Prevention and treatment of mastitis in dairy cows with bacteriocins produced by *Enterococcus faecalis*

by

Elton Davidse

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Supervisor: Prof. L.M.T. Dicks

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DECLARATION

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

Elton Davidse

Date:

BIOGRAPHICAL SKETCH

Elton Davidse was born on the 19th of October 1975 in Beaufort West. He matriculated from Bastiaanse Senior Secondary School in 1993 and thereafter enrolled at the University of Stellenbosch. In 1997 he obtained his B.Sc. degree with Microbiology, Biochemistry and Psychology as majors. In 1998 he obtained his B.Sc. (Hons.) in Microbiology.

PREFACE

The literature review includes an update on bovine mastitis, with special reference to infections caused by *Staphylococcus aureus*. Lactic acid bacteria (LAB), the bacteriocins they produce and their application in mastitis control are also discussed.

The paper, “Prevention and treatment of *Staphylococcus aureus* mastitis in dairy cows by using the cyclic peptide AS-48”, has been written according to the style of J. Dairy Research.

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SUMMARY

The effect of the bacteriocin-like peptide AS-48, produced by *Enterococcus faecalis* FAIRE 92, was tested against a mastitis isolate of *Staphylococcus aureus* in an *in vivo* and *in vitro* study. During initial tests peptide AS-48 showed no significant activity towards *S. aureus*, even with a ten-fold concentrated cell-free supernatant. Activity was obtained only after purification with Triton X-114 phase partitioning, followed by cation exchange chromatography. Titers for the purified peptide varied between 3200 and 12800 AU/ml. The purified peptide also exhibited activity towards *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, but not against *Escherichia coli*.

The size of peptide AS-48 was determined at 7150 Da, based on electronspray mass spectrometry and SDS-PAGE. Complete inhibition of cell growth was obtained by adding 1ml of the purified peptide (3200 AU/ml) to 100 ml of cells of *S. aureus* in the lag growth phase. When the same concentration of peptide AS-48 was added to a culture of *S. aureus* in mid-exponential growth, a slight decrease in viable cell numbers was recorded, which lasted for only 30 min. Cell growth commenced thereafter.

In situ experiments in cows were done with purified peptide AS-48, encapsulated in liposomes. These *in vivo* studies were conducted by administering peptide AS-48 (6400 AU/ml) to different udder quarters. In a prevention trial, i.e. where quarters were pre-treated with peptide AS-48, a reduction close to 90% in the viable cell numbers of *S. aureus* was recorded relative to the control quarters, which were not treated with the peptide. A 50% reduction in somatic cell count (SCC) was recorded. In the treatment trial, i.e. infected quarters treated with peptide AS-48, a reduction of up to 94% in viable cell numbers of *S. aureus* was recorded. In the same quarters, a reduction in SCC amounted to almost 80%.

A recombinant strain was constructed by conjugating plasmid 92 (p92), encoding peptide AS-48, from *Enterococcus faecalis* FAIRE 92 to *E. faecalis* FA2/Ent, which produces enterocins 1071A and 1071B. Southern blot hybridization experiments revealed the

presence of plasmid p92 in the recipient strain without the loss of plasmid pEF1071, which encodes enterocins 1071A and 1071B. All three antimicrobial peptides, i.e. enterocin 1071A, enterocin 1071B and peptide AS-48, were produced in transconjugant FA2/Ent/AS-48. The spectrum of antimicrobial activity of the transconjugant was greater than that recorded for strains FA2/Ent and FAIRE 92, respectively and included *E. faecalis*, *Bacillus cereus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus sakei*, *Leuconostoc cremoris*, *Leuconostoc pentosaceus*, *Staphylococcus carnosus* and *S. aureus*. These organisms are not inhibited by strain FA2/Ent. However, low levels of peptide AS-48 was produced by strain FA2/Ent/AS-48. Further research in fermentation and gene expression will be needed before the transconjugant *E. faecalis* FA2/Ent/AS-48 may be used in the treatment of mastitis.

OPSOMMING

Die effek van die bakteriosien-agtige, peptied AS-48, geproduseer deur *Enterococcus faecalis* FAIRE 92, is gedurende ‘n *in vivo* en *in vitro* studie teen ‘n mastitiese *Staphylococcus aureus*-isolaat getoets. Aanvanklike toetse met peptied AS-48, selfs tienvoudig gekonsentreerde selvrye supernatant, het geen beduidende aktiwiteit teen *S. aureus* getoon nie. Aktiwiteit is eers verkry na suiwering met Triton X-114 fase-skeiding gevolg deur katioon uitruilingschromatografie. Titers vir die gesuiwerde peptied het tussen 3200 en 12800 AE/ml gewissel. Die gesuiwerde peptied het ook aktiwiteit teen *Streptococcus agalactiae* en *Streptococcus dysgalctiae* getoon, maar nie teen *Escherichia coli* nie.

Peptied AS-48 het ‘n molekulêre massa van 7150 Da, soos bepaal met elektronsproei-massa spektrometrie en SDS-PAGE. Totale inhibisie van selgroei is verkry deur 1 ml gesuiwerde peptied AS-48 (3200 AE/ml) by ‘n 100 ml kultuur van *S. aureus* in die sloerfase te voeg. Dieselfe konsentrasie peptied AS-48, toegevoeg tydens die mid-eksponensiële groeifase, het egter slegs ‘n klein vermindering in die aantal lewende selle teweeg gebring en het ook vir slegs ‘n 30 min geduur. Selgroei het hierna weer normaal voort gegaan.

In situ eksperimente op koeie is uitgevoer met gesuiwerde peptied AS-48, ge-enkapsuleerd in liposome. Hierdie *In vivo* studies is onderneem deur peptied AS-48 (6400 AE/ml) in verskillende kwarte van die uier, kunsmatig of reeds geïnfekteerd met *S. aureus*, toe te dien. In ‘n voorkomings-eksperiment waar kwarte vooraf met peptied AS-48 behandel is, is ‘n verlaging van byna 90% in die lewende seltelling van *S. aureus* relatief tot die kontrole kwarte, sonder behandeling met peptied AS-48, verkry. ‘n 50% verlaging in die somatiese seltelling (SST) is verkry. In die behandelings-eksperiment, waar geïnfekteerde kwarte met peptied AS-48 behandel is, is ‘n verlaging van byna 90% in lewende *S. aureus* selle gevind. In dieselfde kwarte is ‘n verlaging van byna 80% in die SST genoteer.

‘n Rekombinante ras is gekonstrueer deur plasmied 92 (p92), wat kodeer vir peptied AS-48, vanaf *Enterococcus faecalis* FAIRE 92 na *E. faecalis* FA2/Ent, wat enterosien 1071A en 1071B produseer, te konjugeer. Southern-klad hibridisasie het die teenwoordigheid van plasmied p92 in die ontvanger ras, sonder die verlies van plasmied pEF1071 wat enterosien 1071A en 1071B kodeer, getoon. Al drie antimikrobiese peptiede, nl. enterosien 1071A, enterosien 1071B en peptied AS-48, is deur die transkonjugant FA2/Ent/AS-48 geproduseer. Die spektum van antimikrobiese aktiwiteit van die transkonjugant vand die transkonjugant is breër as dié van rasse FA2/Ent en FAIRE 92, onderskeidelik en het ook *E. faecalis, Bacillus cereus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus curvatus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus sakei, Leuconostoc cremoris, Leuconostoc pentosaceus, Staphylococcus carnosus* en *S. aureus* ingesluit. Hierdie organismes word nie deur ras FA2/Ent geïnhibeer nie. Lae vlakke van peptied AS-48 is egter deur ras FA2/Ent/AS-48 geproduseer. Verdere navorsing in fermentasie en geen-uitdrukking is nodig voordat *E. faecalis* FA2/Ent/AS-48 in die behandeling van mastitis gebruik kan word.

CONTENTS

INTRODUCTION

INTRODUCTION

Mastitis, also known as intramammary infection (IMI), occurs in all mammalian species, but is particularly important in dairy cattle (7, 21). Mastitis is caused by both non-infectious (traumatic or toxic) and infectious agents. However, bacterial infections are the main cause of the disease in cattle. The bacteria usually gain entry to the udder through the teat (streak) canal, leading to swelling, fever, redness, pain and abnormal lactation (7).

Bovine mastitis is from an economic viewpoint the most costly disease confronting the dairy industry (8, 14, 17, 21, 36). Approximately 17-20% of dairy cattle world-wide suffer from mastitis and at least 50% of cows suffer at least one outbreak of clinical mastitis per lactation (3, 36). Major losses are experienced as a result of reduced milk production (70%), milk discarded during and after therapy (8%), drugs and veterinary expenses (8%) and death or premature culling (14%) (7, 8, 13, 14, 17, 36). In certain cases one or more quarters of the udder of the infected animals may become permanently damaged. In the United States, financial losses due to mastitis is estimated at 200 dollars per animal per year, with an overall loss of 2 billion dollars per year (1, 8, 14, 36). The estimated costs involved in the treating of mastitis in South Africa was close to R190 million in 1978, which increased to R380 million in 1994. This figure is estimated to increase to R 480 million in 2003 (12).

Streptococcus agalactiae has been the most common cause of mastitis prior to 1940. However, with the increased use of antibiotics, the prevalence of *S. agalactiae* decreased, whilst outbreaks of *Staphylococcus aureus*, coliform bacteria, *Mycoplasma* spp., *Streptococcus uberis* and *Staphylococcus epidermidis* increased (7, 21). In the Western Cape, *Escherichia coli*, *Streptococcus dysgalactiae, S. agalactiae* and *S. aureus* are the main causative organisms of mastitis. Control of the disease involves hygienic practices and infusion of antibiotic drugs into the udder. On average 33 million antibiotic treatments are given each year in the United States (3, 21).

Through the use of lactic acid bacteria (LAB) and specifically small peptides, viz. bacteriocins, a low cost-effective method of controlling and treating mastitis may be

achieved. This should also lead to a direct increase in milk yield. LAB enjoys GRAS (generally regarded as safe) status. Most of the antimicrobial peptides (bacteriocins) they produce are also classified as GRAS.

In this study we determined, *in vitro* and *in vivo*, the inhibitory activity of the cyclic peptide AS-48, produced by *Enterococcus faecalis* FAIRE 92 and classified as a bacteriocin, against a strain of *S. aureus* isolated from mastitic milk. In the *in vivo* studies, peptide AS-48 was liposome encapsulated and administered directly into the teats of cows.

Plasmid p92, encoding peptide AS-48 in *E. faecalis* FAIRE 92, was conjugated to *E. faecalis* FA2/Ent which contains the plasmid encoding enterocins 1071A and 1071B. The rationale behind this was to develop a strain (FA2/Ent/92) with a broader spectrum of antimicrobial activity than *E. faecalis* FA2/Ent. Further research will be conducted to determine if the transconjugant can be used in the prevention of mastitis in dairy cows.

UPDATE ON BOVINE MASTITIS, WITH SPECIAL REFERENCE TO INFECTIONS CAUSED BY *STAPHYLOCOCCUS AUREUS*

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THE BOVINE UDDER

Anatomy of the bovine udder

The udder (Fig.1) is a skin gland and weighs in a full-grown cow between 10-25 kg. It consists of four individual milk glands, also known as quarters. Each quarter functions independently, with no mixing of milk between quarters. The size and shape of each teat that drains a quarter is independent of the size and shape of the udder (28).

The teat canal is situated at the lower end of the teat. It is approximately 0.5-1.0 cm in length and is closed by the teat sphincter muscle. This muscle, Fürstenberg's rosette, prevents contaminants entering the udder and loss of milk inbetween milkings. The teat cistern is at the top of the teat canal and Fürstenberg's rosette, while the gland cistern is found at the top of the teat cistern and situated in the udder. Since there is no definitive separation, the teat and gland cistern are continuous. Five to twenty lactiferous (milk) ducts branch out of the gland cistern that in turn branches out into very fine lactiferous tubules which eventually end in an alveolus. The alveolus consists of a single layer of milk producing cells on the inside of a base membrane. This layer of epithelial cells surrounds the lumen or cavity inside the alveolus into which the milk is secreted (28).

Resistance mechanisms of the bovine udder

For mastitis to develop, bacteria must be able to penetrate the teat canal, progress to the milk-producing tissues, and induce inflammation. The tissues of the teat have a marked influence on the ability of udder pathogens to establish an infection. Mammary defenses ensure that most infections persist as chronic rather than acute mastitis. The teat canal and associated tissue (Fig.1) provide the first barrier to mammary pathogens and thus have an important role in mastitis control (25).

There are three primary defense mechanisms provided by the teat canal. This includes adsorption of bacteria to keratin, removal of bacteria-coated keratin during machine milking (19), and drying out of the canal lumen which allows the resealing of keratanized surfaces (25). Bacteria adsorb strongly to keratin and up to a million organisms can be

removed by the teat canal at any given time. The sphincter muscle functions by maintaining a tight closure of the canal, thereby limiting bacteria to enter at the teat orifice (opening). Loss of muscle tone may increase susceptibility to IMI, i.e. more infections occur in quarters with leaky teat canals (24, 25). Studies have shown that teat canals of quarters that are large in diameter and with a thinner keratinous canal lining are more susceptible to infections (6).

