze-drying and stored in cryotubes at -80°C.

Antimicrobial activity of nisin F against the target strains listed in Table 1 was determined by using the agar-spot test method [22]. Plates were seeded with 18 h-old cultures diluted as follows: Separate test tubes with 10 ml sterile saline were each inoculated with 100 µl of culture. From this suspension, 1 ml was used to inoculate 10 ml sterile soft agar (1%, w/v), supplemented with antibiotics as listed in Table 1. Freeze-dried nisin F was suspended in 500 µl sterile water to a final concentration of approximately 54 mg/ml, of which 10 µl (±32 Au) was used to spot onto each of the seeded agar plates. Formation of inhibition zones was recorded after 18 h of incubation at 37°C.

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Intragastric Injection

Eighteen mice were anaesthetized with 2% (v/v) isoflurane (Isofor, Safe Saline Pharmaceuticals, Florida, South Africa). Six mice in group 1 were intragastrically infected with 200 µl (1 x $10^7$ cfu) *S. aureus* Xen 36 and six mice in group 2 with 200 µl (3 x $10^7$ cfu) *L. monocytogenes* EGDe to represent an acute form of infection. Six mice in group 3 received 200 µl sterile saline administered with a straight oral dosing cannula (20 g x 76 mm, Harvard Apparatus Ltd., Edinbridge, UK).

Fecal samples were collected before infection and 2, 4, 6, 8, 24 and 48 h after infection. Samples were then weighed, dissolved in sterile distilled water and plated onto BHI Agar (Biolab), supplemented with 200 mg/ml kanamycin, to detect *S. aureus* Xen 36 and onto BHI Agar, supplemented with 75 mg/ml chloramphenicol, to detect *L. monocytogenes* EGDe. Samples collected from mice in group 3 were plated onto BHI Agar supplemented with kanamycin and chloramphenicol, respectively. Plates were incubated at 37°C for 18 h and then scanned for bioluminescence using the IVIS® 100 *In Vivo* Imaging System (Caliper Life Sciences). Photons emitted at 490 nm were quantified with Living Image® software, version 3.0 (Caliper Life Sciences) by measuring the average radiance in photons/ $cm^2$/ second/ steridian (p/s/$cm^2$/sr) from automatically created regions of interest (ROIs).

In a separate experiment, one mouse infected with *L. monocytogenes* EGDe for three consecutive days was imaged in the IVIS® 100 Imaging System for 14 consecutive days. On the last day, the animal was intraperitonealy administered with 200 mg/kg of pentobarbitone sodium (Euthapent, Kyron Laboratories (Pty) Ltd., Benrose, South Africa) and killed by exsangination (cardiac puncture). Sections of the duodenum, ileum and jejunum were each placed in 1 ml sterile distilled water, crushed with a sterile spatula and 100 µl plated out onto BHI Agar, supplemented with 200 mg/ml kanamycin. Plates were incubated overnight at 37°C and imaged for the presence of bioluminescent colonies as described before.

Intraperitoneal Injection

Three mice were injected intraperitoneally with 200 µl (1 x $10^7$ cfu) *S. aureus* Xen 36, using size 27 g needles. The mice were imaged for the presence of bioluminescent bacteria before injection, 15 min after injection, and again after 2, 4, 24 and 48 h.

Intravenous (Tail Vein and Penile Vein) Injection

Two animals were infected in the dorsal tail vein and one in the penile vein with 200 µl (1.4 x $10^8$ cfu) *S. aureus* Xen 36, using size 27 g needles. Veins were dilated by exposure to infrared light. Bioluminescent images were taken every 24 h for 120 h.

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Intramuscular Injection

The right thighs of two mice were injected with 50 µl (3.6 x $10^7$ cfu) of *S. aureus* Xen 36, followed by another injection 2 h later. The mice were scanned for the presence of bioluminescent bacteria before and immediately after both injections, and 72 and 144 h after the first injection.

Optimizing the Infection Dosage

Eight mice (two per group) were treated as follows: Mice in group 1 were immune compromised by feeding them dexamethazone (2.5 mg/l in drinking water) for a week and then intraperitoneally injected with 200 µl (2 x $10^7$ cfu) *S. aureus* Xen 36. Mice in group 2, not immune compromised, were injected with the same number of *S. aureus* Xen 36. Mice in groups 3 and 4 were injected with 200 µl *S. aureus* Xen 36, containing 1 x $10^8$ cfu and 4 x $10^8$ cfu, respectively. The cell numbers were chosen to represent three different levels of infection. The mice were scanned for the presence of bioluminescent bacteria before injection, and 24 h and 72 h after injection.

Treatment with Nisin F

Six mice were intraperitoneally injected with 200 µl (1 x $10^8$ cfu) *S. aureus* Xen 36, left for 2 h and then treated as follows: Two mice were injected with 200 µl (640 Au) nisin F intraperitoneally, two mice were injected with the same concentration nisin F subcutaneously, and two mice were left untreated. The mice were scanned for the presence of bioluminescent bacteria after 2 h of infection with *S. aureus* Xen 36 and then treated with nisin F. Images were recorded 2, 22 and 46 h after treatment with nisin F.

**Results**

Spectrum of Antimicrobial Activity (*in vitro*)

Growth of *S. aureus* strains Xen 29, Xen 30, Xen 31 and Xen 36, and *L. monocytogenes* EGDe were inhibited by nisin F, with the best antimicrobial activity recorded against *S. aureus* Xen 36 (not shown).

Intragastric Infection

No bioluminescence was recorded during the 48 h the mice were scanned (Fig. 1B). However, bioluminescent, thus metabolically active, cells were observed in the feces collected from these mice. High numbers (3.3 x $10^5$ cfu/mg feces) of *L. monocytogenes* EGDe were secreted 8 h after infection (Fig. 1A). *S. aureus* Xen 36, on the other hand, was mostly secreted (6.2 x $10^5$ cfu/mg feces) 4 h after infection (Fig. 1A). None of the strains were secreted 48 h after infection (Fig. 1A). Bioluminescent

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bacteria were also observed in the jejunum, duodenum and ileum of mice 14 days after intragastric administration with *L. monocytogenes* EGDe (not shown).

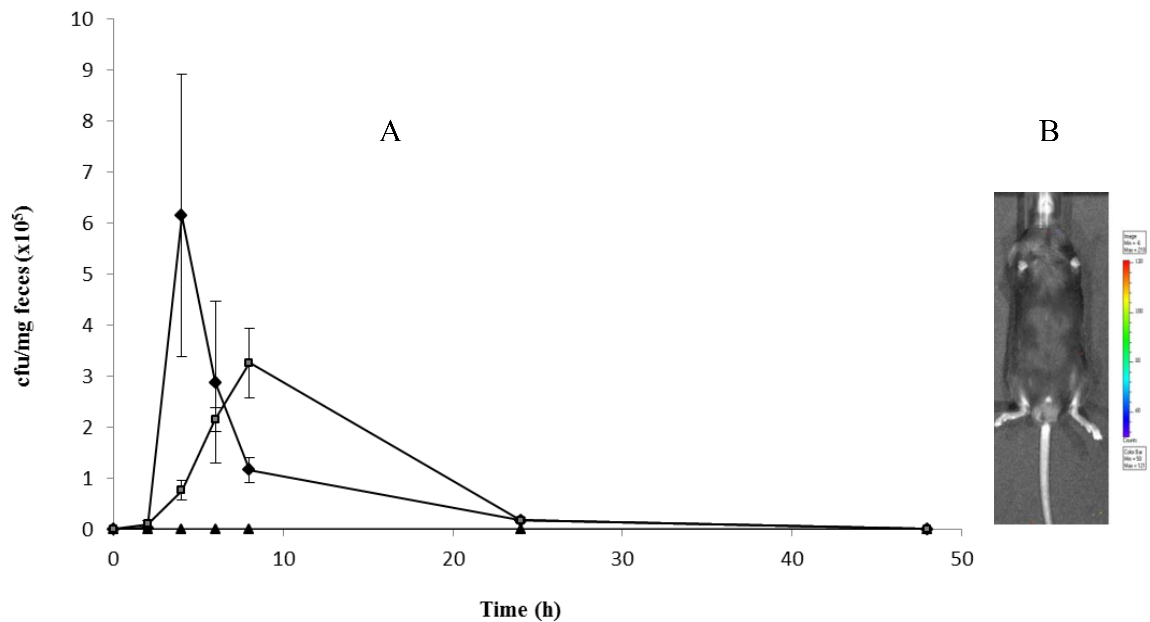


**Fig. 1** **a** Number of viable cells recorded per gram feces collected at specific time points after intragastric inoculation with *S. aureus* Xen 36 (♦) and *L. monocytogenes* EGDe (■). Values represent an average recorded for the six mice in each group. ▲ control (inoculated with sterile saline). **b** Bioluminescent image of a mouse after 48 h of intragastric infection with *L. monocytogenes* EGDe and *S. aureus* Xen 36

Intraperitoneal Infection

Cells of *S. aureus* Xen 36 were recorded in the peritoneal cavity immediately after injection (not shown) and after 2 h (2.3 x $10^4$ p/s/cm$^2$/sr). This was followed by more localized infection after 4 h (4.2 x $10^4$ p/s/cm$^2$/sr), which gradually changed over the next 44 h. Average radiance levels of 2.7 x $10^4$ p/s/cm$^2$/sr were recorded at 24 h and 2.3 x $10^5$ p/s/cm$^2$/sr at 48 h (Fig. 2). Throughout the course of all experiments, a few mice showed no signs of infection over 8 days (shown in Fig. 3A). However, when dissected, bioluminescent bacteria were detected in the peritoneal cavity (Fig. 3B), associated with the stomach, ileum, duodenum and jejunum, and the heart and kidneys (Fig. 3C).

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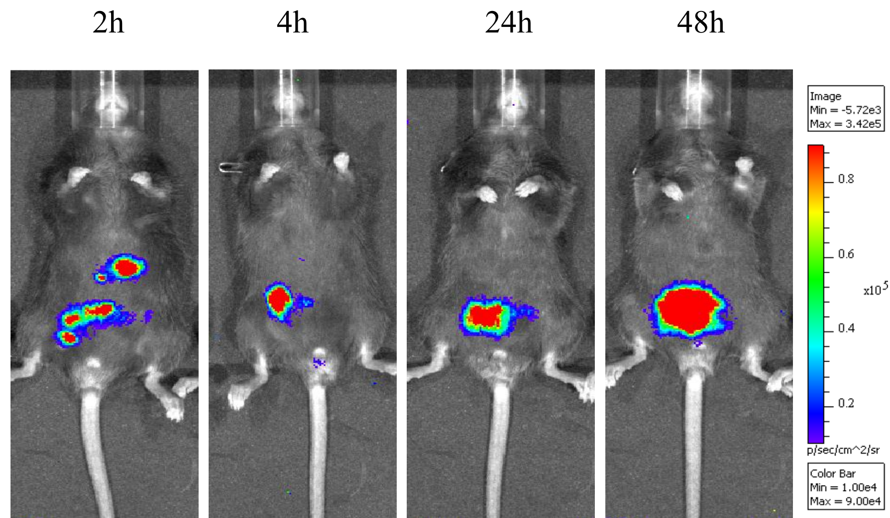


**Fig. 2** Bioluminescence recorded when mice were intraperitoneally injected with 200µl (1x$10^8$ cfu) *S. aureus* Xen 36. Images were recorded over 48

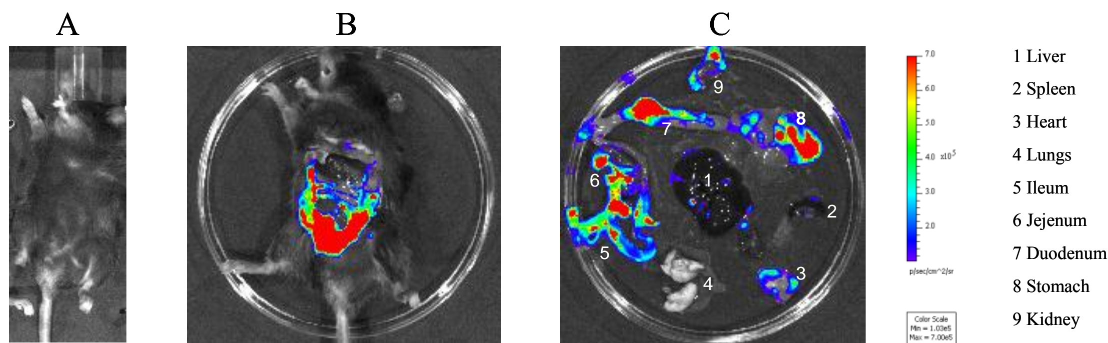


**Fig. 3** Bioluminescence recorded when mice were intraperitoneally injected with 200µl (1x$10^8$ cfu) *S. aureus* Xen 36. **a** Image taken of a diseased mouse after 8 days of infection. **b** The same mouse dissected, with the peritoneal cavity exposed. **c** Individual organs of the same mouse

Intravenous Infection

Injection of *S. aureus* Xen 36 in the tail and penile veins resulted in localized tissue infection for the first 120 h (results not shown). Average radiance values for penile vein injection ranged from 1.4 x $10^6$ p/s/cm$^2$/sr immediately after injection to 1.8 x $10^5$ p/s/cm$^2$/sr 5 days after infection. Bacteria injected into the dorsal tail vein showed an average radiance of 3.5 x $10^5$ p/s/cm$^2$/sr immediately after injection to 1.7 x $10^5$ p/s/cm$^2$/sr after 120 h.

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Intramuscular Infection

A faint bioluminescent signal of 5.2 x $10^3$ p/s/$cm^2$/sr was recorded immediately after injection of *S. aureus* Xen 36 into the thigh, but lasted for only 15 min. No bioluminescence was observed after a second injection with *S. aureus* Xen 36 in the same area 2 h later (results not shown).

Optimal Infection Dosage

No bioluminescent cells of *S. aureus* Xen 36 were recorded when mice were intragastrically infected (not shown). Clear bioluminescent signals with an average of 1.1 x $10^4$ p/s/$cm^2$/sr were recorded on the first day after intraperitoneal injection with 2 x $10^7$ cfu *S. aureus* Xen 36 (Fig. 4).