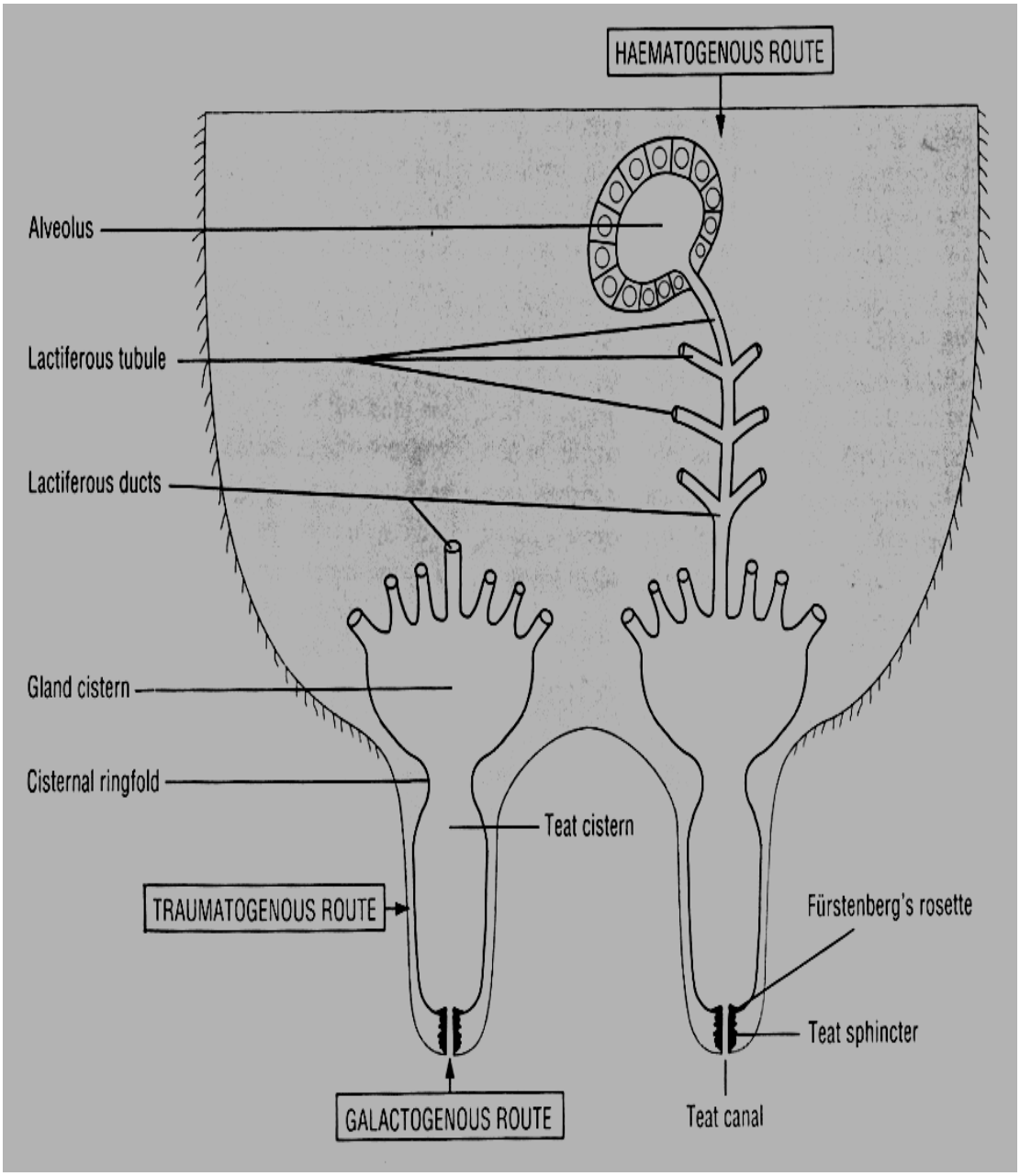

Fig.1. Schematic diagram of the bovine udder

The second barrier of the bovine udder is formed by the somatic cells in milk (22). Somatic cells consist of many types such as neutrophils, macrophages, lymphocytes, eosinophils, and various epithelial cell types of the mammary gland (14, 15). Milk producing cells also wear off and die during the milk production process and through normal aging. It is then secreted in the milk contributing to the milk somatic count (29).

When the mammary gland is infected, the number and predominant types of somatic cells undergo a rapid transition. The relative proportion of cell types shifts to neutrophils, being more than 95% (14, 15, 18, 24). This transition, resulting in a higher somatic cell count (SCC) and showing clots or flakes, takes only a few hours and is part of the normal host defense mechanism (15, 18). The function of neutrophils in milk is to engulf and digest the invading bacteria. When the bacteria are destroyed, the recruitment of neutrophils into the gland ceases. Only a mild inflammatory episode is required to restore health in the gland (14, 15, 24).

Sometimes the innate defense mechanism of the mammary gland loses the battle with the bacteria. The bacteria multiply and release large quantities of toxins. Various and larger quantities of soluble factors are then released by many of the various cell types in the mammary gland. This elicits a massive recruitment of additional leukocytes, mostly neutrophils, into the gland (14, 15, 16).

BOVINE STAPHYLOCOCCAL MASTITIS

Epidemiology of *S. aureus*

Mastitis is caused by a variety of bacteria, including aerobic, facultatively anaerobic, micro-aerophilic and anaerobic genera, and mycoplasmas, yeasts, fungi, moulds, algae, viruses, and rickettsias. Micro-organisms other than streptococci and staphylococci play a minor role (7, 14).

The genus *Staphylococcus* consists of more than 23 species (38). In cattle *S. aureus* survives in different environments, such as the skin, bedding, milk and milk secreting

tissue (4, 38). The species is, however, not commonly found on healthy teat skin, but readily colonize or grow in the teat canal. This is especially the case with lesions or sores in the teat orifice (7, 14, 37). The other major source of infection is infected milk (38). *S. aureus* may, however, persist for long periods of time in other parts of the body, such as the nose, vagina, and infected tonsils. Spreading of the organism occurs during milking (7).

Other organisms include *Streptococcus* spp. and coliform bacteria. They also occur on the surface of materials and objects, including bedding, milking machines, and milker's hands that have been contaminated with milk (36).

Pathogenesis of *S. aureus*

Some strains of *S. aureus* are tissue invaders and are extremely damaging to the udder parenchyma due to the release of toxins. The adherence of *S. aureus* to fibronectin is an important step of localization. Initially, the bacteria damage the epithelium lining of the teat and udder cisterns. They then move to the parenchyma, where deep-seated foci of infection are established. This is followed by abscessation, with fibrous encapsulation being a mechanism for restricting infection to localized areas (7).

During the course of staphylococcal mastitis, the extent and distribution of tissue damage is highly variable; sometimes only small areas of the gland are involved. In such areas, necrotic epithelial cells lining alveoli or ducts are cast off and obstruct the duct system, causing involution of remaining functional alveoli and formation of scar tissue. Obstructed ducts may reopen and release staphylococci, which then infect other areas of the gland. This process may be repeated, causing cycles of infection and reinfection in different sites in the quarter. When microorganisms remain within the obstructed ducts and damaged tissue, abscesses may develop, leading to lumps (7).

Pathology of *S. aureus*

Staphylococcal mastitis may be peracute, acute, subacute or chronic, the latter being the most common. Peracute to acute gangrenous mastitis, which may be restricted to the teat

or a specific quarter, is characterized by dark red to black and dull tissue. Necrosis and venous thrombosis, which are the main features of the lesion, may extend throughout the gland or be restricted to foci within it. If the infected animal remains alive for some time, the gangrenous tissue is excreted through the teats within a week, leaving a raw, purulent surface (7).

In less severe forms of staphylococcal mastitis, the onset is subtle and results in the development of pyogranulomas. Bacteria are present within the lesions and throughout the affected tissue. As the lesions progress, a marked granulomatous mastitis with fibroplasia develops. This process is referred to as botryomycosis (7).

SOMATIC CELL COUNT (SCC)

The SCC in milk is an indication of the level of infection in the udder (27). Various factors affect the SCC, as discussed below.

1. Infection status

This is the single most important factor affecting SCC in milk. Major pathogens such as *S. aureus*, *S. agalactiae* and coliform bacteria cause higher average SCC than minor pathogens such as *Corynebacterium bovis* and the coagulase-negative staphylococci (14, 26).

2. Age

Apart from the infection status, lactation number, i.e. age, has the greatest effect on SCC. Older cows have a higher SCC, probably because they have been exposed to a greater variety of microorganisms. Furthermore, infections in older animals lasts longer and generally causes more extensive tissue damage (14, 26).

3 Stage of lactation

The SCC in milk of non-infected cows is high at calving and at drying off, with lower counts from peak to midlactation (14, 26).

4. Season

The highest SCC generally occurs during summer with the lowest counts in winter. The cell counts of summer milk herds are usually 43% higher than winter milk herds (16). The high SCC is most probably caused by higher humidity and less sound management practices. Variation in SCC should, however, not be linked to weather conditions only (14, 26).

5. Stress

Cows harboring subclinical mammary infections respond to stress like isolation, weather change, agitation, thermal stress and gathering before milking with significant increases in milk SCC. Uninfected cattle, however, do not appear to respond in any significant proportion (14, 26).

6. Diurnal variations

Significant fluctuations in milk SCC occur depending on the time of sampling. Somatic cell counts are the highest 1 to 3 hours after milking, followed by a steady decline until the next sampling. Milk sampled in the afternoon usually has twice the number of SCC compared to milk sampled in the morning. SCC in quarter milk samples may vary as much as five-fold in uninfected cows, depending on when samples were collected (14, 26).

7. Day-to-day variation

Daily variation in individual cows is considerably more in infected than in uninfected animals. Herds with a high mastitis incidence also have greater variation in either daily or monthly SCC (26).

8. Somatic Cell Count Testing Methodology

Sample collection, storage, transport, and test procedures all influence SCC results. Recently, considerable effort has been made to standardize SCC test procedure and calibration of cell counting instruments (26).

9. Management

Consistent use of a teat dip, dry cow therapy, individual towels to wash and dry teats, milking order, type of housing, bedding and stall maintenance, milking system design and maintenance, and manure handling have a great impact on herd SCC (26).

10. Breed difference

Recently there have been reports on breed differences in SCC (26). The variability in SCC within a breed is greater than differences in SCC among breeds (14). However, further research is needed to substantiate these findings.

FACTORS AFFECTING THE INCIDENCE OF MASTITIS

Various factors affect the incidence of mastitis in a dairy herd. Main factors are the environment (housing, bedding, etc.), the animal and the milking machine.

1. Housing

When cows are housed, adequate space is important in minimizing infection of the udder (16). Overcrowding induces problems of sanitation, availability of nutrients and feeding space, and the possibility of stress in some cows. Crowding has also been reported to increase the SCC in milk. Adequate housing implies light, airy buildings free from drafts, stalls of adequate size, plenty of bedding, daily removal of manure, and exercise yards or drylots maintained free of wire, stones, or sharp objects (5).

2. Milking machine

The milking machine affects the incidence of mastitis by injuring the mammary tissue by overmilking. The rate of infection is also increased when quarters are left unmilked for a period of time. Incomplete milking aggravates existing mastitis infections. The milking machine may also transfer infections from one cow to another. Other factors influencing mastitis are pressure changes in the teat cup, slipping of liners and airflow (7, 16).

3. Cow

Individual animals vary greatly in their resistance to mastitis, a phenomenon which may be linked with udder conformation. Large pendulous udders are more susceptible to infections due to more frequent injuries. The size and shape of a teat are not significantly related to infection rate, but the width of the teat sphincter is closely associated with both milking rate and susceptibility to udder infections. Excessive hard milkers and cows which run milk freely are the most likely candidates to become infected, but milkability and resistance to mastitis are not incompatible. Cows can inherit resistance to this disease (5**,** 16).

4. Other conditions that affect the incidence of mastitis

i) Milk yield

The possibility of a positive connection between high milk yields and the incidence of mastitis is dubious. In certain instances there is no significant association between the level of milk production and the incidence of mastitis, while in others there is a positive connection between a high level of milk production and the incidence of infection. Although there are doubts whether there is a connection between milk yield and mastitis, it is a sound policy to pay particular attention to the health of the udders of high yielding cows (16).

ii) Stage of lactation

Increases in the incidence of clinical mastitis in cows have been reported to occur during 31 days post-partum to the 30th day of the next pregnancy inclusive (16). The greatest exposure to IMI is during the milking phase of the lactation cycle, although new IMI occur 7 to 10 times more frequently in the dry period. The highest frequency of new IMI is recorded during the early dry period. IMI decreases during the mid period and increases as calving approaches. Nearly all IMI acquired during this period persist into the next lactation. At cessation of lactation, changes take place that may increase susceptibility to infection. This include (i) the flushing of milk through the teat canal, which eliminates colonized bacteria, is stopped (ii) increased internal udder pressure, which induces teat canal dilation, allows bacterial penetration and (iii) discontinuation of teat dipping, which may lead to the increase of bacterial populations on the exterior skin. The increase in infection in the early dry period is transient, since an effective keratin seal

appears to develop after 10-14 days (24). Degenerative, nonmicrovilliated epithelia of the teat cistern mucosa become less prevalent as the udder returns to its normal size (involution). Most of the udder pathogens adhere preferentially to the epithelia, leading to an enhanced resistance to IMI. During involution, citrate, which competes with lactoferrin for iron, is reabsorbed whereas bicarbonate increases in milk; therefore conditions for the inhibitory activity action of lactoferrin are optimal (24).

iii) Weather conditions

The relationship between weather factors and mastitis is difficult to assess, since abrupt seasonal changes in weather are accompanied by other changes on the farm. The incidence of mastitis appears to be higher in cows just turned out to pasture in wet years than in cows in a similar period in dry years. Wet weather thus contributes to the mastitis problem (5). There is a higher incidence of mastitis infections during the summer than in the cool, dry winter months. Moisture and temperature can have a profound influence on yield and nutritive value of crops. Weather could thus have an indirect effect through its effect on quality and quantity of pastures. Temperature also has an effect on insect populations. Flies are important vectors of bacterial diseases, and biting flies contribute greatly to anxiety and unrest in a herd of cows. The effects of heat stress vary among breeds of cattle and some cattle are able to adapt to extremes of weather better than others. The major effect of heat stress is a decrease in milk production. Cold also decreases milk production because of the overall energy demand, reduced blood circulation in the mammary gland, and direct effect of cooling on milk synthesis and secretion (5).

iv) Age

The incidence of mastitis increases with age. A more rapid increase per lactation of the mild form of the disease is observed in older animals. Susceptibility to mastitis also increases with age. Various other factors may also weaken the natural resistance of the tissues of older udders to infection (16).

CLASSIFICATION OF MASTITIS

Mastitis can be classified into subclinical and clinical forms according to severity, duration, distribution, nature of the exudate and primary cause. It can also be classified based on aetological, clinical and pathological symptoms and the degree to which the udder is affected. A normal quarter shows no outward signs of disease, produces milk free of pathogenic organisms and with a SCC of less than 5 x 10^5/ml (7, 12).

Subclinical mastitis

The affected udder quarter is characterized by inflammation, but without any visible signs thereof. Because of this, infections can persist for months due to the fact that they are undetected by the stockmen. The majority of mastitis cases are subclinical. At any time subclinical infection can affect up to 50 times more quarters than is evident from clinical mastitis. Approximately 70 to 80% of the losses through mastitis could be attributed to subclinical mastitis. Milk production of the infected quarter may be 5 to 10% lower than normal and the composition of the milk is altered (1, 13). Since subclinical mastitis occurs more frequently, this reduction in milk production is economically more important than that caused by clinical mastitis. Most cases of subclinical mastitis are caused by contagious organisms such as *S. agalactiae* and *S. aureus*. Other cases may be attributed to environmental organisms such as *S. uberis* or *S. dysgalactiae*. The milk appears normal, but the SCC is considerably higher (more than 5 x 10^5/ml milk) with pathogenic bacteria and inflammatory products present in the milk. The pH and salt content of the milk is also higher (12, 14, 17).

Chronic (recurrent) mastitis

In this situation the inflammatory process persists for many months, or even from one lactation to the next. Chronic mastitis (Fig. 2) is usually clinical, but may establish itself in a subclinical form and periodic “flare-ups” are very common. This is very often the case with a *S. aureus* infection (7, 12).

Fig. 2. A chronic mastitic udder.

MASTITIS CONTROL

Mastitis cannot be eradicated and, although it is possible to deal effectively with infections caused by some pathogens, little progress has been made in the control of others. Total elimination of mastitis is not a realistic goal, but a reduction in the incidence of mastitis may be realistic (2).

Control must be based on the prevention of new infection and the elimination of existing infections. Programs likely to find acceptance among dairy farmers must be economical, practical, effective under most management conditions, and must reduce the incidence of clinical mastitis. Possible methods for control include eradication of the causative agent, immunization, therapy, breeding resistant cows, or by improved management. However, in practice success has been achieved only with the latter (32).

The first practical control methods were based on specific hygienic methods coupled with the use of improved antibiotics. This was followed by better designs of milking equipment and improved housing. By implementing procedures of postmilking teat disinfection and total dry cow therapy with effective antibiotics, it is possible to eradicate or reduce infections caused by *S. agalactiae* and *S. dysgalactiae*. Control of *S. uberis* is much less affected and coliform mastitis is unaffected. This varying degree of success is due to basic differences in the various types of infections. Control of infections caused by pathogens that emanate from sources other than the mammary gland, e.g. bedding material, is much more difficult (9, 32).

Mastitis infections have similarities, but their differences are distinct, which in turn has important consequences for the development of control mechanisms. The pathogens emanate from various sources, the period the animal is subjected to exposure of pathogens differ, the route of infection differs, and the chances of recovery from an established infection varies. Due to the complexity of mastitis infection, we cannot expect to discover a single technique to augment or replace control methods currently in place. However, by adding to existing control methods, or replacing them with more sophisticated (and environmentally safer) medication, mastitis infection may be better controlled (2).

Drugs used in treatment

1. Penicillins

The majority of isolates of haemolytic staphylococci from bovine milk samples are resistant to penicillin, whereas virtually all species of streptococci are sensitive to the antibiotic (7). The activity of penicillin is decreased only slightly in milk. Penicillin is well distributed throughout the udder and diffuses relatively well into mammary tissue in both normal and mastitic glands, except in large areas of necrosis or fibrosis (30). Cloxacillin is a narrow-spectrum, semi synthetic penicillin which is resistant to staphylococcal penicillinase. In lactating cows, infusion of cloxacillin is as effective as benzylpenicillin against streptococcal and staphylococcal infections. Ampicillin is a semi-synthetic penicillin with a broad spectrum of activity which diffuses into the udder at higher concentrations than benzylpenicillin (7, 30).

2. Dihydrostreptomycin

This antibiotic is seldom used on its own in intramammary treatment, but is often used in combination with penicillin. Dihydrostreptomycin, one of the aminoglycosides, has a basic pH and is unsuitable for systemic treatment of mastitis as it is unlikely that even in very high doses it ever reaches therapeutic levels. The aminoglycosides have fairly low minimal inhibitory concentrations for staphylococci and for some Gram-negative mastitis pathogens, but their activity against streptococci is even lower. The activity of dihydrostreptomycin is also markedly decreased in the presence of milk and has a very uneven distribution within the udder, taking up to eight hours to become widely dispersed throughout the udder parenchyma (7, 30, 37).

3. Tetracyclines

Oxytetracycline and chlortetracycline are partially inactivated in milk. Injectable oxytetracycline has limited bio-availability after being administered intramuscularly and does not reach therapeutic levels in milk. Oxytetracycline is a local tissue irritant and is very unevenly distributed in normal udder tissue following intramammary injection and is therefore not recommended for the treatment of mastitis (7, 30).

4. Neomycin

Neomycin has limited penetration in the udder, which lessens its potential usefulness in both parenteral and local treatment of mastitis. However, it is being used as the main ingredient in combination drugs for intramammary mastitis therapy because of its wide antimicrobial spectrum (7, 30).

5. Erythromycin

The macrolide antibiotics, which include erythromycin, tylosin, lincomycin and spiromycin, pass effectively from the blood into the udder, but their antibacterial spectra are limited to Gram-positive pathogens. They are the drugs of choice when attempting to eliminate persistent Gram-positive udder infections. In the treatment of acute mastitis caused by Gram-positive pathogens, combined parenteral and intramammary application is recommended (7, 30, 37).

6. Chloramphenicol

Chloramphenicol has limited bio-availability when administered intramuscularly and should rather be administered intravenously. In certain countries the use of chloramphenicol in food-producing animals is strictly forbidden because its use constitutes a potential human health hazard (7, 30).

7. Sulphonamides and trimethoprim

Sulphadimidine, when administered intravenously at the correct dosage is sufficient to eliminate both streptococcal and staphylococcal infections. Trimethoprim has a rather short half-life which, in cattle, varies between 50 and 100 minutes, thereby limiting its application (7, 30).

8. Cephalosporins

Cephalosporins, which possess broad-spectrum activity against many Gram-negative pathogens and beta-lactamase-producing staphylococci, can be used instead of combinations of antibiotics. Cephalosporins have a limited distribution in the udder after parenteral and intramammary therapy. Cephoxazole is bactericidal and resistant to destruction by staphylococcal penicillinase, and by binding to penicillinase produced by

Gram-negative bacteria, allows penicillin to act on these otherwise-resistant pathogens. Cephoxazole and penicillin have a mutually potentiating effect (7, 30).

9. Metronidazole and clindamycin

Metronidazole is the only drug that shows consistently good bacteriological activity against *Bacteroides fragilis*. However, there is no intramammary preperation available to date that contains metronizadole for mastitis therapy and its efficacy in parenteral treatment of mastitis requires further research. Clindamycin is active against anaerobic Gram-negative bacilli, such as *Bacteroides*, *Eubacterium* and *Peptococcus* spp. (7, 30).

Studies have shown that 5- or 4-day courses of parenteral therapy produce higher bacterial cure rates than 3- and 2-day courses of treatment. Ampicillin penetrates into the udder to produce concentrations to inhibit most mastitis pathogens for 24 hours after a dose of 20 mg/kg. Doses of 25 mg/kg of cloxacillin and 12.5 mg/kg cephalosporin produce effective concentrations for only 4-8 hours. At doses of 12.5 mg/kg, erythromycin and tylosin is sufficient to maintain the milk at antibiotic levels greater than the MIC's for staphylococci. For effective levels of streptomycin to be reached in the udder, doses of 10-12 mg/kg must be injected every 6 to 12 hours. Penethamate hydriodide is hydrolysed in milk to produce benzylpenicillin, and reaches a concentration five to seven times greater in milk than in blood. Injectable tetracycline at a dose rate of 20 mg/kg and amoxycillin/clavulanic acid at 8.75 mg/kg yield adequate concentrations in the udder. Preparations using a polyvinypyrrolidone base yield good antibiotic concentrations in milk (7, 30).

Antibiotic treatment of mastitis

Clinical cases

Therapy in clinical cases assists the cow's defenses to overcome the infection, aids in the regression of clinical signs to permit the animal's milk to be sold, limits udder tissue damage and prevents further spread of infection. However, there have been concerns to minimize antibiotic residues in milk. This has put pressure on dairy farmers to treat clinical mastitis only to produce clinical improvement. Subsequently, the amount of milk being discarded are minimized and farmers would also avoid financial penalties for

positive antibiotic residue milk test results. These pressures have encouraged the marketing of short-duration or even single-dose intramammary treatments. This may lead to increased subclinical infection if accompanied by less rigorous application of teat disinfection and dry cow therapy; and cost-cutting reductions in the standards of milking time hygiene and milking equipment cleaning and maintenance (10, 17).

Dry cow therapy mastitis control program

Dry cow therapy is presently the most effective means for eliminating infections. Products on the market remove between 70 and 98% of infections, depending upon the formulation and causative organism. Another value of dry cow therapy is the prevention of new infections. Products currently marketed are capable of reducing the new infection rate from approximately 14% to 7% of quarters. Persistence of antibiotics in the gland provides protection against new infections. Since treatment efficacy of products presently available is relatively high, the greatest potential gain could be derived from the development of products that would remain in the udder through the first few milkings of the next lactation and be more effective in preventing new infections. The antibiotic residue would be removed with the colostrum with little risk of contaminating the milk supply. These formulations also have a role to play in the prevention of summer mastitis in dry cows and pregnant heifers (2, 10).

Lactation therapy

Therapy during the lactation often is looked upon unfavorably, not only because of an inferior effectiveness compared to dry cow therapy, but also because of the economic losses associated with discarded milk and udder damage, which may remain throughout lactation (27, 37, 44). Treatment response of quarters with clinical mastitis is highly variable with positive responses in the range of 40 to 70%. The value of lactation therapy in mastitis control is limited further since only 40% of all new infections that become clinical are being identified and treated. Subclinical infections have a higher rate of response to lactation therapy than do clinical infections. Treatment of subclinical infections in early lactation is less successful than treatment later in lactation. Increasing the dose of antibiotic during this time usually has no effect on the cure rate. Effective therapy of subclinical infections during lactation therefore requires relatively long periods of treatment and many countries require that milk from cows under treatment be withheld

from sale during treatment (10, 21, 23). Antibiotic treatment of *S. aureus* mastitis during the lactation period is not economically attractive because of the volumes of milk that is being discarded, low bacteriologic cure rate, and a lack of economically beneficial increase in production following treatment. A program of early identification, culling, and segregation is probably the best management approach for controlling these infections. Early identification of new infections would allow for early treatment and a more favorable response to antibiotics. This may allow the affected quarter to return to normal production in the present lactation (10, 17, 23).

Decreasing susceptibility

Stimulation of the immune response through vaccination should be the ultimate goal if complete control is considered essential. However, the large number of different organisms causing mastitis makes preparation of an effective vaccine a challenging task. Studies have provided evidence that the mammary gland can produce an immune response. The greatest economic benefits would be obtained through development of vaccines against environmental organisms. There are several management and environmental factors related to decreasing susceptibility but are these are usually not considered as control procedures. They include the prevention of teat injury, proper sanitary care of teat cannulae and treatment materials, and reduction of stress. These factors must not be overlooked if maximum benefit is to be gained from control procedures (23).

Reasons for failure of mastitis treatment

One of the biggest problems confronting the milk producer is that cows treated for mastitis respond poorly or not at all. Many mastitic quarters are treated properly, but after 2 or 3 weeks mastitis is once again observed in the apparently cured quarter. According to Giesecke (1995), reasons for the failure of mastitis treatment resort under four main groups, *viz*.:

1. Bacterial factors (factors related to bacteria causing mastitis),
2. Udder pathology (factors related to the damage caused in the udder by mastitis),
3. Pharmaco-dynamics of mastitis remedies (the properties and action of the mastitis remedies administered),

4. Poor animal care and veterinary practices.

1. Bacterial factors

Bacteria such as *S. aureus* penetrate deep into udder tissue. Additional connective tissue is formed and the bacteria are encapsulated. Antibiotics administered reach the bacteria in very low concentrations, or not at all. The udder's connective tissue also has a very weak blood supply and even if antibiotics are administered intravenously or intramuscularly, the antibiotic concentrations in these tissues are so low that the bacteria are usually not killed. Some mastitis producing bacteria produce bacteria without cell walls, while others are able to change into forms without cell walls. Thus, antibiotics that inhibit cell wall formation are not able to inhibit/kill the bacteria and mastitis treatment fails (11, 37). A superinfection of the infected udder with a second mastitis-causing microorganism results because of unhygienic practices. The organisms secondarily contaminating the udder cause a further infection, because they are not sensitive to the antibiotics with which the mastitis was initially treated (11). Antibiotics introduced through the teat canal do not necessarily eliminate the bacteria already established in the teat canal. These bacteria may again cause mastitis when treatment is stopped. Also, the widespread use of antibiotics has raised the question of antibiotic-resistant strains of bacteria. These antibiotics only eliminate susceptible organisms while the resistant strains are allowed to flourish (11, 37).

2. Blockage of milk tubes, clinical cure and necrosis of the udder tissue

Udder swelling resulting from mastitis infection closes off the milk tubes. Antibiotics applied through the teat canal do not reach the infected udder tissue in sufficient concentrations and may thus not kill the bacteria. This may happen despite the fact that the bacteria are sensitive to the antibiotics (11, 37). Mastitis treatment is often stopped when a clinical cure is observed. If treatment is stopped when a clinical cure is apparently achieved, there are still many viable bacteria present in the udder and a repeat attack after one to three weeks is very common. Treatment must therefore be continued for the prescribed or recommended period. Some mastitis cases are accompanied by necrosis of some parts in the udder. This results in a poor blood supply to these areas. Administered antibiotics do not properly reach bacteria in these necrotic tissues (11).

3. Pharmaco-dynamics of mastitis antibiotics

Some antibiotics that are used intramuscularly are poorly absorbed and not well distributed to the udder tissue. Insufficient concentrations reach the udder and the causative organisms are not killed. These antibiotics should preferably be administered intravenously (11). Other antibiotics penetrate the blood udder barrier more difficult than others. These antibiotics have lower concentrations in the milk and can thus not kill the mastitis bacteria efficiently (11, 37). Certain antibiotics have a poor fat solubility and can not migrate in sufficient concentrations through tissue to reach the bacteria. The problem occurs when insufficient quantities are administered. Connective tissue or abscess encapsulation prevents the antibiotics from reaching the bacteria (11, 13). Tetracyclines become partially inactive in milk, as they bind with magnesium and calcium. Other antibiotics have a very short half-life, and if they are not administered hourly or six- to eight-hourly, the concentrations that should be present in the udder for a certain time are not maintained and the treatment fails or is ineffective (11). The prime consideration in antibacterial treatment of any infectious disease should be given to the host-parasite relationship and the effect of the drug on the parasite. Antibiotics alter the balance in favor of the host, whose own clearing mechanisms should then be able to eliminate the infection. Some antibiotics interfere with the host's defense mechanisms and are harmful to the phagocytic clearing mechanism of the host (37).

4. Poor animal care and veterinary practices

In case of severe mastitis, the animal may die if very good supportive treatment is not given, despite antibiotics being administered. Initial incorrect or inadequate antibiotic treatment may result in death, the prognosis being poor or the infection may lead to chronic mastitis. If the teat canal is damaged by unhygienic or harsh local mastitis treatment, it may result in secondary mastitis over and above the mastitis already present (11, 37).