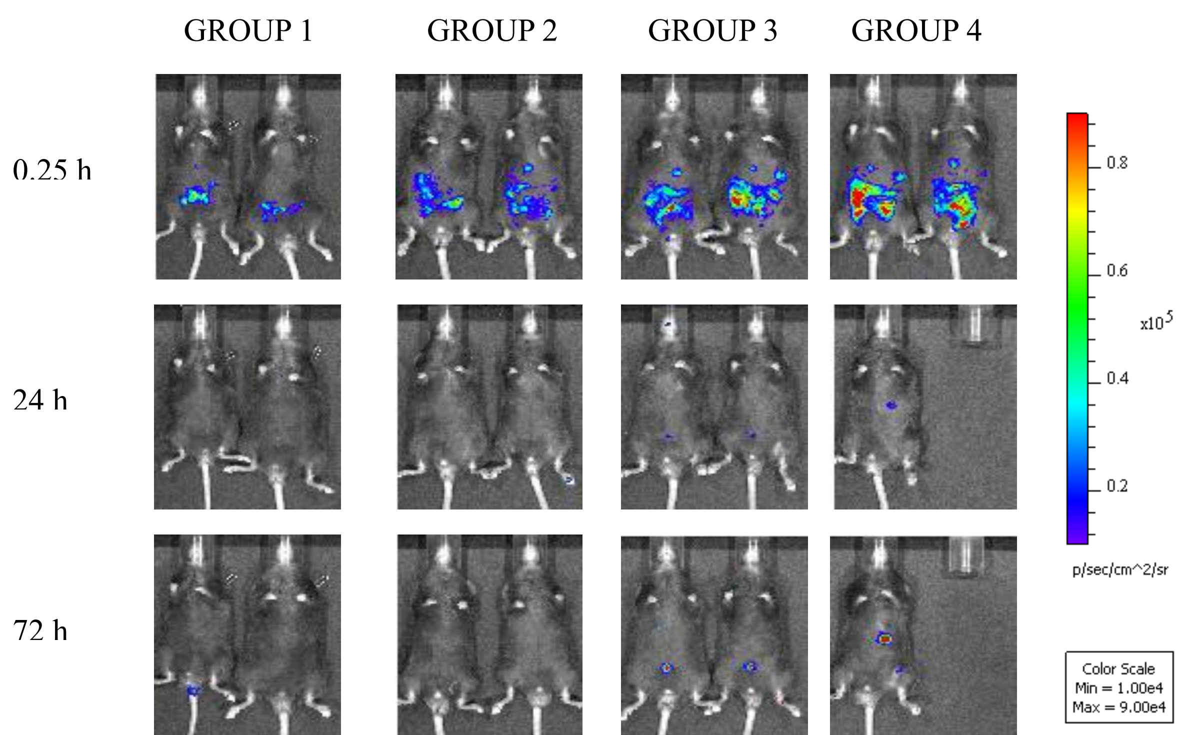


**Fig. 4** Mice intraperitoneally infected with *S. aureus* Xen 36 and imaged over 72 h. *Group 1* Immune compromised with dexamethazone (2.5 mg/l drinking water) for a week before infection with 2 x $10^7$. *Group 2* Not immune compromised and also infected with 2 x $10^7$ cfu. *Groups 3 and 4* Infected with1 x $10^8$ cfu and 4 x $10^8$ cfu, respectively. The mice were scanned for the presence of bioluminescent bacteria before infection, 15 min after infection and again at 24 h and 72 h

A slightly larger spread of infection, but with more or less the same intensity (9.4 x $10^3$ p/s/$cm^2$/sr), was observed in mice that have not been pre-treated with dexamethazone (Fig. 4). Injection with higher cell numbers (1 x $10^8$ cfu and 4 x $10^8$ cfu) of *S. aureus* Xen 36 resulted in more intense

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infection (1.5 x $10^4$ and 2.4 x $10^4$ p/s/cm$^2$/sr, respectively) of the peritoneal cavity (Fig. 4). After 96 h, bioluminescent images (6.1 x $10^4$ p/s/cm$^2$/sr) were only recorded in mice infected with cell numbers higher than 1 x $10^8$ cfu (not shown). Within 24 h after infection, one of the mice in group 4 died.

Treatment with nisin F

Since infection could not be accomplished with intragastric, intravenous or intramuscular routes of administration, all mice were injected with *S. aureus* Xen 36 in the peritoneal cavity. Nisin F injected to the same area of infection resulted in a slight decrease from 2 x $10^6$ to 3 x $10^5$ p/s/cm$^2$/sr after 2 h (4 h, Fig. 5). Similar results (a decrease from 1.9 x $10^6$ to 2.9 x $10^4$ p/s/cm$^2$/sr) were recorded when nisin F was injected subcutaneously. A slight decrease in infection 1.2 x $10^6$ to 3.6 x $10^4$ p/s/cm$^2$/sr was recorded in mice not treated with nisin F, but the levels of infection increased to 1.3 x $10^5$ p/s/cm$^2$/sr whilst no ROIs could be recorded for mice that have been treated (24 h, Fig. 5). However, infection slightly re-emerged during the following 24 h (48 h, Fig. 5).

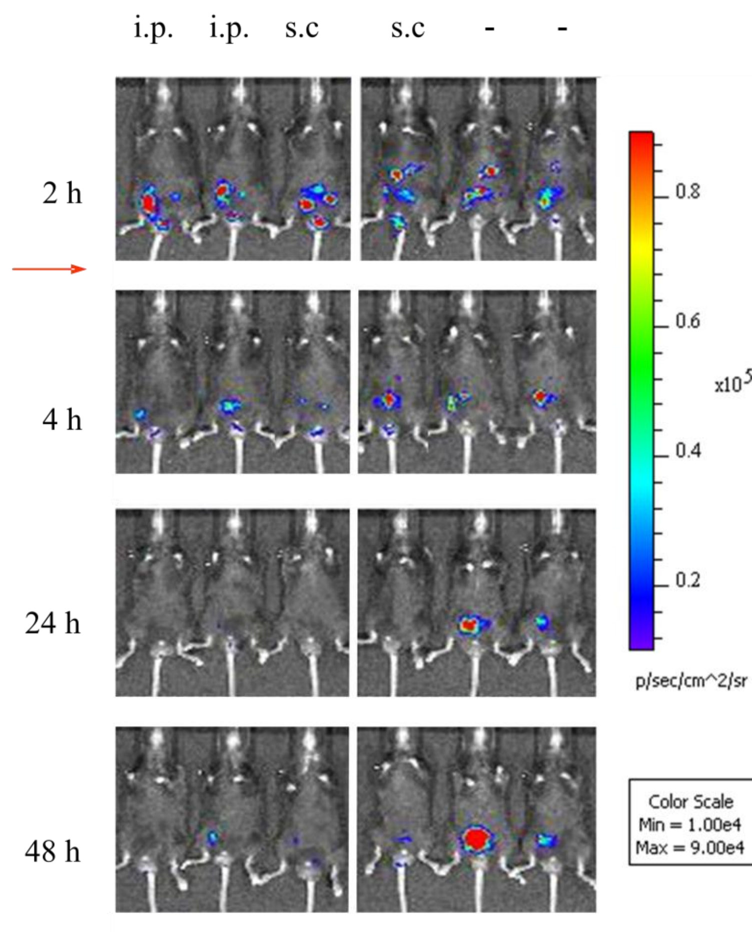


**Fig. 5** Mice infected with *S. aureus* Xen 36 (1 x $10^8$ cfu) for 2 h, treated with nisin F intraperitoneally (i.p.) and subcutaneously (s.c.), respectively, and monitored for 46 h. The *arrow* indicates nisin F administration

**Discussion**

Nisin F showed more antimicrobial response against *S. aureus* Xen 36 and *L. monocytogenes* EGDe growth, rendering these two strains the most appropriate to use in *in vivo* infection studies. *Staphylococcus aureus* Xen 36 and *L. monocytogenes* EGDe survived conditions in the GIT, as clearly indicated by the presence of bioluminescent, and thus metabolically active, cells in the feces collected from intragastrically infected mice (Fig. 1). *Staphylococcus aureus* Xen 36 was secreted more rapidly compared to *L. monocytogenes* EGDe (Fig. 1), suggesting that *L. monocytogenes* colonized the gastro-intestinal tract (GIT) more effectively. This is not surprising, as *S. aureus* is more associated with topical, airway and systemic infections, whereas *L. monocytogenes* causes severe food-borne infections [3]. Colonization was mostly found in the jejunum and to a lesser extent in the ileum and duodenum (not shown), suggesting that conditions in the jejunum are optimal for the survival and growth of *L. monocytogenes.*

The inability to record bioluminescence of *S. aureus* Xen 36 and *L. monocytogenes* EGDe in mice that have been infected intragastrically (Fig. 1B) may be due to a number of reasons. Maximum levels of bioluminescence were recorded in the presence of oxygen. Although expression of the *luxABCDE* operon is not regulated by oxygen, oxygen is required by the luciferase enzyme [4, 23]. It may also be that the signal emitted was too low to penetrate through the GIT and the abdomen.

Of all routes of infection studied, intraperitoneal injection (Fig. 2) proved to be the most successful. The absence of bioluminescence in some mice may be due to a common, unavoidable, randomly occurring error in the administration technique where the inoculum ends up subcutaneously, in an organ or in the intestines as shown in Fig. 3. Similar findings have been reported by Steward et al. [19], Miner et al. [15], Arioli and Rossi [1] and Das and North [5].

Infection of tissue surrounding the tail and penile veins is a clear indication that intravenous injection was not successful, most probably due to the small size of the veins. Based on the results we have obtained, cells injected into the thigh muscle are rapidly transferred to other areas, resulting in no detection after 15 min. It is, however, interesting that injection into the same area 2 h later did not elicit infection. The reason for this is not clear and need to be investigated.

*Staphylococcus aureus* Xen 36 levels of 2 x $10^7$ cfu caused infection, but lasted for one day only (24 h, Fig. 4). Much higher cell numbers (4 x $10^8$ cfu) is considered lethal, because one of the mice died within 24 h after infection. Cell numbers of 1 x $10^8$ cfu yielded a high bioluminescent signal immediately after infection, which was still visible 72 h later (Fig. 4), suggesting that this is the initial cell number required for sustainable infection without killing the animal.

Treating of infected mice with nisin F, either intraperitoneally or subcutaneously, lowered the cell numbers during the first 2 h after treatment, as reflected by a decrease in bioluminescence (4 h, Fig. 5). Control of infection was maintained for at least a further 20 h (24 h, Fig. 5), suggesting that the cell numbers of *S. aureus* Xen 36 were too low to emit a readable bioluminescent signal. Brand et al. [2] have shown that a single dosage of nisin F administered in the peritoneal cavity controlled the growth of *S. aureus* for at least 15 min. Re-appearance of the infection 24 h later (48 h, Fig. 5)

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suggests that some bacteria became resistant to nisin F or that the activity of nisin F decreased to below MIC levels after 46 h in the peritoneal cavity or when injected subcutaneously. Although no significant differences were recorded between infected animals treated intraperitoneally and subcutaneously (Fig. 5), intraperitoneal administration is considered more favorable because subcutaneous administrated substances are taken up slower due to the presence of lymphatic capillaries and regional lymph nodes [17]. Future studies will include prophylactic treatment of mice before infection, and also long-term treatment of *S. aureus* infections to evaluate the therapeutic properties of nisin F.

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# Chapter 4

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# The ability of nisin F to control *Staphylococcus aureus* infection in the peritoneal cavity, as studied in mice

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**Running headline:** Nisin F against *Staph. aureus*

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**Abstract**

**Aims:** To determine the ability of nisin F to control systematic infection caused by *Staphylococcus aureus,* using C57BL/6 mice as a model.

**Methods and Results:** Twelve mice were intraperitoneally injected with 1 x $10^8$ viable cells of *Staph. aureus* Xen 36 containing the modified *Photorhabdus luminescence luxABCDE* operon on plasmid pAUL-A Tn*4001*. After 4 h, six mice were intraperitoneally injected with 640 AU (arbitrary units) nisin F and six were injected with sterile saline. Six mice, not infected with *Staph. aureus*, were treated with nisin F and six not infected were left untreated. The viability of *Staph. aureus* Xen 36 was monitored over 48 h by recording photon emission levels. Nisin F suppressed *Staph. aureus* for 15 min *in vivo*. No abnormalities were recorded in blood analyses and internal organs of mice treated with nisin F.

**Conclusions:** Nisin F suppressed the growth of *Staph. aureus* in the peritoneal cavity for at least 15 min. Re-emergence of *Staph. aureus* bioluminescence over the next 44 h suggests that nisin F was inactivated, most probably by proteolytic enzymes.

**Significance and Impact of the Study:** A single dosage of nisin F administered in the peritoneal cavity controlled the growth of *Staph. aureus* for at least 15 min *in vivo*.

**Keywords**

*Staphylococcus aureus*, nisin F, bioluminescence

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## Introduction

*Staphylococcus aureus* causes food poisoning and a number of diseases (Lowy 1998). The rapid emergence of multidrug resistant strains of *Staph. aureus* (Awad *et al.* 2007) necessitates the search for novel antimicrobial compounds. Nisin F, a newly described nisin variant produced by *Lactococcus lactis* subsp. *lactis*, has *in vitro* activity against *Staph. aureus* (de Kwaadsteniet *et al.* 2008).

This study was conducted to determine whether nisin F could be used to control *Staph. aureus* infection *in vivo*. The target strain used in the study contains a plasmid with the *luxABCDE* operon that produces luciferase in the presence of ATP (Contag *et al*. 1995). Bioluminescence is thus used to record metabolic activity. The same reporter system has been used by Francis *et al*. (2000) and de Kwaadsteniet *et al*. (2009, 2010).

## Materials and methods

### Microorganisms

*L. lactis* subsp. *lactis* F10, producer of nisin F, was cultured at 30°C in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa). *Staph. aureus* Xen 36 (Bioware™ Microorganisms, Caliper Life Sciences, Hopkinton, MA, USA) was used as target strain. Strain Xen 36, originally isolated from a bacteremia patient and deposited as *Staph. aureus* ATCC 49525, was genetically modified by cloning the *luxABCDE* operon of *Photorhabdus luminescence* into a single integration site on a native plasmid containing the kanamycin resistance gene (Francis *et al*. 2000). *Staph. aureus* Xen36 was cultured at 37°C in brain heart infusion (BHI, Biolab), supplemented with 200 $\mu$g ml$^{-1}$ kanamycin to keep the plasmid under selective pressure. Active-growing cells were harvested (15 300 ***g***, 10 min, 4°C), washed twice with sterile saline (15 300 ***g***, 10 min, 4°C) and resuspended in sterile saline to an optical density ($A_{600nm}$) of 0·4 (measured from a ten-fold dilution).

### Preparation of nisin F and *in vitro* activity tests

*Lactococcus lactis* subsp. *lactis* F10 was cultured at 30°C in 1 L MRS broth (Biolab) for 18 h. The cells were harvested (11 300 ***g***, 20 min, 4°C) and nisin F precipitated from the cell-free supernatant with 80% ammonium sulphate and dialised according to Sambrook *et al*. (1989). The peptide was concentrated by freeze-drying and suspended in sterile distilled water. Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) and antimicrobial activity expressed as AU mg$^{-1}$ protein. The purity of nisin F was determined by SDS-tricine gel electrophoresis, according to Van Reenen *et al*. (1998).

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The antimicrobial activity of nisin F was determined by using the agar-spot test method as described by de Kwaadsteniet *et al*. (2008). *Staph. aureus* Xen 36 (1 x $10^6$ CFU) was imbedded in BHI soft agar (1%, w/v), supplemented with 200 $\mu$g $ml^{-1}$ kanamycin.