Control of *S. aureus* infections

Traditionally, treatment has been limited to intramammary infusion of dry cows and lactating cows with clinical mastitis. *S. aureus* is highly resistant to therapy. Quoted cure rates for lactating (40%) and dry cows (65%) are suspect, and actual cure rates following

intramammary infusion may be lower. Explanations for this *in vivo* resistance include the extensive fibrosis and absessation that often follow infection, antibiotic resistance, and the conversion of bacteria to cell-wall deficient variants (13, 35).

A promising avenue of therapy for subclinical *S. aureus* mastitis is the use of antibiotics affecting the whole body (systemic antibiotics), either as sole therapy or in conjunction with intramammary infusions. The antimicrobial spectrum, distribution properties, and long half-life of norfloxacin make this compound a promising candidate for treatment of subclinical mastitis. However, this compound is not currently approved for use in food producing animals. Estimates of penicillin resistance and beta-lactamase production in *S. aureus* vary widely among surveys (0-82%) and geographical variations are likely. Penicillin resistance is associated with decreased cure rates following intramammary dry cow therapy and questions have been raised as to whether *in vitro* resistance or tolerance is correlated with *in vivo* efficacy against *S. aureus* mastitis (13, 35).

Cell-wall defective L-forms are not recovered or identified using standard milk microbiological methods. Cattle harboring these L-forms will be presumptively diagnosed as being cured. The likelihood of conversion to L-forms following therapy with many antibiotics, the intermittent shedding pattern of cattle infected with *S. aureus*, and the dramatic increase in the numbers of isolates recovered using enrichment techniques, raise serious questions concerning reported cure rates. Cure rates have been based upon standard microbiologic methods. These methods are likely to miss cattle that shed L-forms, intermittently shed bacteria, or shed bacteria in reduced numbers. At least one of these three groups of cattle is likely to be an important reservoir of infectious bacteria (13, 35).

IMMUNIZATION

Vaccination of mastitis is defined as the injection of a suspension of sensitized, attenuated, or killed bacteria into the body or udder to induce immunity against the same species of bacteria or their toxins. Autogenous vaccines are developed from bacteria cultured from the cow to be inoculated, whereas a vaccine made from any other virulent

strain/s of the same bacterial species is a stock vaccine. In response to vaccination, a cow should develop an antibody titer in the blood and milk against particular bacterial strains or their toxins. Also, successful vaccination or immunization should prevent a majority of new infections caused by the bacterial strains for which the vaccine was intended (30).

A number of problems are uniquely associated with vaccination of dairy cows against mastitis. First, mastitis is usually an immune response of the gland to invasive agents; i.e. the disease is equal to the immune response. Therefore, specific enhancement of the immune response may exacerbate the disease. Secondly, because of the large volume of milk in the udder, there is a dilution of the immune components available to fight infection, including immunoglobulins, lymphocytes, phagocytes, and the complement system. Similarly, the enormous surface area of the secretory epithelium greatly complicates immune surveillance of the gland. Thirdly, milk components, particularly fat and casein, greatly reduce the phagocytic and bactericidal activity of phagocytes within the milk and gland (37).

The organisms that induce mastitis are numerous and heterogenous. More than 135 agents responsible for mastitis, most of which are bacteria, have been identified. Because there are so many causative organisms and since the opportunities for infection with a large variety of these are so great, immunization is difficult (37).

In addition to these difficulties, the success or failure of a mastitis vaccine is often difficult to define. Ideally, a vaccine should reduce severity and frequency of mastitis, prevent new infections and eliminate existing infections. However, this is more than expected of most other successful vaccines, since because most vaccines do little more than prevent disease. Recently available vaccines do appear to reduce the incidence of clinical disease in a significant and economically efficient manner. Therefore, immunization procedures is best seen as adjuncts rather than replacements for traditional mastitis control programs (34, 37).

S. aureus mastitis vaccines

Commercially available bacterial vaccines for *S. aureus*-induced mastitis are generally of dubious efficacy. This is because the vaccines may not be efficient when IMI is the

primary criterion. However, vaccination may provide some management advantage by increasing spontaneous cure rates, thereby reducing the frequency of subclinical mastitis in the herd (30, 34, 37).

Exotoxins or toxoids like β-toxin, leucocidin and α-toxin are usually included in *S. aureus* vaccine preparations. The rationale for this is, that these exotoxins are involved in virulence of *S. aureus* in the mammary gland. In addition, milk and serum antibody titers to α- and β-toxins increases significantly in cows infected with *S. aureus* (30, 34, 37).

Polymorphonuclear neutrophils (PMN) are mainly responsible for eliminating *S. aureus* infections. Protective immunization results in enhanced capacity of the mammary PMN and an accelerated PMN response to infection. Elevated opsonic and cytophilic IgG_2, but not IgG_1 antibody, concentrations are necessary for this enhanced PMN activity (ruminant PMN lack IgG_1 receptors). In addition, certain antigens expressed *in vivo*, but not always under standard *in vitro* culture conditions, may be important in protection from staphylococcal mastitis. At least one of these antigens expressed *in vivo* appears to be an antiphagocytic microcapsule or pseudocapsule (30, 34, 37).

A surface antigen, probably the pseudocapsular material, and perhaps the α- or β-toxins, are important protective antigenic components for improved *S. aureus* mastitis vaccines. Most mastitis-inducing strains of *S. aureus* produce a diffuse polysaccharide slime layer or a pseudocapsule. This capsular polysaccharide (CPS) is antiphagocytic, and antibodies to the CPS are opsonic. Although antibodies in the serum and milk directed at the pseudocapsule could be detected with CPS-enhanced bacterins, infection by *S. aureus* alone, however, do not result in a detectable antibody response to CPS (30, 34, 37).

Approximately 70% of *S. aureus* strains isolated from bovine mastitis produce CPS serologically classified as type 5 or 8. Purified types 5 and 8 CPS are not immunogenic. This lack of immunogenicity has led to conjugation of types 5 and 8 CPS to carrier proteins. These preparations induce antibody responses to CPS that are of a high titer and T-cell dependent. Conjugated CPS, combined with an adjuvant to induce opsonic IgG_2 antibody in the cow, may well provide protection from *S. aureus* mastitis (30, 34, 37).

Another recent approach to provide a vaccine for *S. aureus* mastitis has been to use one of the presumed staphylococcal adhesion proteins as a vaccine. *S. aureus* has a fibronectin-binding protein on its surface that binds to a specific portion of the N-terminal region of fibronectin, which is one of the specific receptors for its adhesion and colonization. rDNA-produced fibronectin-binding protein have been used to immunize against *S. aureus*-induced mastitis. This rDNA-produced fusion protein reduced the incidence of clinical mastitis in dairy cows. However, more tests will have to be conducted to evaluate the effectiveness of a vaccine based on the fibronectin-binding protein or other *S. aureus* adhesion proteins (30, 34, 37).

A polyvalent *S. aureus* mastitis vaccine is commercially available in the USA. However, immunization with either this product or experimental vaccines does not uniformly prevent infection. Since *S. aureus* mastitis is a contagious udder pathogen, vaccine failures are of particular concern. Infected cattle will remain a potential reservoir of contagious bacteria. Use of this or similar products may provide a useful adjunct to traditional control programs. Any benefits likely will be the greatest in herds with a high prevalence of infection and a high incidence of peracute mastitis in which eradication is not practical. This vaccine apparently provides no protection against infection by other staphylococcal species (30, 34, 37).

IMMUNOTHERAPY

A family of glycoproteins responsible for the regulation of leukocyte proliferation, maturation and release into the peripheral blood have been recognized, isolated, and characterized. Collectively, these glycoproteins are termed colony stimulating factors (CSF). Among this class of compounds are the neutrophil (granulocyte) colony stimulating factor (GCSF), the macrophage colony stimulating factor, and the granulocyte-macrophage colony stimulating factor (GMCSF) (34).

Parenteral injection of exogenous CSF has caused profound neutrophilia within two days that persisted for three days following the termination of CSF administration. Although dramatic increases were observed in peripheral blood neutrophil counts, only modest and

statistically marginal increases in milk somatic cell counts were observed in CSF-treated cows. A decreased rate of IMI has also been observed following the challenge with *S. aureus* in cows pretreated with GCSF. GCSF-treated cows also had a shortened duration of IMI with *Klebsiella pneumoniae* following experimental infection. However, in these studies small numbers of cows were used and further studies are needed before conclusions may be drawn concerning the possible therapeutic role of these compounds (34).

No leukopoietic factor is approved for use in lactating dairy cattle. A variety of other agents, including levamisole, thiabendazole, avridine, isoprinosine, glucan, and ascorbic acid have been investigated as potential immunotherapy agents in cattle. Although several agents have demonstrable effects on *in vitro* measurements of immune response function, none of the aforementioned agents has demonstrated clear efficacy in either the treatment or prevention of bovine mastitis. Although no immunostimulant of demonstrated efficacy is available for mastitis treatment and control, it remains an active field of study and efficacious immunostimulants may become available in the near future (34).

Intramammary devices

Intramammary devices (IMD) have been investigated as a means by which leucocytosis could be induced in culture-negative mammary glands. In one study, insertion of a smooth polyethylene IMD in lactating dairy cows, resulted in a rapid, transient increase in milk SCC that subsided within 1 week to SCC concentrations 50% higher than either the pre-insertion concentrations or concentrations observed in control quarters. Similar increases in milk SCC were not observed when the IMD was inserted at drying off. The observed increase in SCC was associated with an increased resistance to experimental infection. Most IMD designs successfully induce a milk leukocytosis but quarters with IMDs have prominent histologic changes. Quarters treated with some IMD models also have significant decreases in milk production. In subsequent studies, insertion of several IMD models induced a milk leukocytosis, but only one device was deemed protective against IMI (34).

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LACTIC ACID BACTERIA (LAB), THE BACTERIOCINS THEY PRODUCE AND THEIR APPLICATION IN MASTITIS CONTROL

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INTRODUCTION

Lactic acid bacteria (LAB) are united by morphological, metabolic and physiological characteristics (100). They comprise a diverse group of Gram-positive, anaerobic, microaerophyllic or aero-tolerant nonspore-forming bacteria. They occur as cocci or rods and lack catalase, although pseudo-catalase has been found in rare cases (118). They are fastidious, non-motile, acid tolerant, devoid of cytochromes and do not reduce nitrate. They are chemo-organotrophic and grow only in complex media. Fermentable carbohydrates are used as energy source. Hexoses are degraded mainly to lactate (homofermentative metabolism) or to lactate and additional products such as CO_2, formate, succinate as well as acetate or ethanol depending on whether aerobic or anaerobic conditions prevail (heterofermentative metabolism) (100, 117, 118). Recent taxonomic revisions suggest that LAB comprise the following genera: *Aerococcus*, *Alloiococcus*, *Bifidobacterium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (21, 100, 118).

LAB are widely distributed in nature. They exist on plant surfaces, in plant decaying material, food products such as milk, fermented meat and vegetables, fish, sour dough, silage and beverages and in the gastrointestinal, genital and respiratory tracts of man and animals, manure and sewage (117, 118).

Some of these environments are rich in nutrients and energy sources and thus excellent in supporting the growth of other microorganisms. Because of this, LAB have developed strategies to efficiently compete with other organisms (100). This includes the production of growth-inhibiting substances and large quantities of lactic acid. Peptide antibiotics, antibiotic-like substances, bacteriocins and bacteriocin-like substances are also produced. The production of these antimicrobial proteins is the reason why LAB are so important in the food and feed technology, where they may be used to inhibit the growth of food-spoilage bacteria (21). Other applications of LAB have been directed towards probiotics. Probiotics involve the prophylactic use of microorganisms to help protect the host animal from diseases (100).

ANTAGONISM OF LACTIC ACID BACTERIA TOWARDS PATHOGENIC BACTERIA

Lactic acid bacteria may help promote health and combat the proliferation of bacterial pathogens. The following factors may be involved:

1. Competitive exclusion

This theory proposes the prevention of colonization of some microorganisms (including pathogens) by others. The beneficial bacteria occupy adhesion sites in the gut that would otherwise be populated by harmful bacteria. The precise mechanism involves specific recognition of receptor sites (oligosaccharides) by bacterial fimbrae (lectins). Other mechanisms include competition for nutrients, reduction of the redox potential and non-specific activation of the immune system (29). Competitive exclusion can take place throughout the digestive tract when non-pathogenic organisms such as lactic acid-producing bacteria are present in food. The LAB migrate from the stomach into the small intestine where the production of lactic acid may mediate, at least in part, the inhibition of the development of pathogenic bacteria. Competitive exclusion could also be as a result of aggregation of non-pathogens to pathogens, preventing binding to attachment sites and leading to their removal from the gut (29).

2. Reduction of toxic amine production

Metabolic activity of the intestinal micro-flora produces amines and ammonia that may have deleterious effects on the host. Amines are associated with diarrhea in that it increases peristalsis. The level of amines produced within the gut can be reduced by lactobacilli, e.g. *L. acidophilus* (29).

3. Interactions with bile

Bile salts are surface-active chemicals that aid digestion by forming polymolecular aggregates with water-insoluble lipids and fat-soluble vitamins. Bile is important in blocking the passage of many live organisms into the lower intestine and inhibits the growth of enteric anaerobic bacteria. Specific strains of lactobacilli can release free bile acids into the intestinal tract and could thus influence the balance of the intestinal

bacteria. Unconjugated (free) bile acids are much more inhibitory than conjugated forms (29).

4. Anti-enterotoxic activity

Toxins produced by pathogens bind to epithelial receptors and prevent the colonisation of bacteria. Enterotoxins, produced by a number of *E. coli* strains, that causes fluids to be lost from the intestine, can be neutralised by probiotic bacteria. Anti-enterotoxic activity has been recorded for certain strains of *L. bulgaricus* and *E. faecium* (29).

5. Stimulation of immunity

LAB may stimulate the production of antibiotics and phagocytic activity against pathogens in the intestine and other tissues of the body. Bacteria contain some common antigens that can cross react with some pathogenic microorganisms and thus immunize against an invasion of pathogenic microorganisms (29).

6. Antimicrobial substances

Since LAB are found in widespread environments, they have developed methods to inhibit the growth of competitive organisms. Fermented foods preserve well because of the souring effect that LAB imparts to the food by the conversion of sugars to organic acids (109). LAB are also capable of producing inhibitory substances other than organic acids that are antagonistic towards other micro-organisms. These substances are produced in much smaller amounts and include bacteriocins, hydrogen peroxide, diacetyl, volatile fatty acids and secondary reaction products such as hypothiocyanite generated by the action of the enzyme, lactoperoxidase, on hydrogen peroxide and thiocyanite (11, 109).