Antimicrobial activity of nisin F was also recorded by monitoring changes in bioluminescence of *Staph. aureus* Xen 36. The strain was grown in BHI broth, supplemented with 200 $\mu$g $ml^{-1}$ kanamycin, to an optical density ($OD_{600nm}$) of 1·5. Wells of a sterile titre plate were filled with 100 $\mu$l (3 x $10^7$ CFU) of the culture and then treated with two concentrations of nisin F. Wells A1-3 received 75 $\mu$l (240 AU) nisin F and wells A4-6 150 $\mu$l (480 AU) nisin F. The control received nothing. Bioluminescent readings were taken at 490 nm at $T_0$ (before the addition of nisin F), $T_{0.10}$ (6 min after addition), $T_{24}$ (24 h later) and $T_{48}$ (48 h later), using the Xenogen In Vivo Imaging System (IVIS® 100) of Caliper Life Sciences. Living Image® software, version 3·0 (Caliper Life Sciences) was used to quantify the average radiances (avg. rad.) of photons emitted from regions of interest (ROIs). The ROI was selected by using the free draw method, with the threshold set at zero percent. Values recorded from each well were expressed as $log_{10}$ photons $second^{-1}$ $cm^{-2}$ $steridian^{-1}$ (p $s^{-1}$ $cm^{-2}$ $sr^{-1}$). The correlation between p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ and viable cell numbers (CFU) was obtained from a standard curve. For the standard curve, cells were grown in BHI, supplemented with 200 $\mu$g $ml^{-1}$ kanamycin, to an optical density ($OD_{600nm}$) of 1·5, which represented 3 x $10^8$ CFU $ml^{-1}$. Sterile titre plates were filled with 100 $\mu$l of the cell suspension (3 x $10^7$ CFU) and incubated at 37°C for 48 h. At specific time intervals the number of photons emitted per second was recorded and converted to p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ by the Living Image® software. The number of viable cells (CFU) was determined by plating onto BHI agar.

**Antimicrobial activity of nisin F *in vivo***

Approval to conduct this research on mice was granted by the Ethics Committee of the University of Stellenbosch (ethics reference number: 2008B02003A2).

C57BL/6 mice weighing between 20 and 30 g were fed a standard rodent diet and kept under controlled environmental conditions. Twenty-four mice were anaesthetized with 2% (v/v) isoflurane (Isofor, Safe Saline pharmaceuticals, Florida, South Africa). Twelve mice were intraperitoneally injected with 200 $\mu$l (1 x $10^8$ viable cells) of *Staph. aureus* Xen 36 and divided into two groups of six mice each. After 4 h of infection, mice in group 1 were again anaesthetized and intraperitoneally injected with 200 $\mu$l nisin F (640 AU). Mice in group 2 were also anaesthetized, but intraperitoneally injected with 200 $\mu$l sterile saline. Images were taken at $T_0$ (before infection with *Staph. aureus* Xen 36), $T_{0.25}$ (15 min after infection), $T_4$ (4 h after infection and immediately before treatment with nisin F), $T_{4.25}$ (15 min after treatment), and $T_8$, $T_{24}$ and $T_{48}$ (i.e. 4 h, 20 h and 44 h after treatment with nisin F). Mice in group 3 (not infected with *Staph. aureus*) were treated with nisin F (640 AU) 4 h after infection. Mice in group 4 were not infected and were not treated with nisin F. All mice were

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anaesthetized with the same dosage of isoflurane. Bioluminescent readings of each were taken with the IVIS® 100 at 490 nm, as described before. The avg. rad. of mice in each group was calculated. Where no ROIs could be detected, values of 0 were changed to 0·001 to allow for $\log_{10}$ calculations.

**Histology**

At the end of the two-day experiment the mice were sacrificed by cardiac puncture after an overdose of sodium pentobarbitone (Euthanaze, Centaur Labs) administered intraperitoneally. Samples of the intestinal tract, heart, liver, kidneys, lungs and spleen were kept in 4% (v/v) formaldehyde (PBS) for 24 h at 25ºC, embedded in paraffin, sectioned and then stained with hematoxylin and eosin (H&E). Microscopic analysis was done at Pathcare Veterinary Pathologists (Pathcare, Dietrich, Voigt, Mia and Partners, Goodwood, South Africa).

**Statistical Analysis**

Mixed model repeated measures ANOVA was used to determine the effects of treatments over time on results presented in Fig.1 and Table 1.

**Results**

***In vitro* activity of nisin F**

Nisin F prepared from an 18-h-old culture yielded 3 200 AU $ml^{-1}$, as recorded with the agar-spot method. This correlated to 66·72 AU $mg^{-1}$ protein. Separation by SDS-tricine PAGE yielded a single protein band of 3·5 kDa (not shown), corresponding to the 3 457 Da reported for nisin F (de Kwaadsteniet *et al*. 2008). Bioluminescent readings recorded for *Staph. aureus* Xen 36 treated with 240 AU nisin F (A1-A3) and 480 AU nisin F (A4-A6) changed from 5·75 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ (3 x $10^7$ CFU) before treatment to 4·80 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ (2·5 x $10^7$ CFU) within 6 min (not shown). On the other hand, bioluminescence of the control group (B1-B6), not treated with nisin F, increased from 5·76 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ (3 x $10^7$ CFU) to 5·92 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ (3·1 x $10^7$ CFU) over the same period and to approximately 6·30 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ (3·3 x $10^7$ CFU) over the next 48 h (not shown). Results recorded for *Staph. aureus* Xen 36 treated with nisin F differed significantly ($p < 0·01$) from *Staph. aureus* Xen 36 not treated with nisin F (not shown). A decrease in photons indicates that either fewer *Staph. aureus* cells are present or that the cells present are not metabolically active.

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***In vivo* activity of nisin F**

Bioluminescent images recorded for a representative mouse from each of the 4 groups is shown in Fig. 1.

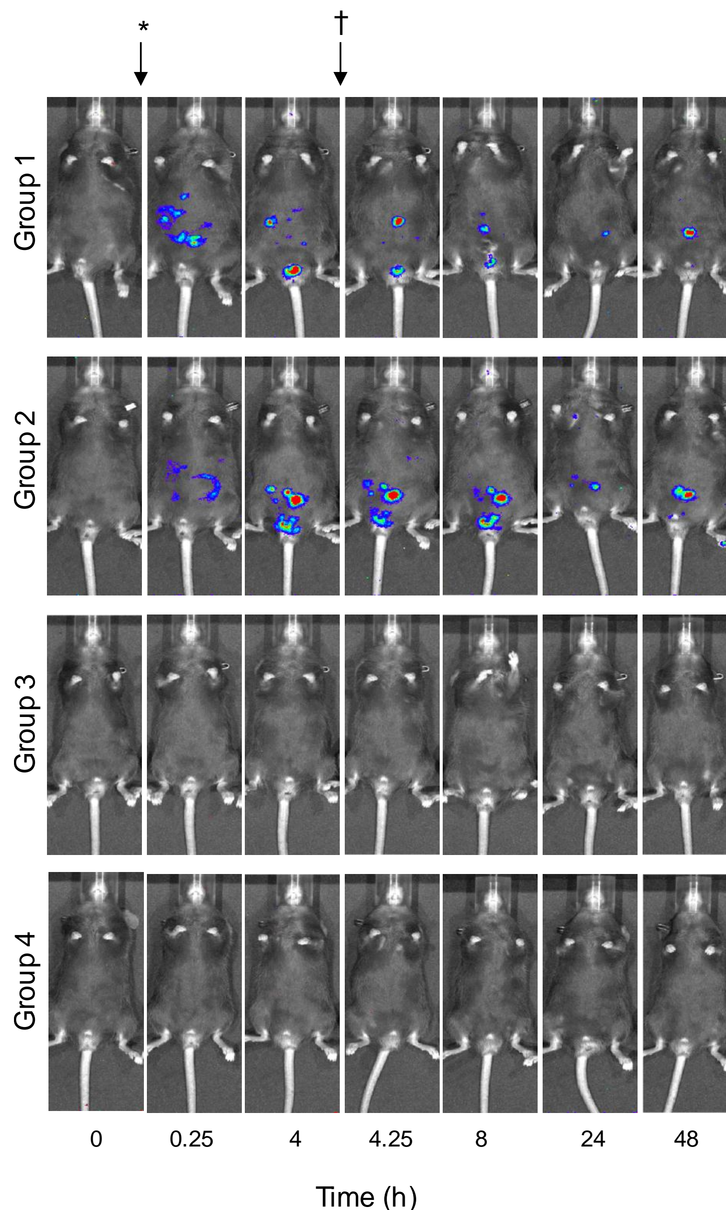


**Figure 1** Bioluminescent images of selected mice from different groups. Group1: infected with 1 x $10^8$ viable cells of *Staph. aureus* Xen 36 for 4 h, treated with 640 AU nisin F, and monitored for a further 44 h. Group 2: infected with the same number of *Staph. aureus* Xen 36 for 4 h, but treated with sterile saline after 4 h. Group 3: Not infected with *Staph. aureus* Xen 36 and treated with 640 AU nisin F. Group 4: Not infected and not treated. Arrows indicate time of infection and treatment. Bioluminescence emitted from regions of interest (ROI) was measured as p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ and the total average radiation for each mouse calculated. Readings are listed in Table 1. Symbols: * = infection, † = treatment.

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Mice injected with *Staph. aureus* Xen 36 (groups 1 and 2) showed clear signs of infection in the upper and lower sections of the peritoneal cavity after 15 min of inoculation ($T_{0.25}$, Fig. 1). In both groups the infection increased over the next 3 h and 45 min, as observed by changes in photon counts (Table 1).

**Table 1** $Log_{10}$ values of average radiance (p $s^{-1}$ $cm^{-2}$ $sr^{-1}$) measured from ROIs in bioluminescent images. Values listed are the average recorded for six mice in each group

| Group* | Time (h) | | | | | |
|---|---|---|---|---|---|---|
| | 0·25 | 4·00 | 4·25 | 8·00 | 24·00 | 48·00 |
| 1 | 3·87 ± 0·28 | 4·73 ± 0·26 | 4·55 ± 0·22 | 4·55 ± 0·25 | 4·04 ± 0·32 | 4·17 ± 0·36 |
| 2 | 3·85 ± 0·21 | 4·88 ± 0·21 | 4·90 ± 0·20 | 4·64 ± 0·53 | 3·48 ± 0·38 | 3·50 ± 0·54 |

*Group 1: infected with *Staph. aureus* Xen 36 and treated with nisin F.

Group 2: infected with *Staph. aureus* Xen 36 and treated with sterile saline (control).

Variations recorded between the different readings are indicated by ± values.

Fifteen minutes after treatment with nisin F ($T_{4.25}$), *Staph. aureus* bioluminescence in the mid to upper section of the peritoneal cavity decreased slightly (an average decrease in photons from 4·73 to 4·55 p $s^{-1}$ $cm^{-2}$ $sr^{-1}$; Table 1). At the same time interval, bioluminescence in the lower section of the peritoneal cavity, closer to the tail, was slightly suppressed (group 1, Fig. 1). *Staph. aureus* bioluminescence gradually decreased over the following 20 h in mice treated with nisin F (Table 1), but bioluminescence re-emerged 24 h later, specifically in the mid to upper section of the peritoneal cavity ($T_{48}$, Fig. 1). Levels of *Staph. aureus* bioluminescence in mice treated with saline (group 2) remained unchanged for 4 h (up to $T_8$), decreased over the next 16 h ($T_{24}$), but re-emerged 24 h later ($T_{48}$) in the mid to upper section of the peritoneal cavity (Fig. 1, Table 1). There were overall no significant differences ($p = 0{\cdot}48$) observed between mice treated with nisin F and mice treated with sterile saline. However, a more significant difference in bioluminescence (post hoc p value of 0·26) has been recorded 15 min after administration of nisin F (Fig. 2).

**Histology**

Lymphoid hyperplasia was observed in the spleen samples of mice from all four groups. Infiltration of polymorphonuclear cells and mild degenerative changes of hepatocytes were observed in the livers of mice from groups 1 and 2 (not shown). Segmented filamentous bacteria were observed in various sections of the intestinal tract of mice in groups 1 and 2 (not shown).

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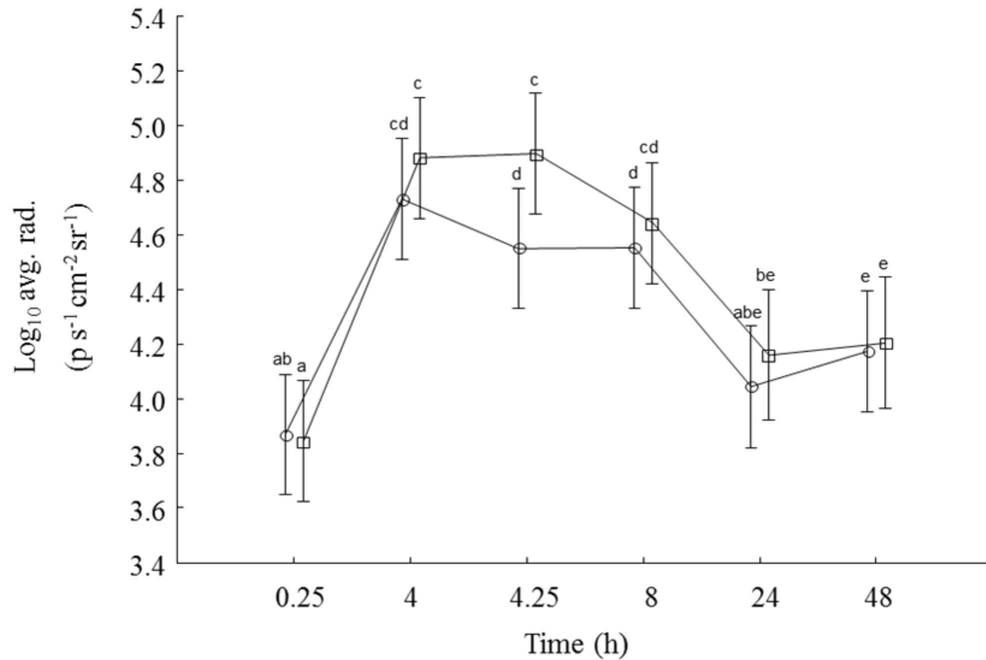


**Figure 2** No significant differences (p = 0·48) were recorded between mice treated with nisin F (plotted as –O–) and mice treated with sterile saline (plotted as –□–). However, according to the post hoc value (p = 0·26) of results 15 min after administration of nisin F, a significant difference has been recorded. The data used is from Table 1. Vertical bars denote 0.95 confidence intervals of Least Squares (LS) means. Letters indicates significant differences on a 5% level (p<0.05) for the post hoc tests. Where associated letters are different (no letters overlap), the difference is significant. Where at least one letter appears above both of the means to be compared, the difference is not significant.

## Discussion

Nisin F inhibits a number of Gram-positive bacteria (de Kwaadsteniet *et al.* 2008) and proved successful in the treatment of *Staph. aureus* infection in the respiratory tract of Wistar rats (de Kwaadsteniet *et al.* 2009). However, nisin F could not control subcutaneous infections caused by *Staph. aureus* (de Kwaadsteniet *et al.* 2010). Based on these results, the authors hypothesised that the innate immune system of mice has been more successful in combating the infection, or that administration of nisin F could have modulated the innate immune system.

Readings recorded with the IVIS® 100 indicated that all cells of *Staph. aureus* Xen 36 have lost their bioluminescence within 6 min of treatment with either 240 AU or 480 AU nisin F, with no recovery towards the end of the 48 h experiment. This suggests that 240 AU nisin F is sufficient to suppress cell growth *in vitro* for at least 48 h.