7. Hydrogen Peroxide

Accumulation of hydrogen peroxide can occur because LAB do not possess catalase. Hydrogen peroxide is produced by several LAB species, including lactobacilli, lactococci and pediococci and it inhibits the growth of many bacteria, especially pathogenic Gram-negative types. The antimicrobial effect of H_2O_2 is based on its oxidative properties that results in irreversible changes in the microbial cell membrane. Peroxide has been implicated in the inhibition of *Staphylococcus aureus*. It can react with other components to form inhibitory substances. In raw milk, hydrogen peroxide can react with endogenous

thiocyanate, which is then catalyzed by lactoperoxidase to form intermediary oxidation products inhibitory to microorganisms. These oxidation products can inhibit bacterial growth, and may be bactericidal at a low pH. This is called the lactoperoxidase antibacterial system (29, 40, 109).

8. Diacetyl

Diacetyl (2,3-butanedione) is a metabolic end product of LAB that is synthesized from the intermediary metabolic pyruvate. It is best known for the buttery aroma that it imparts to cultured dairy products. The compound is produced by a variety of genera, including *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus*. *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* spp. can use citrate in milk to produce diacetyl. Diacetyl is inhibitory to yeasts and Gram-negative bacteria at a level of 200 μg/mL. A level of 300 μg/mL is inhibitory to non-lactic Gram-positive bacteria. LAB are not inhibited at concentrations ≤350μg/mL (40, 109).

9. Acetaldehyde

Acetaldehyde is produced in certain dairy fermentations, notably yogurt, where it arises from the activity of the enzyme threonine aldolase in *L. bulgaricus*. This enzyme cleaves threonine into acetaldehyde and glycine. 44ppm of acetaldehyde are able to inhibit cell division in *E. coli* (109).

BACTERIONS PRODUCED BY LAB

Bacteriocins can be defined as biologically active proteins or protein complexes (protein aggregates, lipocarbohydrate proteins, glycoproteins, etc.), displaying a bactericidal mode of action towards genetically closely related species (21). However, recently the inhibitory spectrum of these molecules has been widened to include various Gram-negative bacteria and food pathogens (109). Bacteriocins form a heterogenous group with respect to the bacterial species responsible for its production, molecular size, physical and chemical properties, stability, antimicrobial spectrum, mode of action (21).

Klaenhammer (1993) defined four distinct classes of LAB bacteriocins: Class I: lantibiotics, Class II: small (<10kDa) heat-stable membrane-active peptides, Class III: large (>30kDa), heat-labile proteins, and Class IV: complex bacteriocins. Class II bacteriocins were further divided into *Listeria*-active peptides with an N-terminal consensus sequence (Class IIa), poration complexes requiring two different peptides for activity (Class IIb) and thiol-activated peptides that require reduced cysteine residues for activity (Class IIc). Currently, bacteriocins are classified as follows (Moll *et al*., 1999; Nes *et al*., 1996). Class I (lantibiotics), which is further divided into type A lantibiotics and type B lantibiotics. Type A lantibiotics are elongated, cationic, pore forming peptides. Type B lantibiotics are compact, with globular structures, are enzyme inhibitors and are immunologically active. Class II, the small heat-stable non-lanthionine peptides, is divided into four groups. Class IIa consists of *Listeria*-active peptides with an N-terminal consensus sequence. Class IIb are two-peptide bacteriocins. Class IIc contains *sec*-dependant bacteriocins, and Class IId contains the small heat-stable non-lanthionine bacteriocins that do not belong to any of the three groups within Class II. Class III consists of large heat labile bacteriocins (73). Most of the bacteriocins belong to Class I or II and most of the research has focussed on these two classes (79).

Bacteriocins produced by LAB either have a bactericidal or bacteriostatic effect on sensitive cells, eventually resulting in cell death. The primary target of bacteriocins produced by LAB is the cytoplasmic membrane. These peptides initiate reactions altering the membrane permeability which disturbes membrane transport or dissipating the proton motive force. Because of this, energy production and the biosynthesis of proteins or nucleic acids are inhibited (21).

Bacteriocins are either chromosomally or plasmid encoded and many bacteriocin determinants appear to be organized in an operon. It has been postulated that the immunity gene, encoding the immunity protein in the bacteriocin-resistant producing cell, forms part of the bacteriocin operon. Furthermore, most of the bacteriocins are produced as precursor proteins (pre-peptides) with helical N-terminal extensions of 18-24 amino acids, which are removed post-translationally to yield the mature bacteriocin. The leader peptide is cleaved off prior to excretion into the microbial environment of the mature peptide. The leader peptide serves as recognition signal and directs the prebacteriocin to

the ABC transporter. Furthermore the leader peptide renders the bacteriocin inactive inside the cell (21)

From an application viewpoint bacteriocins have an interesting potential. They could be used as specific markers in epidemiological studies and as prophylactic tools. The genes of bacteriocin production and immunity could be used for the development of food-grade cloning vectors, which would selectively be retained within the bacterial population. Bacteriocins may also be used food preservatives (21).

Bacteriocins produced by *Enterococcus* spp.

Enterococci belong to the clostridial subdivision of Gram-positive bacteria, together with the other genera of LAB. Since 1984, chemotaxonomic and phylogenetic studies have resulted in the assignment of 19 species to the genus (32). They constitute a large proportion of the autochthonous bacteria associated with the mammalian gastrointestinal tract (GIT). In humans they have also been isolated from the urinary and hepatobiliary tracts and the vagina. They are among the first group of bacteria colonizing the microbial ecosystem in ruminants. Other sources include raw milk, soil, surface waters, plants and vegetables. In food they occur in meats because of contamination by enterococci in the GIT at the time slaughter. They are also found in a variety of cheeses, especially artisanal cheeses produced in Southern Europe and Mediterranean countries (32, 101).

Enterococci produce a wider variety of antimicrobial peptides than has been described for strains of most other LAB genera. Bacteriocins produced by *Enterococcus* spp. belong to class II although some have been characterized as class I or cyclic enterocins (30, 32). However, several bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* such as enterococcin Sf25, enterocin 81, enterocin 01, enterocin EJ97, enterocins 1071 A an 1071B, and enterocin 012 either do not belong in any of the currently described groups, or have not been fully characterized (60). Bacteriocins of some strains of *E. faecalis* are encoded by pheromone responsive conjugative plasmids. All enterocins share a number of common characteristics such as heat stability, stability over a broad pH range and antimicrobial activity towards *Listeria monocytogenes* (101). The anti-*Listeria*

activity may be explained by the fact that enterococci and listeriae are phylogenetically closely related (32).

Enterococcus casseliflavus EC24, *E. faecium* CCM 4231 and *E. faecium* AL86 produce bacteriocins with a broad antimicrobial spectrum including some Gram-negative species. The peptides are heat stable, sensitive to proteolytic enzymes and remain active even after storage at –20^{o}C and/or –80^{o}C. Optimum production was recorded during exponential growth and between pH 4.5 and 7.0 they are bacteriostatic against listeria (62).

Bacteriocins from *E. casseliflavus* strains EC4, EC32, EC5, EC2, *Enterococcus* sp. EV10 and *E. faecalis* strains V24, V6, ES49, EF22, isolated from cattle dung water and/or -waste, have a broad spectrum of activity including acivity against *Escherichia coli* isolated from the hemorrhagic uremic syndrome of children (62).

E. faecium strains EK13 and AL41 produce small peptides that are heat-stable, with optimum production between pH 5.5 and 6.5. Their antimicrobial spectrum includes *Bacillus cereus*, *Enterococcus avium*, *E. caselliflavus*, *E. faecalis*, *E. faecium*, *Enterococcus* sp., *Listeria innocua*, *L. monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus lentus*, *Staphylococcus vitulinus*, *Staphylococcus xylosus* and *Streptococcus bovis* (62).

E. faecalis V24 produces a bacteriocin-like substance active against strains of *Enterococcus durans*, *Enterococcus malodoratus*, *E. casseliflavus*, *E. faecium*, *E. faecalis*, *S. aureus*, *S. xylosus*, *L. monocytogenes* and Gram- negative bacteria such as *Shigella* sp., *Salmonella* sp. and *Enterobacter cloacae*. The substance remains stable for one week at both room temperature and –20^{o}C. A decrease in activity up to 20000 AU/ml is noted at –80^{o}C. Bacteriocin activity is not affected by catalase and decreases after treatment with hydrolytic enzymes, but is not lost. The peptide is resistant at 37^{o}C for 24 h, and at 60^{o}C for 1 h. Activity is also retained after boiling for 30 min and is stable from pH 4 to 7 (61).

E. faecalis strains BFE 1113, BFE 1229 and BFE 1263 isolated from the faeces of minipigs all produce a group II bacteriocin. These strains display a narrow range of antimicrobial activity, mainly towards other enterococci. Enterocin production starts during early exponential growth and maximum production is recorded at the beginning of the stationary phase. The enterocin is sensitive to α-chymotrypsin, proteinase K, pronase, trypsin, papain and pepsin but are not affected by lipase, lysozyme and catalase. Low levels of bacteriocin activity are retained after 15 min at 121^{o}C. The enterocin remain active from pH 2.0 to 11.0 and bacteriocin levels are highest in a pH range from 6.0 to 8.0. The molecular weight of the bacteriocin is 3.4 kDa and a bactericidal mode of action is displayed towards sensitive cells (22).

Bacteriocins JBL1061, JBL1083 and JBL1351 produced by *E. faecium* strains JBL1061, JBL1083 and JBL1351 respectively, are active against several Gram-positive bacteria, including strains of *E. faecalis*, *E. faecium*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus sakei* and *L. monocytogenes*. The inhibitory spectra of the three bacteriocins are relatively similar and with the exception of partial inhibition of strain JBL1061 by JBL1351, the bacteriocins have little effect on each other, suggesting that the bacteriocins may be identical. The antilisterial activities of the bacteriocins were not significantly affected by RNase, catalase, heat (100^{o}C, 30 min) or pH, whereas all three were slightly sensitive to chloroform. The bacteriocins are digested by β-chymotrypsin, ficin, pronase E, proteinase K and trypsin, but not pepsin. Antilisterial activity was also retained after storage at –20^{o}C and 4^{o}C at pH 4.5 and 6.8 for at least 3 months and at 37^{o}C for 24 h at pH 4.5 and 6.8. The apparent absence of plasmids in JBL1061 and JBL1083 suggests that the genes for bacteriocin production and immunity are located on the chromosome of strains JBL1061 and JBL1083. Although strain JBL1351 does possess a plasmid, the genetic determinants related to the listericidal activity are yet to be localized. This listericidal effect is, however, not accompanied by lysis of the cells (2).

E. faecium strains RZS C5 and RZS C13, isolated from cheese and silage, respectively, produce bacteriocins active against *L. monocytogenes* and *L. mesenteroides* subsp. *dextranicum*. Weak inhibition was also measured against *Clostridium butyricum* and *Clostridium perfringens*. The antimicrobial activity was completely lost after incubation with trypsin, α-chymotrypsin, pronase E and proteinase K. With pepsin, activity was lost

partly. Strain RZS C5 contains a 4 kb plasmid, while strain RZS C13 contains a 23 kb plasmid. Heating for 15 min at 100^{o}C at pH 4.5 reduced the residual activity by at least 50%. A similar reduction is encountered at pH 6.5, while this is even more pronounced at pH 8.5. Both bacteriocin preparations remained stable, independent of the pH or the storage temperature for at least 14 days. The molecular weight of the two bacteriocins is estimated at ± 3.0 kDa (115).

E. faecium S-34 produces a bacteriocin of 30 kDa (66). *E. faecalis* E23 produces a bacteriocin with a molecular mass of 290 kDa (66). *E. faecalis* 226 produces a bacteriocin that is encoded on a 5.2 kb conjugative plasmid (33).

Enterocins L50A and L50B

These novel bacteriocins are produced by *E. faecium* strains L50, 6T1a and BFE 1072 (32). Enterocins L50A and L50B resemble the two-peptide bacteriocins of class IIb (22). The bacteriocins are not post-translationally modified and do not require the presence of a leader or signal peptide for secretion. Each peptide has antimicrobial activity on its own, with enterocin L50A being more active. However, when acting together synergistic activity is shown (14, 32). Enterocins L50A and L50B display a broad antimicrobial spectrum, which includes foodborne pathogens, such as *Clostridium botulinum C. perfringens, L. monocytogenes*, *S. aureus*, *B. cereus*, as well as human and animal pathogens such as *Streptococcus pneumoniae*, *Streptococcus oralis*, *Streptococcus parasanguis* and *Streptococcus agalactiae* (13). The bacteriocins share homology with the staphylococcal haemolysins, yet they do not show haemolytic activity (22, 32). Bacteriocins L50A and L50B have a bactericidal, mode of action and are encoded by a 50 kb plasmid, pCIZ1 (26). The mature L50A peptide contains 44 amino acids with a molecular mass of 5190 Da, while peptide L50B contains 43 amino acid with a mass of 5178 Da (32). *E. faecium* L50 also produce enterocins P and a novel unmodified non-pediocin-like bacteriocin termed enterocin Q. In this strain enterocin P is encoded by the same plasmid pCIZ1 that encodes enterocin L50. Enterocin Q is encoded by a second plasmid, pCIZ2, and consists of 34 amino acids with a molecular mass of 3980 Da. The bacteriocin is also synthesized without an N-terminal leader sequence or signal peptide. Antimicrobial activity is lost by protease treatment, but the peptide withstands 100^{o}C for 5 min. Bacteriocin production by *E. faecium* L50 showed that enterocins P and Q are

produced in the temperature range from 16°C to 47°C. Maximal production is at 47°C for enterocin P and between 37°C to 47°C for enterocin Q. Enterocins L50A and L50B are maximally synthesized at 16°C to 25°C and are not detected at 37°C or above. *E. faecium* 27 which harbours both plasmids contains the structural genes for enterocins L50A and L50B as well as enterocin P and Q (13). *E. faecium* B2, isolated from Malaysian tempeh, produce a bacteriocin termed enterocin B2, which is identical to enterocin L50, although the structural genes encoding the bacteriocins are chromosomally located (74).