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Nisin F suppressed a certain percentage of *Staph. aureus* Xen 36 for up to 15 min in the peritoneal cavity (Table 1). Furthermore, the re-emergence of *Staph. aureus* Xen 36 bioluminescence 44 h after treatment suggests that the peptide has been degraded or inactivated, possibly by proteolytic enzymes, or that the strain became resistant to nisin F. Overall, bioluminescence of the cells decreased, but was not eliminated. This suggests that the mode of activity of nisin F against *Staph. aureus* Xen 36 is bacteriostatic *in vivo*. Photon emission readings were taken from the same animal over a short time period (48 h). Thus, changes in p $s^{-1}$ $cm^{-2}$ $sr^{-1}$ readings recorded are directly related to the physiological state of strain Xen 36 in a specific animal and are not influenced by changes in body mass. It is important to realise that photons emitted from strain Xen 36 lose signal strength as they migrate through the tissue. Thus, contrary to readings taken *in vitro*, photons emitted *in vivo* are indirectly related to the level of infection, but no direct correlation can be drawn between photons emitted (p $s^{-1}$ $cm^{-2}$ $sr^{-1}$) and number of viable cells of strain Xen 36 *in situ*.

Growth of segmented filamentous bacteria (SFB) in the intestinal tract could be due to secondary infection induced by *Staph. aureus*. Growth of these bacteria in the gastro-intestinal tract could not be controlled with nisin F, suggesting that the peptide did not migrate across the epithelial cells and mucus of the intestinal wall, or that the SFB are resistant to the lantibiotic. The peptide could also have been degraded. These bacteria naturally colonize the intestinal tract of rodents and their numbers often increase in response to the presence of a pathogen (Davis and Savage 1974). Snel *et al*. (1995) described SFB as autochthonous, strictly anaerobic, spore-forming, Gram-positive bacteria representing a cluster within the *Clostridium* subphylum and proposed the name "*Candidatus* Arthomitus". These bacteria may stimulate the formation of intraepithelial lymphocytes (Umesaki *et al.* 1995; Talham *et al.* 1999) and lead to an increase in the mucosal immune system (Klaasen *et al.* 1993; Umesaki *et al.* 1995). No visible abnormalities were observed in any of the organs that have been studied. This, however, does not rule out the possibility of cell damage or mutations that could have taken place.

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# Chapter 5

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# The effects of continuous in vivo administration of nisin F on *Staphylococcus aureus* infection and immune response in mice

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**Abstract**

Mice were intraperitoneally infected with 2 x $10^8$ cfu *Staphylococcus aureus* Xen 36 and treated with 2130 AU (arbitrary units) Nisin F, a class Ia lantibiotic, over seven days. Nisin F was delivered at a constant rate to the intraperitoneal cavity by using osmotic minipumps. The metabolic activity of *S. aureus* Xen 36, concluded from changes in cell bioluminescence, declined for the first 3.5 h, but increased over the next 24 h and remained constant during the rest of the trial. Similar results were obtained with inactive (autoclaved) nisin F. This suggests that the decline in metabolic activity of *S. aureus* Xen 36 was not due to the presence of nisin F. The drastic increase in neutrophil versus lymphocyte numbers (N:L ratio) observed in mice infected with *S. aureus* Xen 36 and treated with nisin F, opposed to mice not infected and treated with nisin F, suggested that the decline in metabolic activity of *S. aureus* Xen 36 was due to an immune response triggered by the infection. In uninfected mice, treatment with nisin F elicited increased plasma IL-6 and decreased IL-10 levels and the magnitude of these responses was significantly larger in the group treated with active nisin F when compared to inactive nisin F. However, the overall immune response triggered by nisin F was too small to suggest an antigenic immune reaction.

**Keywords:** bioluminescence, *Staphylococcus aureus*, immune response

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## 1. Introduction

*Staphylococcus aureus* is a normal inhabitant of the human skin, nose and mucosal surfaces, but some strains may cause serious infections and even death if cell numbers are not controlled [1]. The prevalence of methicillin-resistant strains of *S. aureus* (MRSA) in hospitals and the community is of global concern and the need to discover novel antimicrobial compounds is of critical importance. Nisin F, a class Ia lantibiotic, showed promising results in the treatment of respiratory tract [2] and intraperitoneal [3] infections, but not in subcutaneous infections [4]. A single dosage of nisin F injected into the intraperitoneal cavity suppressed the metabolic activity of a bioluminescent strain of *S. aureus* (strain Xen 36) for at least 15 min [3]. The subsequent increase in bioluminescence was ascribed to the possible degradation of nisin F by proteolytic enzymes or acquired resistance to the treatment [3]. Nisin F incorporated into bone cement prevented the growth of *S. aureus* Xen 36 in vivo [5]. Although these studies have shown that nisin F may be used to treat *S. aureus* infections, little is known about the effect the lantibiotic has on the immune system.

In this study, nisin F was administered in vivo to BALB/C mice over 7 days. A constant flow of nisin F was delivered into the peritoneal cavity by subcutaneously implanted osmotic minipumps. These osmotic pumps have been used for more than 40 years for the delivery of various compounds including antibodies, cytokines, growth factors, neurotrophic factors, insulin and numerous proteins. To our knowledge this is the first time these pumps were used to deliver a bacteriocin. Implementation of the pumps has various advantages over repeated manual administration, especially in the case of proteins and peptides with short half-lives. Advantages include a maintained therapeutic level throughout the study, convenience, minimized experimental variables, reduced handling and stress to laboratory animals and delivery of agents to most bodily sites.

Changes in the immune system of mice in response to nisin F were monitored by differential blood counts (total white blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils) and by determining the levels of interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor (TNF) and interleukin-12p70 (IL-12p70) in plasma of blood sampled from non-infected mice. These are the most important cytokines (immunomodulating cell-signalling molecules produced by white blood cells, but also various other cell types) involved in the inflammatory response in mice and were therefore chosen for assessment.
In addition, changes in the metabolic activity (bioluminescence) of *S. aureus* Xen 36 was monitored in mice treated with either active or inactive (autoclaved) nisin F.

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## 2. Materials and methods

*2.1 Preparation of nisin F*

*Lactococcus lactis* subsp. *lactis* F10, producer of nisin F, was cultured at 30ºC in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa), as described by Brand et al. [3]. Cells were harvested at 11300 x g (20 min, 4°C). Nisin F was precipitated from the cell-free supernatant with 80% saturated ammonium sulphate [6] and dialysed against sterile distilled water through a 1000 Da cut-off regenerated cellulose membrane (Spectra/Por®, Spectrum Laboratories, Inc. Rancho Dominguez, CA, USA). The dialysate was concentrated by freeze-drying and then redissolved in sterile distilled water. The antimicrobial activity of nisin F was determined using the agar spot-test method [7] and expressed as AU (arbitrary units) per mL. *Staphylococcus aureus* Xen 36, cultured at 37°C in Brain Heart Infusion (BHI, Biolab Diagnostics) supplemented with 200 µg/mL kanamycin, was used as target strain.

*2.2 In vivo antimicrobial activity and immunological effect of nisin F*

*2.2.1 Preparation of osmotic minipumps and polyethylene tubing*

Active nisin F (200 µL per pump, equivalent to 2130 AU) and the same volume of inactivated (autoclaved) nisin F were injected into Alzet® model 2001 osmotic minipumps (Durect, Cupertino, CA, USA). The outer surfaces of the minipumps were sterilized by wiping the outside surface with 70% (v/v) isopropanol. Pumps were placed in sterile petri dishes and incubated at 37°C for 24 h to initialise capillary flow. Sterile polyethylene tubing was attached to the flow moderators of minipumps to deliver nisin F directly into the peritoneal cavity. To prevent adherence of *S. aureus* Xen 36 to the outer surface of the polyethylene tubing, the tubing was coated with a thin layer of nisin F (10650 AU/mL), encapsulated in a combination of poly (D-L) lactide dissolved in dimethylformamide (20% w/v). Inactive nisin F served as control. Three coatings of the nisin F-imbedded polymer were applied, with 2 h of drying (at room temperature) in between. The coated tubes were left in a laminar flow cabinet overnight to dry at room temperature before they were attached to minipumps and surgically implanted into the mice.

*2.2.2 Infection with* S. aureus *and treatment with nisin F*

Ethical approval was granted by the Ethics Committee of the University of Stellenbosch (reference number: 10NMDIC01).

Twenty-four BALB/c mice (20 to 30 g each) were divided into four equal groups and housed in sterilized type II short filter cages under controlled environmental conditions. The mice had free access to water and feed and were anaesthetised with 2% (v/v) isofluorane (Isofor Safeline Pharmaceuticals, South Africa) before all injections or imaging. On day two, the intraperitoneal

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cavities of mice in groups 1 and 2 were infected with 200 µL (2 x $10^8$ cfu) *S. aureus* Xen 36, as described previously [3]. Four hours later, minipumps filled with 200 µL (2130 AU) nisin F were surgically implanted in a subcutaneous pocket on the dorsal side of mice in group 1. Mice in group 2 received minipumps filled with 200 µL inactivated nisin F. The procedure was repeated on two other groups of mice (groups 3 and 4, respectively) that were not infected with *S. aureus* Xen 36. The polyethylene tube attached to each minipump was inserted through a small incision made in the peritoneum. Peritoneum and skin incisions were closed with CliniSorb 1.5 (4/0) sutures (CliniSut, Akacia health, Port Elizabeth, South Africa) and the skin wounds sterilized with 10% (v/v) Betadine® (Purdue Products L.P., Stamford, CT, USA). Mice were injected with the analgesic Temgesic (Schering-Plough, Woodmead, South Africa), 0.03 mg/kg bodyweight, immediately after surgery and 12 h later.

*2.2.3 Bioluminescent imaging*

Bioluminescent images were recorded with an In Vivo Imaging System (IVIS® 100, Caliper Life Sciences, Hopkinton, MA, USA). All mice were imaged at the same time points, i.e. before infection with *S. aureus* Xen 36, 15 min, 3.5 h, 8 h and 24 h after infection, and every 24 h thereafter for a further 6 days. Photons were quantified by manually selecting the border of each mouse and defining the whole body as a region of interest (ROI). Images were captured after 5 min of exposure, analysed with Living Image® software (Caliper Life Sciences) and expressed as average radiance (photons/$cm^2$/second/steridian) per ROI. After 7 days of monitoring the mice were killed by cardiac puncture after an overdose of sodium pentobarbitone (Euthapent, Kyron Laboratories, Benrose, South Africa).

*2.2.4 Immunological analyses*

Blood (ca. 250 µL) was collected from the ventral tail vein of mice in all four groups on days 1, 3, 4, 6 and 8. Blood samples were collected in paediatric $K_2$EDTA Vacutainer® tubes (BD Diagnostics, Franklin Lakes, NJ, USA). Within 4 h after collection, full blood counts were determined using an automated hematology analyser (Cell-Dyn 3700 CS, Abbott Diagnostics, Santa Clara, California, USA) and analysed with HEMO VetSoft v. 1.1 (Veterinary Software, Davis, CA, USA). The remaining blood samples were centrifuged (1500 x g, 10 min, 25ºC) and the plasma stored at –80°C. The effect of nisin F and inactive nisin F on the cytokine (IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70) levels of uninfected mice (groups 3 and 4) was determined using the BD Mouse Inflammation cytometric bead array (CBA) kit (BD Diagnostics). The cytokine levels of infected mice were not analysed, since we were interested in the effect nisin F (active and inactive form) has on the immune response in the absence of additional antigenic agents.

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*2.3 Data analyses*

Data was analysed with Statistica software (v.10, StatSoft Inc, OK, USA). For bioluminescent data, t-tests assuming unequal variances were performed. Results for blood parameters were analyzed for effects of time and treatment using repeated measures ANOVA or one-way ANOVA with Bonferroni post hoc testing. P-values < 0.05 were considered as significant.

## 3. Results

*3.1 Effect of nisin F on* S. aureus *Xen 36*

The average radiance (photons/cm$^2$/second/steridian) values recorded for the six mice in group 1 (infected with *S. aureus* Xen 36 and treated with nisin F) and the six mice in group 2 (infected with *S. aureus* Xen 36 and treated with inactive nisin F), is shown in Fig. 1.

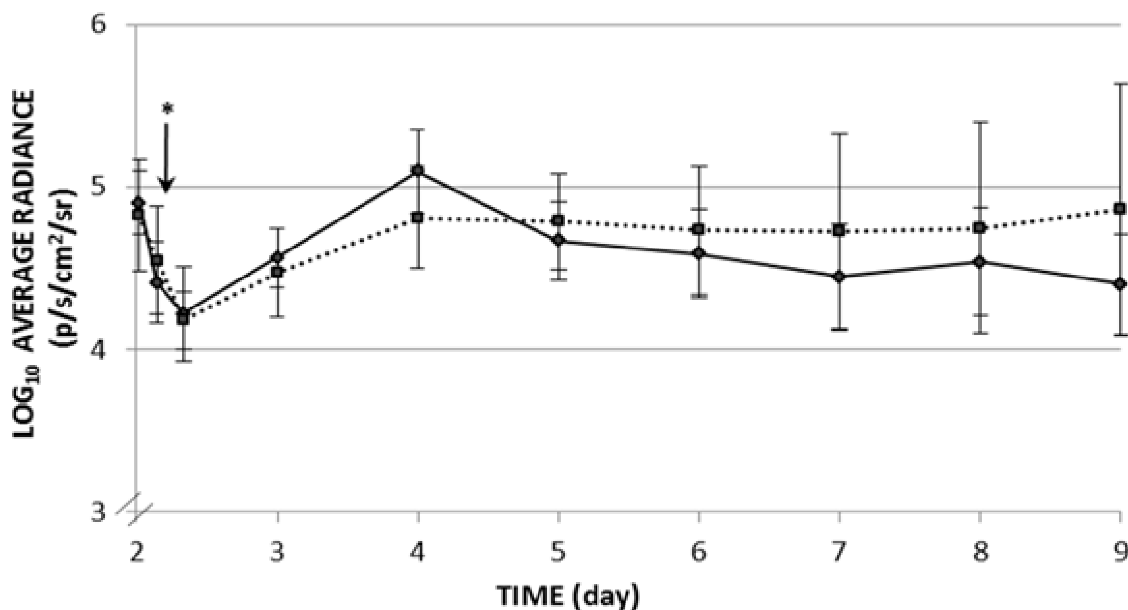


**Fig. 1.** Bioluminescent readings (photons/cm$^2$/second/steridian) recorded from mice infected with *S. aureus* Xen 36 and then treated with nisin F for the following 7 days. Readings were taken before infection (not shown) and 15 min, 3.5 h, 8 h and 24 h after infection, and every 24 h thereafter for a further 6 days. Each data point is an average of readings recorded for six mice in each group. --♦-- = mice infected with *S. aureus* Xen 36 and treated with nisin F (group 1), ··■·· = mice infected with *S. aureus* Xen 36 and treated with inactive (autoclaved) nisin F (group 2). Nisin F was injected via surgically implanted Alzet osmotic minipumps on day 2, 4 h after infection with *S. aureus* Xen 36. The point at which nisin F was injected is indicated by *.

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No significant differences in bioluminescence were recorded between the two treatments. One mouse in group 1 and three mice in group 2 died within 24 h after infection. Since mice in groups 3 and 4 were not infected with *S. aureus* Xen 36, no bioluminescence was observed.

*3.2 Immune response*

Total white blood cell, monocyte, eosinophil and basophil counts were on average within normal ranges, irrespective of the treatment (results not shown). On day 3, 24 h after infection with *S. aureus* Xen 36, the relative neutrophil count in group 1 mice increased from 23% to 53%, which represents an average increase of 130% (Fig. 2A).

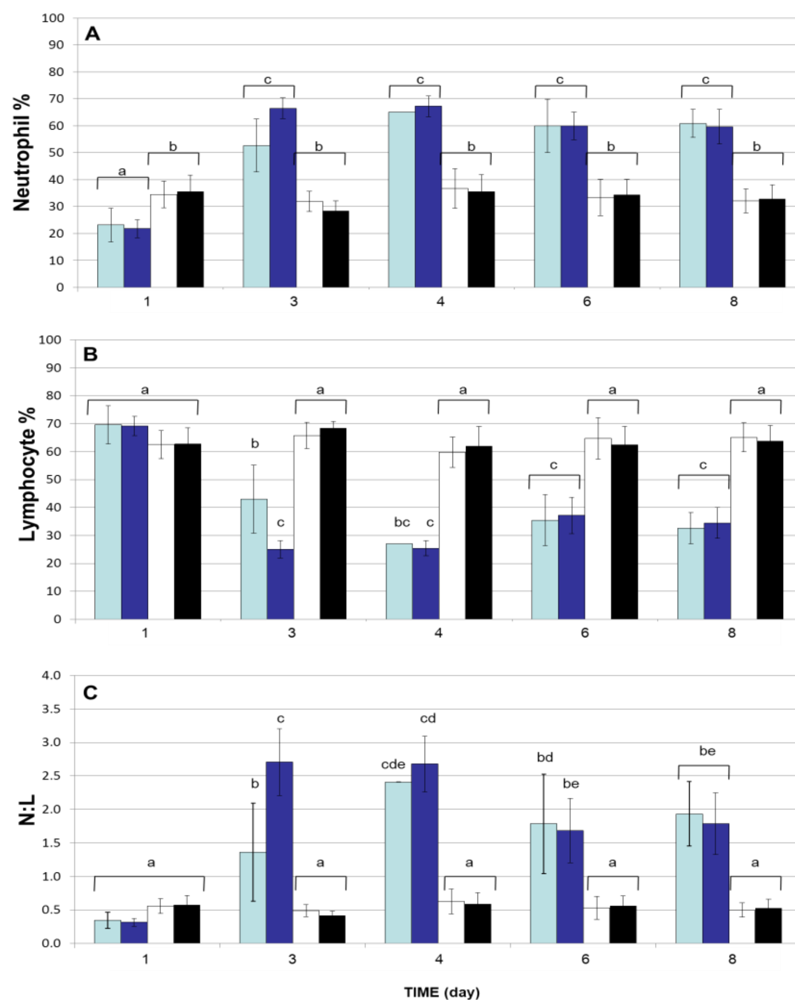


**Fig. 2.** Distribution of neutrophils and lymphocytes in mice recorded on day 1 (before infection with *S. aureus* Xen 36), on day 3 (24 h after infection), and on days 4, 6 and 8. Mice in groups 1 and 3 were infected with *S. aureus* Xen 36 on day 2. ■ = group 1 (infected with *S. aureus* Xen 36 and treated with nisin F), ■ = group 2 (infected with *S. aureus* Xen 36 and treated with inactive nisin F), □ = group 3 (not infected and treated with active nisin F), ■ = group 4 (not infected and treated with inactive nisin F). Data are presented as mean ± SD (n=6/group). Values without common post hoc letters (a-e) differ significantly ($p < 0.05$).

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On the same day, the relative neutrophil count in mice of group 2 increased from 22% to 67%, which represents an increase of 200% (Fig. 2A). On day 4, the relative neutrophil count of group 1 mice increased to a similar level (approximately 65%) as that recorded for group 2 mice (Fig. 2A). The neutrophil count of mice in both groups remained at more-or-less this level for the next four days. The neutrophil levels of mice in groups 3 and 4 basically remained the same throughout the trial (Fig. 2A).

One day after infection with *S. aureus* Xen 36, the relative lymphocyte count in group 1 mice decreased from 70% (day 1) to 43% (day 3), which represents a decrease of 39% (Fig. 2B). The lymphocyte count in group 2 mice decreased from 69% (day 1) to 25% (day 3), which represents a decrease of 64% (Fig. 2B). On day 4, the relative lymphocyte count of group 1 mice decreased to almost the same level (27%) as that recorded for group 2 mice (25%, Fig. 2B). The lymphocyte count of mice in both groups remained at this level for the next four days (Fig. 2B). No significant differences in lymphocyte counts were recorded in mice not infected with *S. aureus* Xen 36, irrespective of whether they received active nisin F (group 3) or inactive nisin F (group 4) (Fig. 2B).

The leukocyte response expressed as a ratio between neutrophil and lymphocyte counts (N:L) is shown in Fig. 2C. The N:L values of mice in group 2 was exactly double compared to that recorded for mice in group 1 on day 3, i.e. 24 h after infection with *S. aureus* Xen 36 (Fig. 2C). However, on day 4, the N:L values in both groups were similar and remained more-or-less equal over the following four days (Fig. 2C). No significant differences in N:L levels were recorded between mice not infected with *S. aureus* Xen 36, irrespective of whether they received active nisin F (group 3) or inactive nisin F (group 4; Fig. 2C).

Significant changes from baseline levels (day 1) were observed on day 3 and 4 for all cytokines tested (Fig 3). Most notably, the average levels of IL-6 in group 3 mice increased from 10.1 pg/mL on day 1 to 30.85 pg/mL within 24 h of nisin administration, and remained similar (32.6 pg/mL) on day 4. The IL-6 levels in group 4 mice showed a slow, but sustained, increase from 7.9 pg/mL to 19.8 pg/mL on day 3, and 19.1 pg/mL on day 4. In contrast, average levels of IL-10 decreased in mice of groups 3 and 4, but to a lesser extend in mice of group 4. The MCP-1 levels in group 3 decreased with 31.38 ± 40.9 %, whilst that in group 4 increased to 16.2 ± 16.7 % from baseline (day 3).

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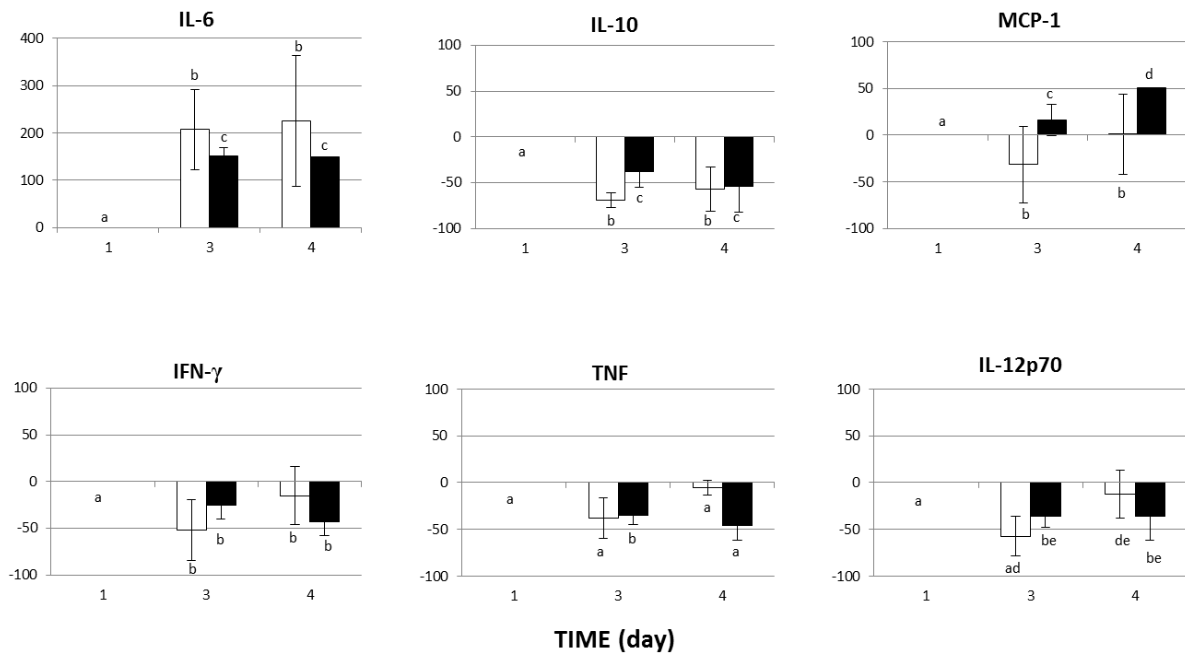


**Fig. 3.** Percentage change from baseline (day 1) in plasma levels of IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70 in mice on days 1, 3 and 4. Results are the average calculated for six mice in each group. □= not infected with *S. aureus* and treated with active nisin F (group 3), ■= not infected with *S. aureus* and treated with inactive nisin F (group 4). Values are displayed as means ± SD and values with uncommon letters (a-e) differ significantly ($p<0.05$).

## 4. Discussion

In a previous study [3], we have shown that a single dose of nisin F (640 AU) to the intraperitoneal cavity reduced the metabolic activity of *S. aureus* Xen 36 (1 x $10^8$ cfu) for 15 min, but that some cells survived the treatment and that their metabolic activity increased over the next 48 h. The conclusion drawn by Brand et al. [3] was that the short-lived antimicrobial effect of nisin F could be attributed to degradation of the peptide by proteolytic enzymes in the peritoneal cavity. With the design of the present study, we have argued that the continuous administration of nisin F to the peritoneal cavity may effectively repress the growth of *S. aureus* for a longer period. Although the metabolic activity of *S. aureus* Xen 36 declined for the first 3.5 h after treatment with nisin F, which is longer than the 15 min recorded by Brand et al. [3], the cells recovered and, after two days, their metabolic activity increased to a level equivalent to that before treatment (Fig. 1). Similar results were recorded when the infection was treated with inactive nisin F ($p > 0.05$) (Fig. 1). These results suggested that the initial growth inhibition of *S. aureus* Xen 36 was not due to the antimicrobial activity of nisin F, but rather an immune response triggered by the bacterial cells. This contradicts our previous findings [3], namely that nisin controlled the growth of *S. aureus* Xen 36 in the peritoneal cavity for as long as the

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peptide remained intact, in other words not degraded by proteolytic enzymes. Concluded from the results in Fig. 1, the immune system showed transient efficiency/activation, lasting only 3.5 hours.

The reason why nisin F did not repress the metabolic activity (and thus growth) of *S. aureus* Xen 36 is not clear. Nisin F could have been degraded by proteolytic enzymes, as speculated by Brand et al. [3]. A variety of nisin resistance mechanisms have been described, which includes degradation by proteolytic enzymes, the export of nisin by ATP-binding cassette (ABC)-transporters and the acquisition of resistance genes from other bacteria [8,9]. A nisin resistance (NSR) protease has been described for *Lactococcus lactis* [10] and a glutamate decarboxylase (gad)-mediated nisin resistance enzyme for *Listeria monocytogenes* [11]. We did, however, not test for the presence of these enzymes in *S. aureus* Xen 36. Another possibility is that *S. aureus* Xen 36 could have adhered to the epithelial cells to form a protective biofilm within the first 4 h before nisin F was injected into the peritoneal cavity. The biofilm could have lasted throughout the 6-day trial. It is also possible that nisin F had been encapsulated by mucus or other proteins in the peritoneal cavity, as suggested by Breukink and de Kruijff [8] and Rink et al. [9]. Another possibility could be that a higher dosage of nisin F is needed to overcome bacterial infection in the complex immunological environment in vivo.

Changes recorded in the neutrophil counts suggest that the immune response was activated during the first 24 h after infection with *S. aureus* Xen 36 (groups 1 and 2, day 3, Fig. 2A). The neutrophil counts of mice in groups 1 and 2 did not differ significantly (Fig. 2A), suggesting that active and inactive nisin F elicited the same immune response. The neutrophil counts in uninfected mice treated with active and inactive nisin F (groups 3 and 4, respectively), were much lower compared to results recorded for infected mice and remained at more-or-less the same level throughout the 7-day trial (Fig. 2A). This suggested that the activation of neutrophils (increase in counts) was not due to a response to nisin F, but rather due to a response to the infection caused by *S. aureus* Xen 36.

In contrast, lymphocytes seemed to exhibit effects in response to both bacterial infection and nisin F. Firstly, the decline in relative lymphocyte counts from pre-infection with *S. aureus* Xen 36 (day 1, Fig. 2B) to 24 h after infection on day 2 and treatment with nisin F (groups 1 and 2, day 3, Fig. 2B) may be due to apoptosis. This is usually observed in the case of severe infection [12]. A decrease in lymphocytes may limit the production of pro-inflammatory cytokines, which in turn leads to a decline in the defence against a pathogen. The lymphocyte counts were much higher and constant in mice that have not been infected with *S. aureus* Xen 36 (groups 3 and 4, Fig. 2B), and also similar to pre-infection counts in mice infected with *S. aureus* Xen 36 (groups 1 and 2, Fig. 2B). Treatment of non-infected mice with active and inactive nisin F had no effect on lymphocyte counts (groups 3 and 4, Fig. 2B). This proved that the major decrease in lymphocyte counts observed in infected mice was due to the presence of *S. aureus* Xen 36. Of interest, however, is that the infection-associated decline in lymphocyte counts were of smaller magnitude after treatment with active nisin F, compared to

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inactive nisin F. This may indicate that nisin F somehow limited the sepsis-induced apoptosis of lymphocytes.

The N:L ratio provides an accurate indication of bacterial infection, as observed with studies on bacteraemia [13]. Similar to the lymphocyte response, the N:L ratio calculated for mice that have not been infected with *S. aureus* Xen 36, but treated with active and inactive nisin F (groups 3 and 4, respectively) remained constant throughout the 7-day trial (Fig. 2C). However, infected animals had hugely increased N:L ratio, although this response was also attenuated (to the benefit of the host) in animals treated with active nisin F (Fig. 2C). The overall immune cell data suggests that not only did nisin F not have any detrimental effect on the immune system, but that it may have protective benefits to this system.

Statistically significant effects of the treatment was seen up to day 4 for cytokines IL-6, IL-10, MCP-1 and IFN-γ and up to day 3 for TNF and IL-12-p70 (Fig. 3). The drastic increase in IL-6 levels of uninfected mice treated with active nisin F (Fig. 3) suggests that the lantibiotic did initiate some degree of an immune response. The increase in IL-6 levels recorded in the presence of inactive nisin F was, however, not that profound (Fig. 3). The increase in IL-6, together with a decrease in IL-10 and TNF levels, suggests that nisin F had an immune boosting effect. Heat treatment breaks the disulphide bridges of nisin [14] and changes the tertiary structure. Concluded from the results shown in Fig. 3, nisin F elicits a stronger immune response in terms of IL-6 release when intact. However, the data presented here suggests that nisin F in its active form may also trigger an IL-6 response. This could be through protection of lymphocytes (allowing them to produce IL-6) in an infection situation, as seen in Fig. 2. Recently, nisin Z was shown to modulate the innate immune system through the stimulation of IL-6 production, together with various other cytokines including MCP-1, IL-8 and Gro-α in human peripheral blood mononuclear cells (PBMCs) ex vivo [15]. Through immunomodulatory stimulation, the peptide (200 µg/mouse) was also able to protect mice against *S. aureus* (2 x $10^8$ cfu/mouse) and *Salmonella typhimurium* (2 x $10^4$ cfu/mouse) infections.