Enterocin P

The bacteriocin is produced by *E. faecium* P13, isolated from Spanish dry-fermented sausage, and other related strains (12, 45). Enterocin P is an amphipatic, cationic pediocin-like peptide composed of 44 amino acids and has a molecular weight of 4493 Da (32, 44, 74). The structural gene of enterocin P is located chromosomally and the bacteriocin is secreted via the *sec* system. Enterocin P is classified as a Class IIc bacteriocin (32, 74). The bacteriocin is unique in having both the consensus sequence YGNGVXC and a wide bactericidal inhibitory spectrum against foodborne Gram-positive pathogenic bacteria, including *S. aureus* and *L. monocytogenes* as well as organisms such as *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *L. sakei*, *L. lactis*, *E. faecalis*, *E. faecium*, *L. innocua*, *B. cereus*, *C. perfringens*, *C. botulinum* and *Propionibacterium* sp (26, 45). Depending on the buffer used, enterocin P dissipates the transmembrane potential. The bacteriocin efficiently elicits the efflux of potassium ions, but not of intracellularly accumulated anions like phosphate and glutamate. This indicates that enterocin P forms specific, potassium ion-conducting pores in the cytoplasmic membrane of target cells (45). Maximum antimicrobial activity is obtained during late logarithmic growth with a drop during stationary growth. No decrease in activity is detected after 5 days at 16°C. Enterocin P is protease sensitive. The peptide retains activity after exposure to heat of 100°C for 60 min and 121°C for 15 min and can withstand exposure to pH values between 2.0 and 11.0, freeze thawing, lyophilization and long-term storage at 4 and –20°C (12). Other enterocin P-producing strains are *E. faecium* AA13 and *E. faecium* G16 (13). *E. faecium* B1, isolated from Malaysian tempeh produce a bacteriocin termed enterocin B1 identical to enterocin P (74).

Enterocins A and B

The bacteriocins are produced by *E. faecium* strains WHE 81, CTC492, T136, BFE 900, BFE 1170 and BFE 1228. Strains WHE 81 and CTC492 were isolated from Muster cheese; strain T136 from fermented Spanish sausage; strain BFE 900 from black olives and strains BFE 1170 and BFE 1228 from the faeces of minipigs. Enterocin A is a class IIa, pediocin-like bacteriocin with a molecular weight of 4833 Da and a pI of 9.07 (5, 10, 27, 32). The bacteriocin remains active from pH 4.0 to 9.0. (28). The antimicrobial is translated as a prepeptide of 65 residues, which is then processed to yield a mature bacteriocin of 47 residues. The 18-residue N-terminal extension is a typical double-glycine leader sequence (27). Enterocin B is a small nonpediocin-like class IIc bacteriocin (5, 32, 76). The bacteriocin is secreted *sec*-independently and is a one-peptide bacteriocin lacking the YGNGVX_{aa}C-motif. The bacteriocin's prepeptide includes a 53-residue sequence and a double-glycine leader sequence of 18 residues in the putative N-terminus. Enterocin B has a molecular weight of 5462.2 Da and a pI of 9.25 (5, 27, 32). The bacteriocin is heat stable and retained some activity after incubation at 100^{o}C for 20 min. Bacteriocins A and B are bactericidal and inhibit spoilage and foodborne pathogens such as *Clostridium tyrobutyricum*, *Clostridium sporogenes*, *Propionibacterium* spp., *L. monocytogenes*, and *S. aureus* (10, 27). Production of enterocins A and B is regulated by an internal induction factor, enterocin F (5). The genes encoding enterocins A and B are chromosomally located. The enterocins produced by *E. faecium* strains T136 and DPC1146, isolated from Spanish fermented sausages, and *E. faecium* BC25, isolated from the rumen of a cow, are suspected to be identical to enterocin A (27, 76).

Enterocin CCM 4231

The bacteriocin is a small, thermostable hydrophobic substance with a broad antimicrobial spectrum against Gram-positive bacteria, including *L. monocytogenes* and Gram-negative bacteria. Enterocin CCM 4231 is produced by *E. faecium* CCM 4231 (58, 59). The bacteriocin controlled *L. monocytogenes* in experimentally contaminated dry fermented Hornád salami and exhibited an anti-listerial effect during Saint-Paulin cheese manufacture (59, 63). Enterocin CCM 4231 lowered the counts of enterococci and staphylococci significantly in a cattle slurry environment, while counts of *E. coli*, listeriae and pseudomonads were also decreased (60). The bacteriocin was able to eliminate enterococci and staphylococci in "bryndza", while a reduction in *E. coli* was also noted.

The level of experimentally innoculated *L. innocua* strain was also reduced, although not completely inhibited (57). The inhibitory effect of enterocin CCM 4231 have also been used to control the growth of *L. monocytogenes* and *S. aureus* in soy milk, although the latter was not fully inhibited. The inhibitory effect against *L. monocytogenes* was bactericidal, while *S. aureus* was influenced bacteriostatically (58). The inhibitory effect of enterocin CCM 4231 has also been observed in environments such as the rumen fluid (60).

Enterocin 1146

E. faecium DPC 1146 produces enterocin 1146 active against *L. monocytogenes*, *L. innocua* and *L. sakei*. Enterocin 1146 has a bactericidal mode of action. The bacteriocin is produced during late logarithmic growth and the highest antimicrobial activity is obtained after 6 h of fermentation at 37^{o}C (21, 33). The optimal pH for enterocin 1146 production is 5.5 (96). Enterocin 1146 samples lose activity rapidly at 37^{o}C and the activity also decreases after 30 min at 80^{o}C and 100^{o}C (21, 22). The activity is stable for long periods (months) at –20^{o}C. The bacteriocin is sensitive to pronase E, pepsin, trypsin, α-chymotrypsin and proteinase K, but insensitive to catalase (21). Enterocin 1146 has a molecular mass of 3000 Da and can thus be classified as a low molecular weight bacteriocin (21, 115). Heating above 60^{o}C causes partial or total loss, depending on the pH of the enterocin preparation. The bacteriocin is more stable at acid pH. Inactivation at neutral or alkaline pH is partially reversible. Elimination of the only plasmid present in *E. faecalis* DPC 1146 do not affect production and immunity of the bacteriocin, which could indicate that these traits are either chromosomally encoded or present on a stable but undetectable plasmid (21).

Enterocin ON-157

E. faecium NIAI 157, isolated from plant materials such as silages and fermented vegetables, produces enterocin ON-157 which is inhibitory towards strains of *Lactobacillus plantarum*, *L. sakei, E. faecalis*, *E. faecium*, *E. hirae, L. mesenteroides*, *P. acidilactici*, *P. pentosaceus* and *L. monocytogenes*. Enterocin ON-157 is produced during logarithmic growth with maximum yield during stationary growth, which is after 6h at a pH of 5.4. Bacteriocin activity is stable at 30^{o}C for 1 h at pH range 2 to 7. However, the activity is unstable at 100^{o}C for 30 min at pH 6 to 7, while it is stable at pH 2 to 5 under

the same conditions. Activity is completely inhibited by α-chymotrypsin and pepsin and partly inhibited by pronase and trypsin. Activity is also completely inhibited by α-amylase and partly by α-glucosidase. Enterocin ON-157 is associated with a 49 kb plasmid (pEN100) and has a molecular weight of 2500 Da. The bacteriocin has a bactericidal mode of action and is described as a glycoprotein (82).

Enterocin I

This novel bacteriocin is produced by *E. faecium* 6T1a, a strain originally isolated from a Spanish-style green olive fermentation. Enterocin I is active against strains of *Lactobacillus*, *Propionibacterium*, *Clostridium*, *Bacillus, E. faecalis, L. lactis*, *P. pentosaceus*, *L. monocytogenes* and *L. innocua*. The bacteriocin is stable at 100^{o}C for 5 min, but was partially inactivated by autoclaving. The inhibitory activity is abolished after treatment with α-chymotrypsin, pronase E, proteinase K, subtilopeptidase A, thermolysin and trypsin. Other enzymes such as α-amylase, catalase, ficin, lysozyme and RNase A, however, did not affect the activity. Maximum inhibitory activity is observed during early stationary growth. Enterocin I consists of 44 amino acids and has a molecular size of 5 kDa. Bacteriocin production and immunity are encoded by plasmid pEF1 (18). However, enterocin I is identical to enterocin L50 (66).

Enterocin 01

E. faecium NA01, isolated from "wara", produces enterocin 01 which is inhibitory towards *Lactobacillus viridescens*, *L. sakei*, *Listeria welshimeri*, *L. monocytogenes*, *L. innocua*, *L. lactis* subsp. *lactis* and *E. faecalis*. The bacteriocin is inactivated by α-chymotrypsin and proteinase K, but not by trypsin and pepsin. The inhibitory activity is stable at 100^{o}C for 5 min and at pH 2.0 to 6.0. However, heating for a longer period at this temperature results in inactivation (85). Production of enterocin 01 is encoded on a plasmid (33).

Bacteriocin 7C5

E. faecium 7C5 produces bacteriocin 7C5, which inhibits the growth of *L. monocytogenes* on the surface of Tallegio cheese (101). The peptide is inactivated by α-chymotrypsin, proteinase K and trypsin. Bacteriocin 7C5 is heat stable and is produced during late logarithmic growth. The bacteriocin has a weak stability at pH 5.0 (33).

Bacteriocin RC714

The vancomycin-resistant *E. faecium* RC714, isolated from a human exudate sample, produces bacteriocin RC714. The bacteriocin shows inhibitory activity against *Listeria murrayi*, *Listeria grayi*, *L. monocytogenes*, *L. innocua*, *Lactobacillus paracasei*, *L. plantarum*, *E. faecalis*, *E. faecium*, *P. pentosaceus* and *Leuconostoc* sp. The bacteriocin is resistant to heat (100^{o}C for 20 min) and is stable from pH 3 to 11. Bacteriocin RC714 is susceptible to trypsin, α-chymotrypsin, papain, alkaline protease and proteinase K, but resistant to lysozyme. Bacteriocin RC714 is a class II bacteriocin. According to the amino acid sequence, bacteriocin RC714 is 88% homologous and 92% similar to bacteriocin 31 from an *E. faecalis* strain. A difference of 5 out of 42 amino acids with respect to bacteriocin 31 is observed. On this basis bacteriocin RC714 could represent a new enterocin different from bacteriocin 31 (20).

Enterocin CRL 35

E. faecium CRL 35, isolated from a regional cheese, produces a pediocin-like Class IIa bacteriocin, termed enterocin CRL 35. The bacteriocin has a molecular weight of 3500 Da and is inhibitory towards foodborne pathogens, including *L. monocytogenes*. Enterocin CRL 35 also has antiviral activity against thymidine-kinase (tk^{+}) and deficient (tk^{-}) strains of herpes simplex (HSV) type 1 and 2 in Vero and BHK-21 cells. The compound inhibits viral multiplication and has no virucidal effect. Antibacterial activity is destroyed by incubation with protease IV or drastic treatment such as raising the pH to a value of 13 or heating at 100^{o}C for 10 min (116).

Enterocins EIA and EIB

The bacteriocins are produced by *E. faecium* strain EI and are active against certain strains of enterococci, *Streptococcus salivarius, Clostridium septicum*, *C. perfringens* and *L. monocytogenes* (55). Enterocin EIA is pepsin-sensitive, while enterocin EIB is resistant to trypsin (21, 55). The MW for EIB is greater than 4000 Da and about 10 kDa for EIA (115). The action of purified enterocin EIA and EIB is primarily bactericidal, but is not accompanied by cell lysis (55).

Enterocin 81

E. faecium WHE 81, isolated from cheese, produces enterocin 81 which is inhibitory towards certain enterococcal strains, *Listeria seeligeri*, *L. monocytogenes*, *L. innocua* and *L. plantarum*. The bacteriocin has a bactericidal, but not bacteriolytic, mode of action. Enterocin 81 is completely inactivated by α-chymotrypsin, ficin, nagarse, pepsin, pronase E, proteinase K and trypsin, but not by catalase. The bacteriocin is equally active at pH values ranging from 4.0 to 8.0. (26).

Enterococcin Sf3

E. faecium 3, isolated from human sources, produces a 9 kDa bacteriocin designated enterococcin Sf3. Production and immunity is encoded on a 5 kb plasmid. Enterococcin Sf3 has a narrow inhibitory spectrum, is thermostable, and is sensitive to proteolytic enzymes (21, 33).

Enterocin CRL 50

The bacteriocin is produced by a *E. faecium* strain, isolated from Argentinian cheese (66).

Bacteriocin Bc-48

E. faecalis S-48 and its mutant B-48-28 secretes bacteriocin Bc-48. The bacteriocin is highly sensitive to temperature. Partial inactivation occurs during incubation at 45^{o}C for 60 min., and all activity is lost upon incubation at 50^{o}C for 60 min. However, bacteriocin preparations are stable at 4^{o}C for 1 week or at –20^{o}C for 3 months without any loss in activity. Bacteriocin Bc-48 is stable in a pH range between 5.5 and 9.0, while a more acidic or basic pH causes inactivation. Bacteriocin activity is resistant to lipase, lysozyme, DNase, RNase, catalase or alkaline phosphatase. However, the proteolytic enzymes trypsin, pronase or protease type XI cause complete inactivation. The inhibitory spectrum of bacteriocin Bc-48 is restricted to related strains. Only strains of *E. faecalis* are inhibited. Not all strains are equally sensitive to bacteriocin Bc-48. The bacteriocin is produced during logarithmic growth, but production stops once the cultures reaches stationary phase. The peptide has a molecular mass of 80 kDa and a pI of 7.0. Bacteriocin Bc-48 inhibits protein synthesis, but does not affect amino acid uptake, which suggests the contribution of bacteriolysis to cell death. The production and immunity of bacteriocin Bc-48 are encoded by a 90 kb plasmid (66).

Peptide AS-48

Another inhibitory agent produced by *E. faecalis* S-48 is the peptide antibiotic AS-48 (21, 66). Peptide AS-48 production occurs during exponential growth (68). Production and immunity are encoded by a 58 kb plasmid, pMB2 (21, 66). The inhibitor is a 8 kDa protein, has a bactericidal effect with a wide inhibitory spectrum including Gram-positive and Gram-negative bacteria (21). Unlike most other bacteriocins, this peptide is a cyclic molecule arising from a 'tail-to-head' linkage that results from post-translational modification of the primary product during secretion (32, 68). Peptide AS-48 is thermolabile and sensitive to proteolytic enzymes (21). It has a molecular mass of 7.4 kDa, a basic isoelectric point and a high stability to acid pH (66). The site of action is the bacterial membrane, and prolonged exposure leads to cell lysis as a secondary effect, due to the activation of autolysins (21). *E. faecalis* 39-5Sa produces bacteriocin 21 which is identical to peptide AS-48. Bacteriocin 21 is encoded on a 59 kb plasmid, pPD1 (48).