In conclusion, nisin F, active or inactive, presented a mild immune boosting effect as indicated by IL-6, IL-10 and TNF levels. The overall immune response triggered by nisin F (administered at 2130 AU over 7 days) was, however, too small to suggest that an inappropriate immune reaction of clinical relevance would occur in the absence of infection. The fact that inactive nisin F has the same effect on the metabolic activity of *S. aureus* Xen 36 than active nisin F is a clear indication that nisin F has no ability to directly control *S. aureus* infection in the peritoneal cavity.

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# Chapter 6

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# Antimicrobial activity of nisin F, nisin A and Nisaplin® in the presence of serum and the effect of these lantibiotics on leukocyte functional capacity

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**Abstract**

Antimicrobial peptides from various origins, including human, animal, fungal and bacterial, have protective properties. Nisin, a lantibiotic, has been used as preservative in the food industry for decades. Recently, nisin has also been evaluated as an antibacterial agent in the control of infections. Here we evaluated the ex vivo functional capacity of rat peripheral blood mononuclear cells (PBMCs) through interleukin-1β (IL-1β) and IL-6 production in response to exogenous stimulus (LPS from *Escherichia coli*) and three nisin variants: nisin F, nisin A and a commercially purified form of nisin A, Nisaplin®. The effect of rat serum on the antimicrobial activity of the three variants was also analysed with a newly developed multi-well bioluminescence assay. The antimicrobial activity of nisin F and nisin A decreased within 10 h of exposure to 40 and 80% (v/v) serum. The activity of Nisaplin®, however, remained unchanged after 18 h in the presence of serum. Nisin F induced IL-1β secretion in PBMCs in a dose-dependent manner and at high concentration (5 x MIC) led to a decrease in the number of viable lymphocytes (cell counts decreased with 82%, without LPS, and 90% with LPS). No significant effect of treatment or dosage was observed for Nisin A or Nisaplin®. These results suggest that the *in sera* effect of nisin is influenced by the preparation of the peptide and that nisin-induced cytotoxicity is variant-dependent.

**Keywords:** nisin, PBMCs, cytotoxicity, serum, bioluminescence

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**Introduction**

Nisin is a lantibiotic, defined as small ribosomally synthesized, posttranslationaly modified class I bacteriocin [1]. Nisin A in purified form, commercially distributed as Nisaplin®, is used as food preservative. Nisin F is structurally similar to nisin A [1] and is also produced by *Lactococcus lactis* subsp. *lactis*. Nisin is active against *Staphylococcus aureus* and *Listeria monocytogenes* and has been studied for its application in the medical field [2-4].

Nisin F controlled *S. aureus* infection in mice for 15 min after one intraperitoneal (i.p.) administration [4]. Continuous administration of nisin F to the i.p. cavity failed to alleviate *S. aureus* infection, despite an increase in immune response elicited by the lantibiotic [5]. The reason for this is not entirely clear. Althought it is an important aspect of drug development, only a limited number of studies have been conducted on the immune response of bacteriocins. Cytotoxicity studies have been done on the bacteriocins pediocin PA-1 [6,7], divercin V41 [8], carnobacteriocins Cbn BM1 and B2 [9], the antimicrobial peptides P40 [10] and P34 [11,12], nisin A [13-16] and nisin Z [17]. The immunomodulatory effect of nisin A [18,19] and nisin Z [17] has been studied and variable results were obtained, which suggests that no general assumptions can be made regarding immune modulation.

It is also important to investigate the degree of protein binding (PB) during drug development [20]. PB has two main negative effects on *in vivo* drug efficiency. Firstly, only the unbound fraction penetrates the site of infection [21] and is cleared from the body [22]. Secondly, PB can have a detrimental effect on the antimicrobial activity of a drug (20,23-25). Various methods have been used to determine the degree of PB. Parameters that should always be taken into consideration are growth media, concentration of the antimicrobial agent, temperature, pH, electrolytes and supplements [26]. Current PB assays involve either the study of bacterial growth (CFU/ml) at different time points that is laborious, or minimum inhibitory concentration (MIC) testing that can be less accurate [26]. The method we have developed allows the simultaneous reading of 96 samples over an extended period. According to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [27], a drug should be able to exert its antimicrobial activity for up to 6 h. In the present study the effect of nisin F, nisin A and Nisaplin® on the growth of the target strain *S. aureus* Xen 36 (expressed as bioluminescence and optical density) was recorded over 18 h.

In this study, the immunomodulatory effect of partially purified nisin F was compared to partially purified nisin A and Nisaplin®, a purified form of nisin A. The ability of peripheral blood mononuclear cells (PBMCs) to produce interleukin-1β (IL-β) and IL-6, two pro-inflammatory cytokines, in the presence of the bacteriocins as well as an additional external stimulus, lipopolysaccharides (LPS) from *Escherichia coli,* was investigated. Lastly, the effect of different concentrations of rat serum on the antimicrobial effect of the bacteriocins was determined with bioluminescence imaging (BLI) using the

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In Vivo Imaging System (IVIS® 100; Caliper Life Sciences, Hopkinton, MA) in a newly developed assay.

**Materials and methods**

**Preparation of nisin F, nisin A and Nisaplin® and MIC determinations**

Nisin F was produced by growing *L. lactis* subsp. *lactis* F10 [1] in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) for 18 h at 30ºC. Nisin A was produced by growing *L. lactis* subsp. *lactis* QU51 (obtained from Prof K. Sonomoto, Kyushu University, Japan) under the same conditions. The two lantibiotics were concentrated from the culture-free supernatant with 80% saturated ammonium sulphate [28]. Nisaplin®, the commercial form of nisin A, was purchased from Aplin & Barret (Dorset, England), dissolved in sterile distilled water to a final concentration of 0.2 mg/ml and centrifuged (3000 x g, 10 min, 4ºC) to remove milk solids and stabilizers. The three suspensions were dialysed against sterile distilled water, concentrated by freeze-drying and stored at -80 ºC.

Freeze-dried peptides were resuspended in sterile distilled water and the antimicrobial activity of nisin A, nisin F and Nisaplin® determined against *S. aureus* strain Xen 36 (Caliper Life Sciences, Hopkinton, MA). Activity was expressed in arbitrary units per ml (AU/ml). The MIC required to inhibit the growth of *S. aureus* Xen 36 was determined using a 96-well microtitre plate. Each well was filled with 100 µl Mueller Hinton (MH) broth (Sigma-Aldrich, St. Louis, Mo., USA) and seeded with 1 x $10^6$ CFU/ml *S. aureus* Xen 36. Nisin F, nisin A and Nisaplin® were serially diluted (2-fold) and 100 µl added to each well. The MIC value was expressed as the lowest concentration (AU/ml) of the lantibiotic required to inhibit the growth of 5 x $10^5$ CFU/ml *S. aureus* Xen 36, as suggested by the NCCLS guidelines [24]. Total protein concentrations (mg/ml) were determined with the colorimetric Pierce® Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Scientific, Waltham, MA, USA).

The purity of nisin F, nisin A and Nisaplin® (125 AU each) was determined by tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Schägger [29], but modified by using 48% acrylamide/ 1.5% bis-acrylamide for the stacking gel, and 46.5% acrylamide/ 1.5% bis-acrylamide for the separating gel. The acrylamide/ bis-acrylamide, gel buffer, 50% glycerol and water were used at a ratio of 1:1:1:0.78 for the SDS separating gel and at 1:3:0:8 for the stacking gel. The gels were polymerized with 10% ammonium persulphate (APS) and TEMED (10:1). The bacteriocins were each mixed with 2x sample buffer, consisting of 0.125 M Tris (pH 6.8), 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue, at a ratio of 1:1 and heated at 95°C for 5 min. The protein samples and a low-range rainbow marker, RPN755E (Amersham International, United Kingdom), were separated at 180V for 4 h. Protein bands were visualised after silver staining, as described by Ilmén *et al*. [30].

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**Effect of nisin F, nisin A and Nisaplin® on leukocyte counts and functional capacity**

Whole blood from healthy male Wistar rats was collected into BD Vacutainer® $K_2$EDTA tubes (BD Diagnostics, Franklin Lakes, NJ, USA), pooled and kept at room temperature. Full blood counts of pooled blood were measured using a Cell-Dyn hematology analyser (Cell-Dyn 3700 CS, Abbott Diagnostics, Santa Clara, California, USA) and HEMO, VetSoft Veterinary Software (Davis, CA, USA). The wells in Multiwell (BD Falcon™) tissue culture plates (BD Diagnostics) were each filled with 200 µl Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St Louis, MO, USA). Nisin F, A and Nisaplin®, each dissolved in 50 µl RPMI medium, was added to wells to correspond to final concentrations of 0.5, 1.0, 2.5 and 5.0 x MIC. Corresponding volumes of RPMI medium were added to control wells. To one set of tissue culture plates, 50 µl RPMI containing lipopolysaccharide (LPS, final concentration 1.5 µg/ml) from *E. coli* 0111:B4 (Sigma-Aldrich) was added per well to stimulate the production of cytokines. The control set received 50 µl RPMI medium without LPS. All reactions were performed in triplicate. Whole blood (200 µl) was added to each well to a final volume of 500 µl and the plates were incubated at 37°C (98% humidity, 5% $CO_2$) for 4 h. The content of each well was centrifuged (1500 x g, 10 min, 25ºC) and the supernatants collected and stored at -80°C. Volumes removed from the wells were replaced with equal volumes of sterile phosphate buffered saline (PBS). The blood cells were resuspended and cell counts determined as described before. The PBMC values were calculated as the sum of the monocyte and lymphocyte counts in each sample. Initial and final values (before and after incubation) were analysed to assess the net effect of changes in cell death and/or proliferation on viable PBMC count. Levels of IL-6 and IL-1β (produced by PBMCs during incubation) in the supernatants were determined using Platinum Enzyme-linked Immunosorbent (ELISA) kits (eBioscience Inc., SanDiego, USA).

**Effect of serum on the antimicrobial activity of nisin A, nisin F and Nisaplin®**

Blood was collected from healthy male Wistar rats in BD Vacutainer® Plus Plastic Serum tubes (BD Diagnostics), pooled and centrifuged at 1500 x g (10 min, 25ºC). Media with different serum concentrations were prepared as follows: To obtain a 40% serum content, two parts of serum was added to one part 5 x MH broth (Sigma-Aldrich) and two parts sterile saline (0.85% NaCl, w/v). Medium containing 80% serum was prepared by adding four parts of serum to one part of 5 x MH broth. Medium without serum was prepared by adding one part of 5 x MH broth to four parts sterile saline. Wells of a microtitre plate were filled with the serum-containing media, respectively. Nisin A, nisin F and Nisaplin®, each suspended in sterile distilled water, were added to media to yield final levels of 1.0 x MIC, 2.5 x MIC and 5.0 x MIC, respectively, in total volumes of 50 µl per well. Sterile distilled water was used as control for the lantibiotics. *Staphylococcus aureus* Xen 36, cultured at 37°C in 1 x MH broth, supplemented with 200 µg/ml kanamycin to maintain selective pressure on the plasmid containing the *luxABCDE* operon, were harvested (11300 x g, 10 min, 4°C), resuspended into MH broth and 50 µl added to each well to yield a final cell number of $3.8 \times 10^5$ CFU/ml. The plates

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were incubated in the IVIS at 37°C for 18 h and bioluminescent images were recorded every 30 min (10 sec exposure) for 18 h. The entire size of each well was selected as one ROI and analysed with Living Image® software (Caliper Life Sciences). Optical density ($A_{600nm}$) readings were recorded for each well immediately after inoculation with *S. aureus* Xen 36 and after 18 h of incubation.

### Statistical analyses

IL-1β and IL-6 production were analysed separately for nisin A, nisin F and Nisaplin®, using one-way ANOVA with Bonferroni post hoc testing (Statistica software v.10, StatSoft Inc, OK, USA). Serum data were analysed with the PROC GLIMMIX mixed model with time as the repeated measure (SAS software v. 9.3, SAS Institute Inc., USA). P-values < 0.05 were considered as significant.

## Results

### MIC levels of nisin A, nisin F and Nisaplin®

The lantibiotic preparations showed different degrees of purity, as observed with trycine SDS-PAGE (not shown). The specific activity of nisin A, nisin F and Nisaplin® (expressed per total protein content) was 766.1 AU/mg, 356.7 AU/mg and 8888.9 AU/mg, respectively, as determined against *S. aureus* Xen 36 grown in MH broth. The MIC values against *S. aureus* Xen 36, were 147.7 µg/ml, 280.0 µg/ml and 11.3 µg/ml for nisin A, nisin F and Nisaplin®, respectively.

### Changes in leukocyte counts and cytokine secretion

The total white blood cell count of pooled blood was 11.7 x $10^6$/ml. The differential cell counts were also within expected normal range, i.e. 9.5% neutrophils, 84.0% lymphocytes, 3.0% monocytes, 1.2% eosinophils and 2.3% basophils. The initial PBMC (sum total of monocyte and lymphocyte) count in each well was 5.3 x$10^6$/ml. The PBMC counts remained unchanged in the presence of 0.5 x, 1.0 x, 2.5 x and 5.0 x MIC levels of nisin A and Nisaplin®, irrespective of the absence or presence of LPS (not shown). Nisin F at 0.5 x, 1.0 x and 2.5 x MIC had no effect on PBMC levels (Fig. 1). However, the highest dosage of nisin F (5 x MIC) caused a significant decrease in PBMC counts (Fig. 1). At 5 x MIC, in the absence of LPS, PBMC counts decreased by 70% (lymphocytes by 82%). At the same nisin dosage, but in the presence of LPS, the decreases in cell counts were substantially greater (PBMC decreased by 86.5% and lymphocytes by 90%).

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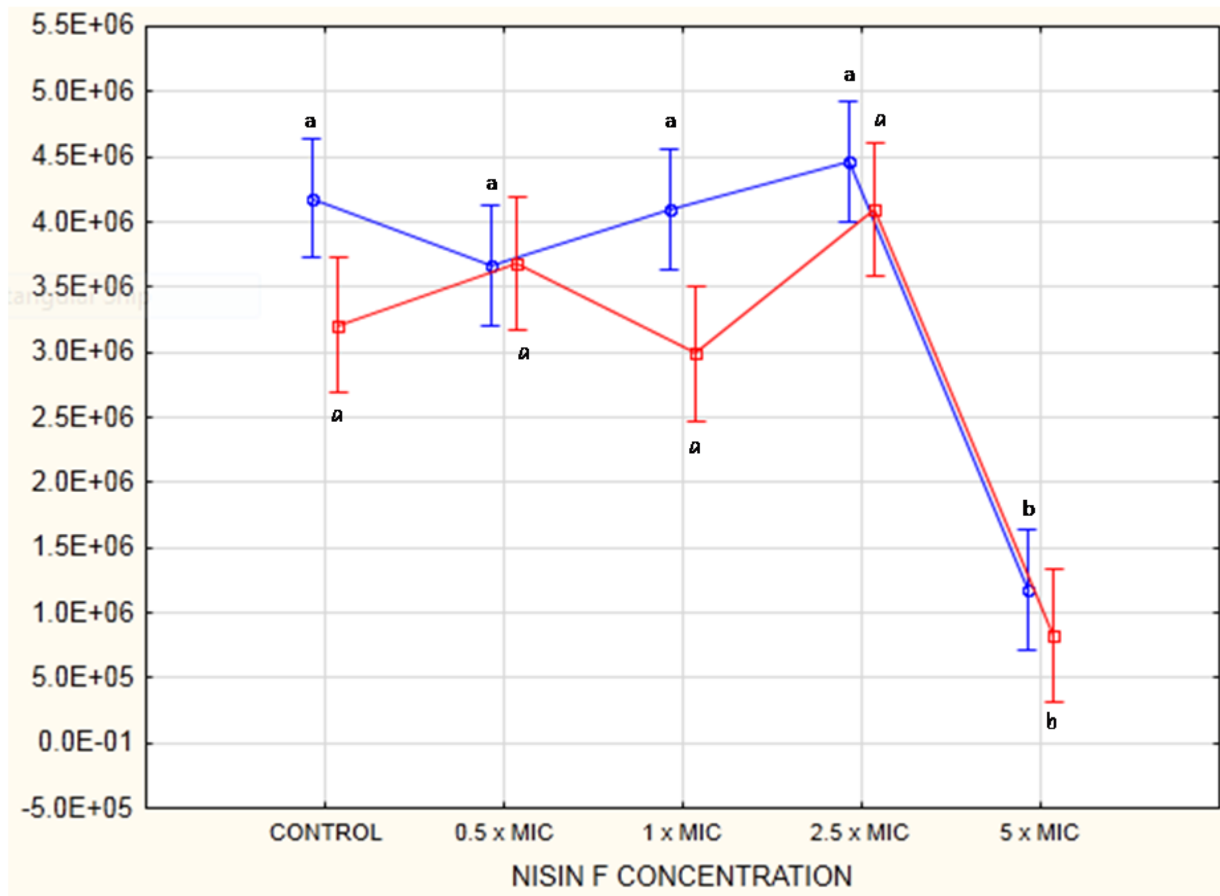


**Figure 1.** Peripheral blood mononuclear cell (PBMC) counts after incubation with different concentrations of nisin F in the presence and absence of *E. coli* LPS. -○- = spontaneous PBMCs/ml; -□- = stimulated PBMCs/ml. Data are presented as means ± SD of PBMCs/ml (n=3). Values without common letters (a-b) differ significantly ($p < 0.001$).

No significant differences in IL-1β and IL-6 levels were recorded in the presence of 0.5, 1.0, or 2.5 x MIC nisin A, nisin F or Nisaplin® when compared to the control (Fig. 2). Nisin F showed a dose-dependent pattern of IL-1β production. Nisin F at 5.0 x MIC resulted in a 50.1% increase of spontaneous IL-1β (Fig. 2A), while no significant difference in stimulated IL-1 β or any IL-6 levels was seen (Fig. 2B). The averages for stimulated production of IL-1 β in response to nisin A were consistently higher than the spontaneous production recorded for all MIC levels, although not statistically significantly (Fig. 1A). A similar consistent effect was not observed for nisin F or Nisaplin®.

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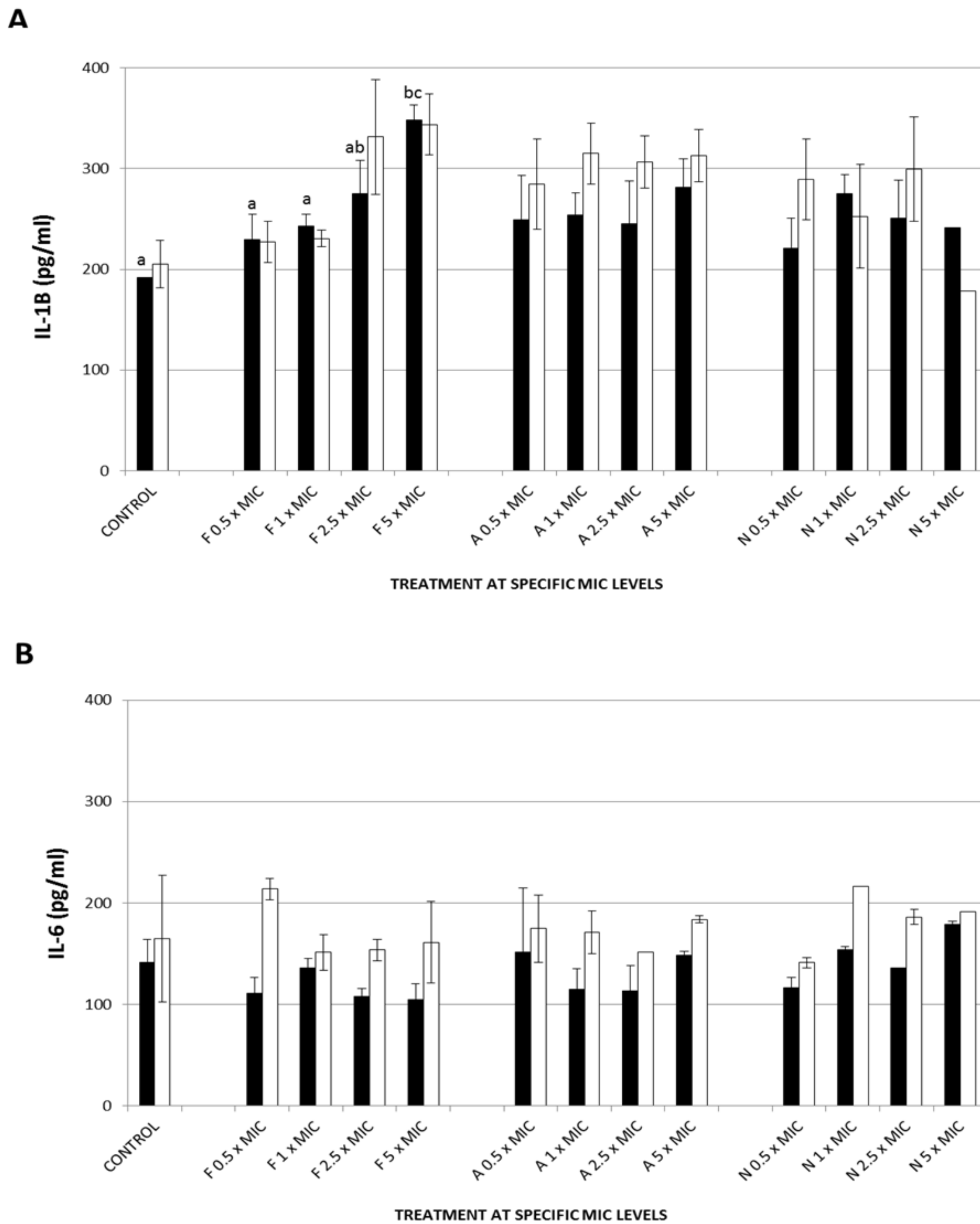


**Figure 2.** Spontaneous (■) and stimulated (□) production of (A) IL-1β and (B) IL-6 by peripheral blood mononuclear cells (PBMCs) after 4 h of incubation in the presence of 0.5, 1, 2.5 and 5 x MIC nisin F, nisin A and Nisaplin® . IL-1β and IL-6 production was stimulated by the addition of 1.5 µg/ml lipopolysaccharide (LPS) from *E. coli*. Data are presented as means ± SD (n=3). Values with different letters (a-c) differ significantly ($p < 0.05$).

**The effect of serum on the antimicrobial activity of bacteriocins**

Ten hours of incubation in the presence of 40% (v/v) serum had no effect on the antimicrobial activity of nisin F at MIC values of 1.0, 2.5 and 5.0 (Fig. 3B). Similar results were observed with nisin F incubated in the presence of 80% serum, except that the antimicrobial activity of 1.0 x MIC nisin F

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decreased rapidly after 4 h of incubation ($p < 0.05$; Fig. 3C). With a further 8 h of incubation in the presence of 40% serum, the antimicrobial activity of 1.0 x MIC nisin F declined whereas the activity of 2.5 x and 5.0 x MIC nisin F remained unchanged (Fig. 3B insert). A further 8 h of incubation in the presence of 80% serum resulted in a decline of 2.5 x MIC nisin F activity, whereas the activity of 5.0 x MIC nisin F remained unaffected ($p < 0.05$; insert, Fig. 3C).

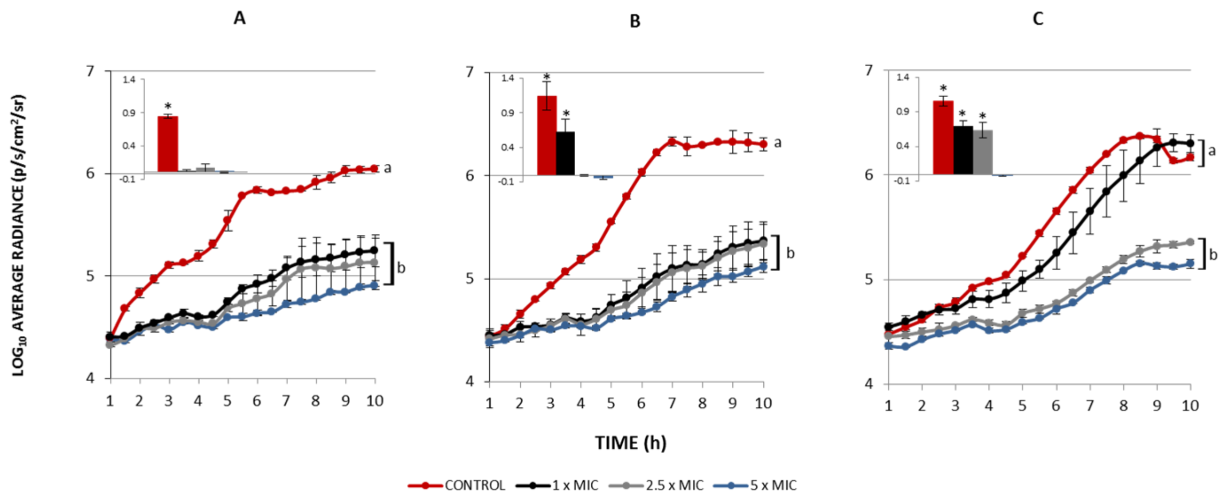


**Figure 3**. Effect of serum on the antimicrobial activity of nisin F. Changes in the metabolic activity (growth) of *S. aureus* Xen 36 were observed by bioluminescence. A: no serum, B: 40% (v/v) serum, C: 80% (v/v) serum. Data are presented as means ± SD (n=3) and values without common letters (a-b) differ significantly ($p < 0.05$). The insert in each graph represents the optical density readings ($A_{600nm}$) recorded 18 h after incubation. Significantly different values ($p < 0.05$) are indicated by *

The activity of 1.0 x MIC nisin A was completely inhibited after 10 h of incubation in the presence of 40% (v/v) and 80% (v/v) serum and remained inactivated for a further 8 h (Fig. 4 B and C, respectively). Higher concentrations of nisin A (2.5 x and 5.0 x MIC) were inactivated after 10 h of incubation in the presence of 40 % serum (Fig. 4E and H). Incubation of 2.5 x and 5.0 x MIC nisin A in the presence of 80% (v/v) completely inactivated 2.5 x MIC, while 5.0 x MIC nisin A was slightly more resistant (Fig 4 F and I). These results were confirmed by optical density readings recorded for *S. aureus* Xen 36 (Fig. 5A). The antimicrobial activity of 1.0 x, 2.5 x and 5.0 x MIC Nisaplin® was not inhibited by 40% serum (Fig. 4 B, E and H). A slight inhibition of 5 x MIC Nisaplin® was observed in the presence of 80% (v/v) serum ($p < 0.05$, Fig. 4 I). According to optical density readings, the growth of *S. aureus* was significantly inhibited for 18 h by all MIC dosages of Nisaplin®, irrespective of serum concentration (Fig 5B).

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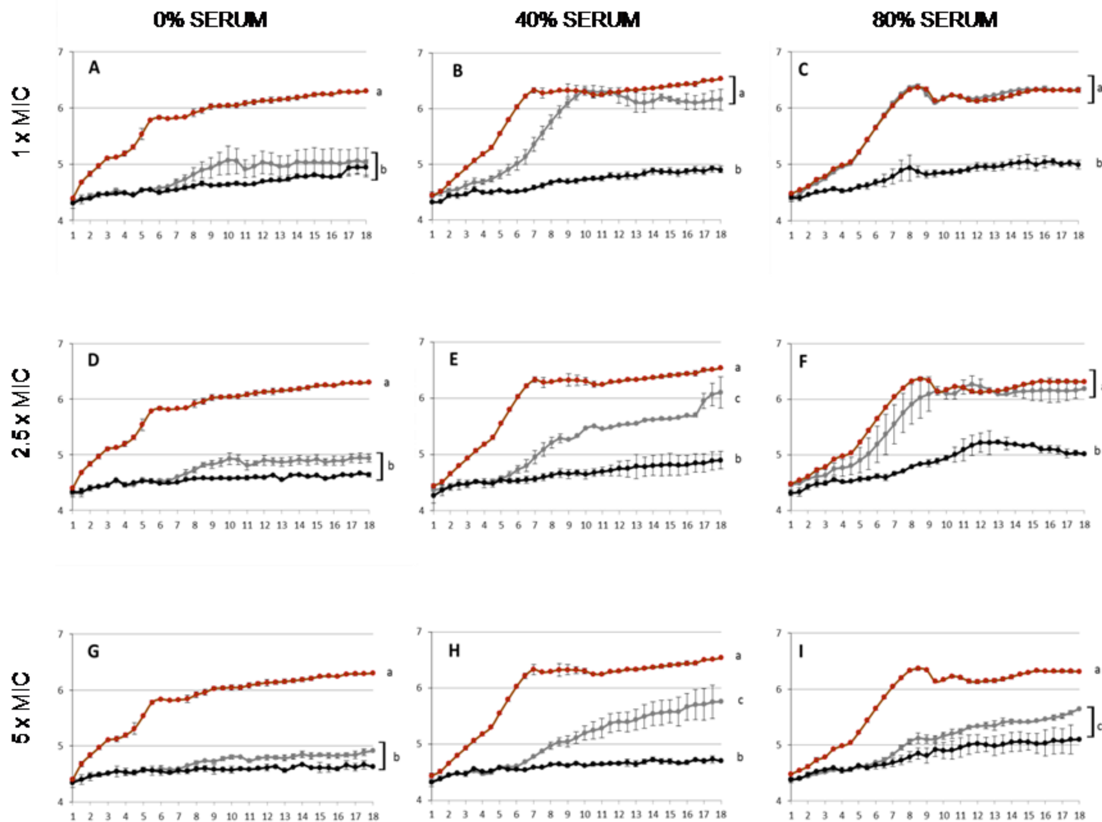


**Figure 4.** Bioluminescence of *S. aureus* Xen 36 incubated with different concentrations of serum (A, D, G: 0%; B, E, H: 40% and C, F, I: 80%) and antimicrobial proteins (A-C: 1 x MIC; D-F: 2.5 x MIC; G-I: 5 x MIC). $Log_{10}$ values of average radiance (p/s/cm$^2$/sr) are shown as mean ± SD (n = 3) and curves without common letters (a-c) differ significantly ($p < 0.05$). Negative control (●), nisin A (●) and Nisaplin® (●).