Enterococcin EFS2

E. faecalis EFS2, isolated from cheese, produces enterococcin EFS2 inhibitory towards *Leuconostoc lactis*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *paramesenteroides*, *Listeria ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. welshmeri*, *L. innocua*, *C. tyrobutyricum*, *L. fermentum*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* and *S. aureus*. Enterococcin EFS2 induces pore formation in the plasma membrane, efflux of K^+ ions and ATP, and cell lysis. Maximum bacteriocin activity is found at the beginning of stationary growth. At pH 7.0 the bacteriocin activity is highest at 35^{o}C and lost at 15^{o}C. The peptide is more active at pH 6.0, 7.0 and 8.0 than at pH 4.5 at 30^{o}C (67). Enterococcin EFS2 is a strongly hydrophobic molecule consisting of 67 amino acids and a molecular weight of 7149.6 Da. The bacteriocin has several features identical to peptide AS-48, including molecular mass, amino acid composition and cyclic structure which could indicate that enterococcin EFS2 might in fact be peptide AS-48 (68).

Enterocin 4

E. faecalis INIA 4, isolated from raw ewe's milk and Manchego cheese, also produce a cyclic peptide, termed enterocin 4, with bactericidal activity against histamine-producing *Lactobacillus buchneri, L. brevis, Salmonella cholerasuis, E. faecalis, E. faecium, L.*

monocytogenes, *L. innocua*, *S. aureus* and strains of *C. tyrobutyricum*. Enterocin 4 is also active against *Bacillus laterosporus*, *Enterococcus durans*, *Corynebacterium laevaniformans* and energized cells of *L. buchneri* St2A (49). Enterocin 4 decreased the counts of *L. monocytogenes* Ohio, but was unable to inhibit *L. monocytogenes* Scott A, at least in the pH range 5.2 to 5.4, during the manufacture and ripening of Manchego cheese (81). Enterocin 4 displays no activity against Gram-negative bacteria (49). However, it is possible that enterocin 4 was not tested at concentrations high enough to inhibit Gram-negative bacteria. The structural gene encoding enterocin 4 is identical to that encoding peptide AS-48, which suggests that enterocin 4 may also be peptide AS-48 (68).

Enterocins 1071A and 1071B

E. faecalis BFE 1071, isolated from the faeces of minipigs, produces enterocins 1071A and 1071B inhibitory towards strains of *C. tyrobutyricum* and *E. faecalis*. The bacteriocins are also active against *Lactobacillus salivarius* subsp. *salivarius, Peptostreptococcus aerogenes*, *Propionibacterium freudenreichii* subsp. *shermanii*, *E. durans*, *L. innocua*, *Micrococcus* sp. DF 132 and *S. agalactiae*. Enterocins 1071A and 1071B are resistant to heat of 100^{o}C for 60 min. Approximately 50% of the antibacterial activity is retained after 15 min at 121^{o}C. The enterocins are not drastically affected by incubation at pH values ranging from 3 to 12, but they are sensitive to α-chymotrypsin, papain, pepsin, pronase, proteinase K and trypsin. The genes encoding enterocins 1071A and 1071B are located on a 50 kb plasmid (pEF1071) giving rise to 39- and 34 amino acid peptides, respectively. Enterocins 1071A and 1071B are 4.825 kDa and 3.899 kDa in sizes, respectively (6).

Enterocin EJ97

E. faecalis EJ97, isolated from municipal waste waters, produces enterocin EJ97 inhibitory to *E. faecalis, E. faecium* and *Bacillus stearothermophilus*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus megaterium*, *Bacillus subtilis*, *Paenibacillus macerans*, *Listeria grayi*, *L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. murrayi*, *L. welshmeri* and *S. aureus*. Enterocin EJ97 is produced during exponential growth with maximum activity obtained after 7 to 8 h. The bacteriocin retained 100% of its initial activity in a pH range from 2.0 to 9.5, and at temperatures between 50 and 80^{o}C, and after being stored for 1 week at 4^{o}C or for 6 months at –20^{o}C or –70^{o}C. A 30% loss in activity of occurs after

heating at 100°C for 30 min. Enterocin EJ97 is totally inactivated by trypsin and pronase but is not affected by EDTA or by SDS and Tween 80. No loss of bacteriocin activity is recorded after being heated in the presence of DL-dithiothreitol or 2-mercaptoethanol. Enterocin EJ97 is an ampiphatic, hydrophobic molecule with a molecular mass of 5.340 kDa (35).

Enterocin 226NWC

E. faecalis 226, isolated from natural whey cultures, produces enterocin 226NWC inhibitory towards *L. monocytogenes*, *L. innocua*, *E. faecalis* and *E. casseliflavus*. Enterocin 226NWC has a bactericidal effect. The bacteriocin is produced after 6 h during mid-logarithmic growth with maximum activity reached after 10 h in late logarithmic to early stationary growth. The bacteriocin is stable at 90°C for 30 min, with 50% activity remaining after 30 min at 100°C. The peptide is completely inactivated after autoclaving (121°C for 15 min) and after treatment with α-chymotrypsin, trypsin, pronase E, papain and proteinase K. Treatment with pepsin, lysozyme, catalase, lipase (type VII) and α-amylase also have no effect on the antibacterial activity of enterocin 226NWC. Production of the bacteriocin is encoded by a 5.2 kb conjugative plasmid. The peptide is 5800 Da in size (113).

Enterocin SE-K4

E. faecalis K-4, isolated from grass silage, produce enterocin SE-K4 with bactericidal activity against *Bacillus subtilis*, *Clostridium beijerinckii, E. faecium*, *E. faecalis* and *L. monocytogenes*. Enterocin SE-K4 is produced during exponential growth. Since *E. faecalis* SE-K4 is a thermophilic *Enterococcus*, maximal concentrations of the bacteriocin is produced at 43 to 45°C. It has a molecular mass of 5362.2 Da. The bacteriocin has high heat stability with 50% residual activity obtained after heating at 100°C for 60 min. More than 25% of its maximal activity is retained between pH 3.0 and 11.0 (23).

Haemolysin/bacteriocin

E. faecalis subsp. *zymogenes* DS16 produces a haemolysin/bacteriocin, which is a two component bacteriocin with both haemolytic and bacteriocin activity (32, 48). The

bacteriocin is encoded on a 58 kb conjugative plasmid, pAD1 (6). It contains β-methyllanthionine residues, suggesting that it is a lantibiotic (32, 48).

Streptocin E-1

E. faecalis E-1, isolated from clinical material (conjunctiva), produces streptocin E-1 which is particularly active towards *Diplococcus pneumoniae*. The peptide also inhibits non-hemolytic strains of enterococci and strains from the Viridans group of streptococci. The bacteriocin is heat labile (80^{o}C, 20 min), chloroform- and trypsin-resistant and has a molecular mass of approximately 30 kDa (21).

Bacteriocin 31

E. faecalis YI717 produces bacteriocin 31 which inhibits *E. hirae*, *E. faecium* and *L. monocytogenes*. The bacteriocin is heat stable, is encoded by a 57.5 kb conjugative plasmid, pYI17, and contains 43 amino acids. Bacteriocin 31 is similar to enterocin P. It is a pediocin-like, class II bacteriocin secreted by the *sec*-pathway (32, 107).

Mundticin

Enterococcus mundtii ATO6, isolated from fresh chicory endive, produces mundticin which prevents the outgrowth of spores and vegetative cells of toxin-producing strains of *C. botulinum.* Mundticin inhibits the growth of *Carnobacterium piscicola, Pediococcus dextrinicus*, *P. pentosaceus, Leuconostoc paramesenteroides*, *L. mesenteroides*, *L. salivarius*, *L. sakei*, *E. faecalis*, *E. hirae*, *L. innocua*, and Gram-negative bacteria and fungi. Mundticin is a class IIa bacteriocin. Proteolytic treatment of the bacteriocin results in total loss of the activity. The antimicrobial activity of the peptide is not affected by heating for 15 min at 100^{o}C, but prolonged heating for 1 h at 100^{o}C results in a 50% loss of activity. The peptide is stable from pH 1 to 10 for 14 h at 4^{o}C. Mundticin is a positively charged, hydrophobic, 43 amino acid peptide, with a highly conserved YGNGV motif at positions 3-7 with an average mass of 4278.8 Da. It exerts its action on the cytoplasmic membrane, which involves the dissipation of the membrane potential and a depletion of the intracellular ATP pools. The observed reduction of the ATP pool size can be explained by the accelerated consumption of ATP to regenerate the decreased PMF and/or by a shift in the ATP hydrolysis equilibrium resulting from phosphate efflux (7).

Enterocin 012

Enterococcus gallinarum 012, isolated from the duodenum of ostrich, produces enterocin 012 which inhibits *Lactobacillus acidophilus*, *L. sakei*, *Propionibacterium acidipropionici*, *Propionibacterium* sp., *E. faecalis*, *L. innocua*, *C. perfringens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *E. coli*. The bacteriocin has a bactericidal and bacteriolytic mode of action. Enterocin 012 is produced during mid-logarithmic growth with further increases into stationary growth. No bacteriocin activity is lost after heat treatment (30 min at 60^{o}C). A loss of 50% and 75% was recorded after 30 min at 80^{o}C and 121^{o}C for 15 min, respectively. Incubation at pH 6.0 to 10.0 at 4^{o}C did not result in any loss of activity. A loss of 50% activity was recorded when incubated at a pH range of 1.0 to 5.0 and at pH 11.0 and 12.0, respectively. Enterocin 012 is resistant to treatment with lysozyme, catalase, lipase and papain, but resistant to proteinase K, α-chymotrypsin, trypsin and pepsin. The peptide is hydrophobic with a molecular mass of 3.4 kDa (48).

Bacteriocins produced by *Lactobacillus* spp.

Lactobacilli are widespread in nature and consist of 64 valid species (21, 100). They are present in a variety of habitats, such as mucosal membranes of man and animal, e.g. the oral cavity, intestines and vagina (118). Animals such as pigs, chickens, dogs, mice, rats and hamsters harbour predominantly *Lactobacillus* flora in their intestines (117). They are also found on plants or material of plant origin, in manure and man-made habitats such as sewage and fermenting or spoiled food (118). Bacteriocin-producing *Lactobacillus* spp. are present in many foods, including salads and cheese as well as dry and fermented sausages. Most of the characterized bacteriocins from *Lactobacillus* are classified as Class I or II (21). A number of them are inhibitory to *Listeria* spp. (111).

L. plantarum BF001, isolated from spoiled cattle fish, produces plantaracin F which inhibits several strains of LAB and foodborne pathogens such as *Pseudomonas aeroginosa, L. monocytogenes*, *S. aureus*, and salmonellas (34, 87).

L. plantarum strains Lb 75 and Lb 592, isolated from fresh meat and different meat products, inhibits *Lactobacillus divergens, L. sakei*, *L. curvatus* , *L. monocytogenes* but not *L. plantarum* (102).

L. plantarum strains CTC 242, CTC 244, CTC 272, CTC 305, CTC 306, CTC 316 produce antagonistic activity. *L. plantarum* ATCC 8014 is inhibited by compounds produced by strains CTC 242, CTC 244, CTC 305, CTC 316. *L. plantarum* DSM 20174 is inhibited by all six strains. A laboratory strain of *L. curvatus* is inhibited by strains CTC 242, CTC 244, CTC 306 while *L. sakei* is inhibited by strains CTC 242, CTC 244 and CTC 272 (36). Strains CTC 305 and CTC 306 inhibit *L. monocytogenes* and a laboratory strain of *E. faecalis* (102). Both peptides have a bactericidal action against *L. monocytogenes* (110).

Plantaracin EF and JK

L. plantarum C-11, isolated from a natural cucumber fermentation, produces two two-peptide bacteriocins termed plantaracin EF and JK. Both peptides are Class II bacteriocins (21).

Plantacin B

Plantacin B is produced by *L. plantarum* NCFB 1193 and inhibits *Pediococcus damnosus, L. plantarum*, *L. mesenteroides*, *L. mesenteroides* subsp. *mesenteroides* and (21). The peptide is also produced by *L. plantarum* NCDO 1193 (111).

Plantaracin S and T

L. plantarum LPCO-10, isolated from a green olive fermentation, produces plantaracin S and T. Plantaracin S inhibits *E. faecalis*, *C. tyrobutyricum* and several *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Micrococcus*, and *Propionibacterium* spp. Plantaracin T exhibits a lower level of inhibition against the various organisms tested for plantaracin S (111).

Plantaracin 423

The bacteriocin is produced by *L. plantarum* 423 isolated from sorghum beer and is inhibitory to several food spoilage bacteria and food-borne pathogens, including

Staphylococcus carnosus, B. cereus, *C. sporogenes*, *E. faecalis*, several *Lactobacillus* sp., (including *L. plantarum*), *Propionibacterium acidipropionici*, *Propionibacterium* sp., *Oenococcus oeni*, *L. innocua*, *L. monocytogenes*, *P. acidilactici*, *P. pentosaceus* and *S. thermophilus*. Plantaracin 423 has a bactericidal mode of action and is a class IIa (anti-*Listeria*) bacteriocin (111).

Plantaracin ST31

The bacteriocin is produced by *L. plantarum* ST31 isolated from sourdough. The bacteriocin inhibits strains of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Bacillus* and certain foodborne pathogens such as *S. aureus* (106).

Plantaracin C19

The bacteriocin is produced by *L. plantarum* C19 isolated from fermented cucumbers. It is inhibitory towards several Gram-positive pathogenic and spoilage bacteria, but the activity is weak or nonexistent. Plantaracin C19 is a class II bacteriocin (4).

Plantaracin C

This peptide, classified as a lantibiotic, is produced by *L. plantarum* LL441, isolated from Cabrales cheese, and is inhibitory towards several strains of lactobacilli, leuconostocs, pediococci and *S. thermophilus* (38).

Plantaracin UG1

The bacteriocin is produced by *L. plantarum* UG1 isolated from dry sausage. The peptide inhibits several strains of lactobacilli and lactococci, and certain foodborne pathogens, such as *B. cereus*, *L. monocytogenes*, *C. perfringens* and *C. sporogenes* (25).

Plantacin 154

The bacteriocin is produced by *L. plantarum* LTF 154 isolated from fermented sausage. It inhibits *E. faecalis*, various *Lactobacillus* sp. such as *L. acidophilus*, *L. casei*, *L. fermentum* and *L. plantarum, P. acidilactici* and *P. pentosaceus*, *S. lactis* and *S. thermophilus, Propionibacterium jensenni*, *Propionibacterium theonnii* and *P. acidipropionici* (52).

Plantaracin KW30

The bacteriocin is produced by *L. plantarum* KW30, isolated from fermented corn. Its inhibition spectrum is restricted to *L. brevis*, *L. delbrueckii* subsp. *lactis* and *L. plantarum* strains (53).