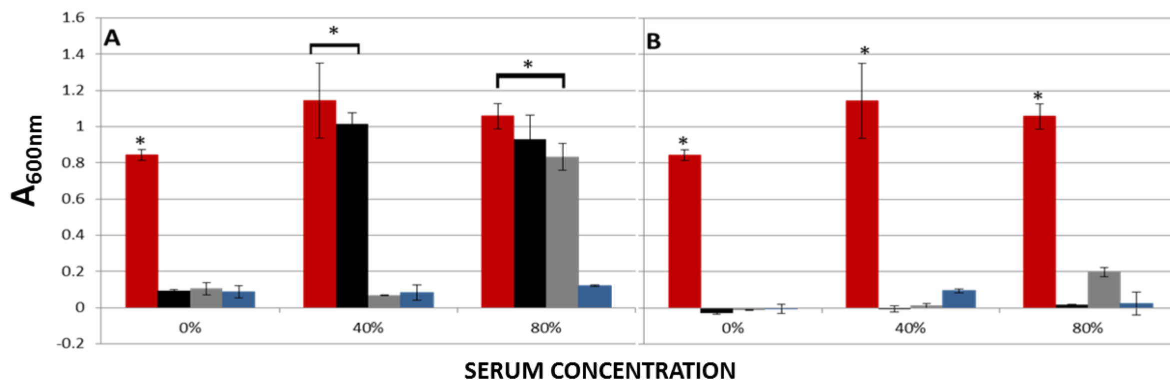


**Figure 5.** Optical density ($A_{600nm}$) of different concentrations control ■, 1 x MIC ■, 2.5 x MIC ■ and 5 x MIC ■ of nisin A (A) and Nisaplin® (B) incubated with *S. aureus* Xen 36 and different concentrations of rat serum (0, 40 and 80%). Values were obtained after 18 h of incubation and are presented as means ± SD (n = 3). Asterisks (*) indicate values with significant differences ($p < 0.05$).

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When comparing the three nisin variants at 1 x MIC in media containing 80% (v/v) serum, clear differences in antimicrobial activity can be observed (Fig. 6). The activity of nisin F and nisin A was totally inhibited ($p < 0.05$), while Nisaplin® (commercially purified) was not affected. Optical density ($A_{600}$) data obtained after 18 h confirmed results obtained by BLI (insert, Fig. 6).

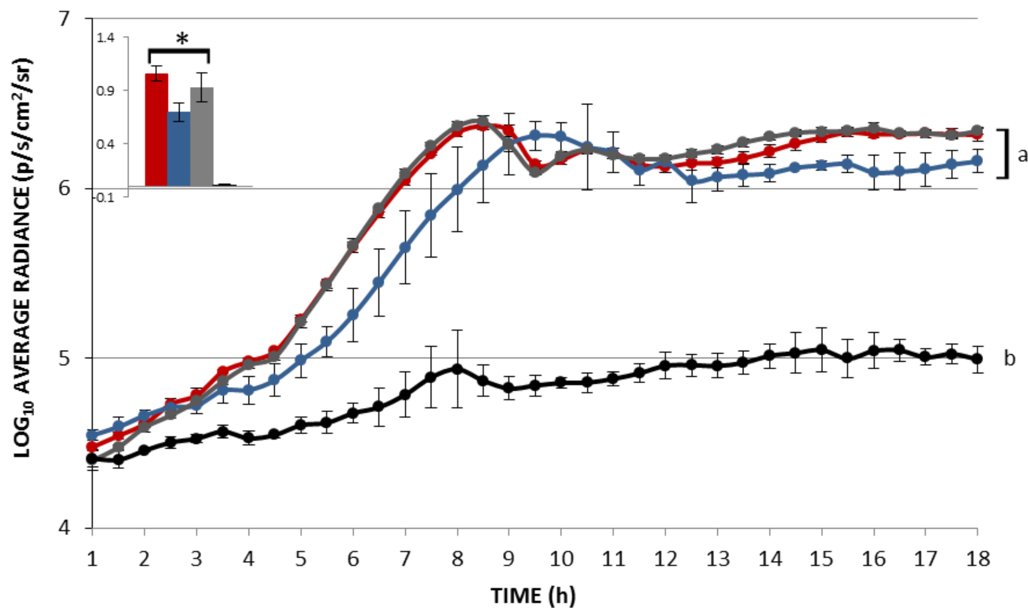


**Figure 6.** Bioluminescent values of nisin F (●), nisin A (●), Nisaplin® (●) and water as negative control (●) during incubation with *S. aureus* Xen36 over a period of 18 h. The proteins were added to a final concentration of 1 x MIC and media contained 80% serum. Curves without common letters (a-b) differ significantly ($p < 0.05$). $A_{600}$ values of the same samples 18 h after incubation are shown in the insert graph. Samples with significantly the same values ($p < 0.05$) are indicated with an asterisk (*).

## Discussion

Of the three nisin preparations, Nisaplin® was the purest. This is also evident from the low concentration Nisaplin® required, compared to the other preparations, to yield the same antimicrobial activity.

Only the highest dosage of nisin F was cytotoxic to PBMCs (Fig. 1). The decrease in cell counts was mainly due to lymphocyte death. In a study by Bedge *et al*. [16], cytotoxicity towards lymphocytes and neutrophils was observed after exposure of human whole blood to nisin [16]. When continuously administered to uninfected mice, nisin F did not cause any significant changes in relative neutrophil or lymphocyte counts [5]. However, the lantibiotic, in its active form, partly protected *S. aureus* infected mice against lymphocyte death. In another study, De Pablo *et al*. [34] observed an increase in CD4

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and CD8 T-lymphocytes and a decrease in B-lymphocytes in mice orally administered with Nisaplin® in their diet for 30 and 75 days. The effects were, however, neutralized over time with long-term administration of Nisaplin® (100 days). The phagocytic activity of peritoneal cells also increased after long-term administration [31].

Based on lactate dehydrogenase (LDH) secretion, purified nisin Z as high as 200 µg/ml did not cause toxicity (through membrane permeabilisation) to human PBMCs [17]. The same concentration of gallidermin, on the other hand, caused roughly 40% cytotoxicity. The authors did not assess leukocyte viability. Nisin showed an immunostimulatory effect through head kidney macrophages after i.p. administration to fish [18]. The purity of the nisin used in previous studies are however not clear. An exception is an *in vivo* toxicity study done by Gupta *et al.* [15] where RP-HPLC purified nisin (free of milk solids and high salt concentration usually found in commercial versions of nisin) was applied as a vaginal microbicide in rats. No toxic effect was observed. In the case of toxicity, the host will often break down foreign peptides into nontoxic amino acids that can be metabolized [32]. Nisin [33], pediocin [34] and peptide AS-48 [35] also showed an immunogenic effect in antibody studies.

Recently, nisin Z was proposed to be similar in action as the natural host-defense peptide (HDP) and synthetic innate defense peptide (IDR) [17]. Nisin was able to enhance innate immunity both *ex vivo* in human PBMCs as well as *in vivo* in mice. Nisin Z (50 µg/ml), and not Pep5 or gallidermin, caused human PBMCs to produce the cytokines monocyte chemoattractant protein-1 (MCP-1), gro-α and IL-8 in a dose-dependent manner after 24 h incubation. In the prescence of 2 ng/ml LPS from *Pseudomonas aeruginosa*, 50 µg/ml nisin Z caused a significant reduction in production of the pro-inflammatory cytokine, TNF-α, by PBMCs after 24 h incubation. More significantly it was determined that stimulation of IL-6 production together with various pathways modulates the immune system. When administrated 1 h post-infection, these immunomodulatory properties offered partial protection against infection by Gram-negative organisms such as *E. coli* and *Salmonella thyphimurium* in mice, even though nisin Z is not active against these organisms. In another study, the bacteriocin CBT-SL5 also suppressed IL-8 production in human keratinocytes exposed to *Propionibacterium acnes* [36]. In our case, cell death was preceded by a burst of IL-1β production (Fig. 2), which is also a pro-inflammatory cytokine. This might suggest that nisin F has a different mechanism of action, compared to nisin Z, although conditions differed greatly from this study.

The inhibitory effect serum has on the antimicrobial activity of nisin F and nisin A (Figs 3-6) is contradicting to the study done by Ghobrial *et al*. [37] where the MIC of the lantibiotic MU114 against *S. aureus* decreased from 6.4 µg/ml to 1.6 µg/ml in media containing 25% and 50% human serum. Purified nisin A (Nisaplin®) was less affected by serum. MIC dosages are usually determined based on visible changes recorded in bacterial growth. Because the IVIS used in this study is based on bioluminescence (metabolic activity), it is more accurate. Even though bacterial cells might not be

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visible with the eye, metabolic activity will produce a bioluminescent signal that can be detected with the IVIS. This might explain the slight increase in bioluminescence observed at the end of incubation (Fig. 3, 4, 6) even though all dosages were equal to or higher than 1 x MIC. These facts further illustrate the sensitivity of the assay developed here. Binding and inactivation of nisin F by serum could limit the *in vivo* therapeutic application of the lantibiotic. Future studies could include development of ways to overcome this problem.

From bioluminescent results we can conclude that preparation (as seen between Nisaplin® and the semi-purified nisin A and F) plays a significant role in PB in serum (Fig. 6). However, cytotoxicity was more dependent on the type of nisin (Fig. 1). The semi-purified form of nisin F, which was used in all our previous studies [4,5,38], showed similar SDS-PAGE separation profiles and antimicrobial activity in rat serum (Fig. 6) as nisin A. Thus, further purification of nisin F might decrease PB and antigenicity of the lantibiotic, allowing more effective *in vivo* application.

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# Chapter 7

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# General Discussion and Conclusions

The global pharmaceutical market experienced rapid growth over the last ten years and was estimated at €614,583 million ($855,500 million) in 2011 (EFPIA 2012). Although the annual growth rate of research and development (R&D) in leading countries like Europe and the United States of America suffered a decrease of 81.8 and 75.9%, respectively, over the last ten years (EFPIA), South Africa has been ranked as one of seventeen "Pharmerging" countries with high-growth pharmaceutical markets (IMS 2011). Unfortunately this economic asset also comes with several drawbacks. The increase in novel antibiotics leads to over-subscription of antibiotics by medical practitioners and that leads to increased antibiotic resistant pathogens, which in turn lead to an even greater need for novel antibiotics - in essence, the start of a vicious cycle.

Antimicrobial peptides like nisin F have various attributes rendering them attractive for use as pharmaceuticals. Because they are small, they can reach infected areas more easily and increase possibilities for drug engineering. They also show high specificity and potency. Peptide pharmaceuticals are widely used in fields like oncology, diagnostics, diabetes, obesity, arthritis, cardiovascular disease and central nervous system disorders (Ayoub and Scheidegger 2006). One major disadvantage of such peptides is the short half-life and rapid degradation by proteolytic enzymes like aminopeptidases and carboxypeptidases, followed by endopeptidases cleaving to specific sites within the peptide (Rink et al. 2010).

From *in vitro* work in this study, it was determined that the lantibiotic nisin F has the best activity against *S. aureus* Xen 36, compared to other pathogens in our bioluminescent culture collection. After exploring different *in vivo* routes of infection (i.g., i.p., i.m., i.v.), we concluded that intraperitoneal administration was the most effective in establishing systemic infection and treatment (Chapter 3, Brand et al. 2011). Dosages for infection and treatment applied in subsequent studies were also optimized. C57BL/6 mice were used to determine the *in vivo* antimicrobial effect of nisin F against *S. aureus* Xen 36 infection. Results showed that nisin F suppressed *S. aureus* growth in the peritoneal cavity for at least 15 min (Chapter 4, Brand et al. 2010). In addition, no abnormalities were observed in histological analysis of the hearts, lungs, livers, spleens, kidneys and intestinal organs of the mice.

The *in vivo* stability of nisin F was further examined in BALB/c mice intraperitoneally infected with *S. aureus* Xen36 (Chapter 5). Four hours after infection, mice were surgically implanted with Alzet osmotic minipumps filled with active and inactive nisin F. Nisin F-coated polyethylene tubing was connected to each minipump and inserted through an incision in the peritoneum of the mouse to ensure the delivery of nisin F inside the peritoneal cavity and its uptake into the bloodstream. Infection was monitored with bioluminescent imaging (BLI) for seven days, and no significant differences could be recorded between groups treated with active and inactive nisin F. Differential blood counts

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(specifically neutrophils and lymphocytes) suggest that nisin F might significantly enhance the inflammatory response in the presence of a pathogen. Similarly, but at much lower magnitude, nisin F also caused an increase in IL-6 and decrease in IL-10 levels 24 h after initiation of treatment in the absence of an added pathogen. Taken together, these data suggest that while the lantibiotic itself does not cause an inappropriate immune response or intolerance when administered in the absence of bacterial infection, it may indeed have immune boosting functions in the presence of bacterial infection.

The interpretation of the *in vivo* cytokine response (Chapter 5) is further substantiated by the results of the *ex vivo* assay in Chapter 6. PBMCs incubated with nisin F produced IL-1β in a dose-dependent way. Cytotoxicity to leukocytes (especially lymphocytes) observed after exposure to the highest concentration (5 x MIC) of nisin F could be overcome through further purification of the peptide. Cells cultured with nisin F and an added pathogen, *E. coli* LPS, were still able to elicit an immune response similar to controls (without nisin F).

A clear difference was observed between the purity of semi-purified extracts of nisin F and nisin A when compared to Nisaplin®, a commercial form of nisin A (Chapter 6). These results were confirmed when the three above-mentioned proteins were incubated with *S. aureus* Xen 36 and media containing three different serum concentrations (0%, 40% and 80%). The metabolic activity of *S. aureus* in the presence of serum and different dosages of the proteins (1 x MIC, 2.5 x MIC and 5 x MIC) was measured with a bioluminescent assay. The results were confirmed with optical density measurements. Serum tolerance was significantly less in semi-purified nisin F and nisin A, compared to Nisaplin®. Because they are foreign to the animal/patient's body, peptides are often degraded or lysed (Rink et al. 2010). The combination of bacteriocins and other treatments like pulsed electric fields, high hydrostatic pressure and organic acids, enhances potency of the bacteriocins and treatment of Gram-negative bacteria (Taylor et al. 1985; Mazzotta and Montville 1997; De Martinis et al.1997). Nanotechnology can be applied to protect peptides or antibiotics inside nanoparticles or polymer coatings that will enable slow release of the compounds (Guillaume et al. 2012) FDA- approved water-soluble polymers can be used and manipulated for sustained release.

This was the first time the *in vivo* antimicrobial effect and stability of nisin F was monitored in the bloodstream. After determining a model for systemic infection and treatment, we established that nisin F controlled *S. aureus* growth in the peritoneal cavity for at least 15 min after a single administration. However, after administration of the peptide for seven days, active nisin F had the same effect on *S. aureus* growth as inactive nisin F, according to bioluminescence. Blood counts of infected mice showed that active nisin F exerted protection against lymphocyte death. The immune boosting effect of nisin F in terms of IL-6 and IL-10 when administered in the absence of infection shows that the

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peptide might act in an immunomodulatory way to aid its host against infection. Future studies could involve further purification of the peptide, followed by novel application methods.

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