Plantaracin-149

The bacteriocin is produced by *L. plantarum* NRIC 149 isolated from pineapple. The peptide inhibits strains of *Pediococcus cerevisae, P. acidilactici, L. plantarum*, *L. delbrueckii*, *L. helveticus*, *L. casei*, *L. fermentum*, *L. mesenteroides*, *E. hirae* and *L. lactis* (50).

Plantaracin LC74

Plantaracin LC74 is produced by *L. plantarum* LC74 isolated from crude goat's milk. It inhibits strains of *Bacillus stearothermophilus, L. plantarum*, *L. brevis, L. buchneri* and *L. mesenteroides* (91).

Plantaracin SA6

The bacteriocin is produced by *L. plantarum* SA6 isolated from fermented sausage. The peptide inxhibits several LAB including *L. plantarum*, *L. brevis*, *L. buchneri*, *L. paramesenteroides* and *Listeria grayi* (92).

Plantaracin D

The bacteriocin is produced by *L. plantarum* BFE 905 isolated from "Waldorf" salad. The peptide has a narrow spectrum of inhibition, being antagonistic against only one strain of *L. plantarum*, one strain of *L. sakei* and several *L. monocytogenes* strains (31).

Plantaracin BN

L. plantarum BN produces plantaracin BN inhibitory towards *C. botulinum* (83).

Plantaracin NA

An isolate of *L. plantarum* sp. from vegetable origin produces plantaracin NA which inhibits *L. monocytogenes* (84).

Plantaracin K

The bacteriocin is produced by *L. plantarum* DK9 isolated from "fufu", a fermented cassava product. It inhibits strains of *Lactobacillus*, *Leuconostoc* and *Enterobacter* (83).

Other bacteriocins produced by *L. plantarum* include plantaracin A, plantaracin F, plantaracin 154, plantaracin 406, and produced by *L. plantarum* strains BN, LB75, LB592, CTC305, respectively (12). *L. plantarum* TMW 1.25 produces plantaracin 1.25α and plantaracinβ (93).

Lactocin S

L. sakei L45, isolated from fermented dry sausage, produces lactocin S. The bacteriocin is active only towards selected species of the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus* (46, 54). Lactocin S is a lantibiotic (21).

Sakacin P

L. sakei LTH673 produces sakacin P, which is active towards closely related lactobacilli, *Carnobacterium* spp., *E. faecalis* and *Listeria* spp (21).

Sakacin 674

The bacteriocin is produced by *L. sakei* Lb674. The peptide inhibits the growth of several *Lactobacillus* spp. and *L. monocytogenes*. Subsequent studies have shown that sakacins P and 674 are identical. It is a class IIa bacteriocin (21).

Sakacin A

L. sakei Lb 706, isolated from different types of meat and meat products, produces sakacin A which is active against *L. monocytogenes*, *L. ivanovii*, *L. innocua* and a variety of LAB, including lactobacilli, enterococci and carnobacteria. An inhibition of *Aeromonas hydrophila* has also been observed. The bacteriocin has a bactericidal mode of action (21).

Bavaracin MN

The bacteriocin is produced by *L. sakei* MN. Bavaracin MN is a Class IIa bacteriocin (21).

L. sakei CTC 472 produces a bacteriocin, which inhibits *Listeria* spp. Many *L. sakei* strains isolated from meat produce antimicrobial substances while strain Lb 706 inhibits *L. monocytogenes* (21).

Lactacin B

L. acidophilus N2 produces lactacin B inhibitory towards *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *lactis* ATCC 4797, *L. delbrueckii* subsp. *lactis* NCDO 970, *L. delbrueckii* subsp. *bulgaricus* NCDO 1489, *L. acidophilus* ATCC 6032 and *L. helveticus* NCDO 87. The production of lactacin B appears to be genomically encoded (21).

Acidophilucin A

L. acidophilus LAPT 1060, isolated from infant faeces, produces acidophilucin A inhibitory towards *L. delbrueckii* subsp. *bulgaricus* and *L. helveticus.* Acidophilucin A is a Class III bacteriocin (21).

Acidocin A

Acidocin A, produced by *L. acidophilus* TK9201, a starter organism for fermented milk, is inhibitory towards several closely related LAB and *L. monocytogenes* (21).

Acidocin B

This bacteriocin is plasmid-encoded and produced by *L. acidophilus* M46. The peptide is inhibitory towards *Brochothrix thermosphacta L. monocytogenes*, *C. sporogenes*, *L. fermentum* and *L. delbrueckii* subsp. *bulgaricus*, but inactive against most other *Lactobacillus* spp (21).

L. acidophilus JCM 1132 produces the two-peptide bacteriocin, acidocin J1132. Acidocin 8912 is produced by *L. acidophilus* TK9812 (21).

Curvacin A

Curvacin A was the first bacteriocin identified and characterized from a strain of *L. curvatus* (LTH 1174), isolated from fermented sausage. It has a bactericidal mode of action, including cell lysis, and is effective against closely related lactobacilli as well as organisms causing food spoilage of meat, including *E. faecalis, Carnobacterium* spp.,

and *Listeria* spp. The growth of micrococci and strains of *S. carnosus*, which are constituents of meat starter preparations, was not affected (21). Later studies showed that the bacteriocin is identical to sakacin A (110).

Other bacteriocins produced by *L. curvatus* include curvaticin FS47 produced by strain FS47, isolated from meat, and curvaticin 13, produced by strain SB13 (37, 104).

Lactocin 27

Lactocin 27 is produced by the homofermentative *L. helveticus* LP27, and shows a narrow spectrum of activity limited to *L. acidophilus* and *L. helveticus*. It imparts its bacteriostatic effects by inhibiting protein synthesis and by interfering with sodium and potassium transport (21, 100).

Helveticin J

The bacteriocin is produced by *L. helveticus* NCDO 481 and shows a narrow inhibitory spectrum which includes strains of *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. Helveticin J is a Class III bacteriocin. It has a bactericidal mode of action and its genetic determinants are chromosomally localized. Helveticin V-1829 is produced by *L. helveticus* V-1289 (21).

Lactacin F

Lactobacillus johnsonni VPI11088 produces lactacin F. The bacteriocin is a hydrophobic, heat-stable peptide belonging to the Class II bacteriocins. It is inhibitory towards *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus, L. helveticus, L. fermentum*, *E. faecalis*, *S. aureus* and *Aeromonas hydrophila* (21, 64).

Caseicin 80

L. casei B 80, isolated from wine, produces caseicin 80 that is active only towards another strain of *L. casei* (B 109) (90). Another caseicin-producing *L. casei* strain (LHS) with a wide spectrum of activity was also isolated from sherry (19). Caseicin is a class III bacteriocin (21).

L. casei CRL 705, isolated from dry sausages, produces lactacin 705 (112).

Lactacin A

L. delbrueckii subsp. *lactis* JCM 1106 and JCM 1107 produce lacticin A which is active towards *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *delbrueckii*. The peptide is a Class II bacteriocin (21).

Lactacin B

L. delbrueckii subsp. *lactis* JCM 1248 produces lacticin B which is active only towards *L. delbrueckii* subsp. *bulgaricus* NIAI yB-62. The peptide is a Class II bacteriocin (21).

Brevecin 27

The bacteriocin is produced by *L. brevis* SB27 and mainly inhibits strains of closely related *L. brevis* and *L. buchneri* (8).

Brevecin 286 is produced by *L. brevis* VB286 isolated from vacuum-packaged meat (16). Brevicin is produced by *L. brevis* 37 (90).

Reutericin 6

Lactobacillus reuteri LA 6, isolated from infant faeces, produces reutericin 6 which is inhibitory towards *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. Reutericin is another bacteriocin produced by *L. reuteri* LA6 (21).

Bacteriocins from *L. salivarius* are salavaricin A produced by *L. salivarius* 20P3, salavaricin B produced by strain M7 and salavacin 140 produced by *L. salivarius* subsp. *salicinius* T140 (3, 97, 105).

Bacteriocins produced by *Lactobacillus amylovirus* include lactobin A, produced by strain LMG P-13139 isolated from corn steep liquor, and amylovirin L471 produced by strain DCE471 (9, 15).

Pentocin D

Lactobacillus pentosus DK7 isolated from “ogi”, a fermented maize product, produce pentocin D which inhibits strains of *Lactobacillus*, *Leuconostoc* and *Enterobacter* sp. (86).

Bavaracin A

The bacteriocin is produced by *Lactobacillus bavaricus* MI401. Bavaracin A has a bactericidal mode of action against several *L. monocytogenes* strains (56).

L. fermentum 466 produces bacteriocin 466. Fermenticin is another bacteriocin produced by this species (21).

Brevecin 37

L. brevis produces brevicin 37 which is active against many other LAB and *Nocardia corallina* (21).

Gassericin A is produced by *Lactobacillus gasseri* LA39 (51).

Bacteriocins produced by *Lactococcus* spp.

The ability of some lactococci to produce inhibitory substances other than organic acids has been known for quite some time. The genus *Lactococcus* includes *Lactococcus garviae*, *Lactococcus plantarum*, *Lactococcus raffinolactis, L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae* (21). Most lactococci occur in the dairy industry although other sources do exist for different species (118). The *Lactococcus* genus has been known to produce two types of bacteriocins: the first type only affects closely related species whereas the second one is effective against many Gram-positive genera (77).

Nisin

This polypeptide is produced by *L. lactis* subsp. *lactis* (21, 100). Nisin-producing lactococci have been isolated from raw and pasteurized milk and cheese, human and bovine faeces as well as the human throat. Other sources where nisin-producing lactococci have been isolated include fermented carrots and also a commercial sauerkraut tank. The active protein consists of 34 amino acids and has a molecular mass of 3500 Da. It can exist in multimeric forms. Nisin contains four unusual amino acids, namely dehydroalanine, dehydrobutyrine, lanthionine and β-methyl lanthionine. Because of the

presence of these unusual amino acids, nisin is classified as a lantibiotic. Nisin is ribosomally synthesized as a prepeptide and postranslationally converted into a mature peptide (109). Nisin displays a rather broad inhibitory spectrum. It inhibits the majority of Gram-positive bacteria of which the lactococci are especially sensitive. It further inhibits several strains of bacilli, clostridia, corynebacteria, lactobacilli, leuconostocs, micrococci, pediococci, pneumococci, streptococci and actinomycetes. *Staphylococcus pyogenes*, *Staphylococcus epidermidis, S. aureus*, *Mycobacterium tuberculosis*, *Erysipelothrix rhusiopathiae* and *L. monocytogenes* are also sensitive to nisin. Nisin has a bactericidal mode of action and is effective not only against the vegetative cells of spore-forming bacilli and clostridia, but even more against their spores (21). In spores, the cytoplasmic membrane is apparently destroyed immediately after germination (100). Nisin Z, produced by *L. lactis* subsp. *lactis* strain NIZO 22186 is a natural variant of nisin A (78).

Lacticin 481

The bacteriocin is produced by *L. lactis* subsp. *lactis* CNRZ 481. It is active against lactobacilli, especially *L. delbrueckii* subsp. *bulgaricus, L. helveticus*, some leuconostocs, pediococci and *S. thermophilus*. In food spoilage, lacticin 481 is active only against *C. tyrobutyricum* and *S. carnosus*. It has a bactericidal mode of action and acts similarly to nisin by forming ion channels in the membrane of sensitive cells. The lantibiotic lactococcin DR, produced by *L. lactis* subsp. *lactis* ADRIA 85LO30 is identical to lacticin 481 (21).

Lacticin 3147

Lacticin 3147 is a broad-spectrum bacteriocin produced by *L. lactis* DPC3147, isolated from Irish kefir grains. The peptide is heat stable, particularly at low pH, and is produced during logarithmic growth but is however not related to nisin (98). It is inhibitory to a wide range of Gram-positive organisms, including strains of *Listeria*, clostridia, *Enterococcus*, *Staphylococcus* and *Streptococcus* (76). Lacticin 3147 is more effective against streptococci than staphylococci when compared to nisin (99). *Streptococcus dysgalactiae* M is the most sensitive (98).

Lactococcins

Lactococcins R9/1, R9/2, R10/1 and R35 are produced by *L. lactis* subsp. *lactis* strains R9/1, R9/2, R10/1 and R35, respectively. These lactococcins displays a broad inhibitory spectrum of activity against *Carnobacterium piscicola*, *S. aureus*, *L. lactis* subsp. *lactis* and *Listeria* strains. Lactococcin R9/2 and R10/1 show the broadest range of inhibitory action (23).

L. lactis subsp. cremoris 9B4 produces lactococcin A, B and M while lactococcin A is produced by strain LMG 2130 (21). The antagonistic activity of strain 9B4 affects the growth of lactococci and some *Clostridium* species. Lactococcin A only inhibits the growth of lactococci. The action of lactococcin A on sensitive cells is bactericidal. Lactococcin A and B are also produced by *L. lactis* subsp. *lactis* biovar. *diacetylactis* WM4 (21).

Lactococcin G, produced by *L. lactis* LMG 2081, is a two-peptide bacteriocin that inhibits various LAB and different clostridia (80).

L. lactis subsp. *lactis* IPLA 972, isolated from homemade cheese, produces lactococcin 972 (70).

Diplococcin

Diplococcin is produced by *L. lactis* subsp. *cremoris*. The inhibitory spectrum of diplococcin is restricted to other *L. lactis* subsp. *cremoris* and *lactis* strains (20).

Lactostrepcins

This is another class of bacteriocins produced by other genera of lactococci, i.e. *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* var. *diacetylactis* and *L. lactis* subsp. *cremoris*.. These antimicrobial proteins are unique in that they are active only in the acid pH range. Five types of lactostrepcins can be defined. Lactostrepcin (Las 5) is effective against *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* var. *diacetylactis*. Besides activity against various lactococci, Las 5 is also active against strains of *Leuconostoc*, *L. helveticus* and *B. cereus*. Las 1 displays high activity against group A, C and G streptococcal and against *L. helveticus* strains. The same strains together with

Leuconostoc strains are highly susceptible to Las 2. Las 3 is effective only against *L. lactis* subsp. *cremoris* 1P5 while Las 4 is active against both *L. lactis* subsp *lactis* and *L. lactis* subsp. *cremoris*. Lactostrepcins exert a strong and rapid bactericidal on sensitive cells during exponential growth (21).

Bacteriocins produced by *Carnobacterium* spp.

The genus *Carnobacterium* accommodates some physiologically, biochemically and chemically atypical lactobacilli (21), isolated from: meat and meat products, poultry, fish and seawater (118). The genus at present includes *Carnobacterium divergens*, *Carnobacterium gallinarum*, *Carnobacterium mobile* and *C. piscicola* (21).

All bacteriocin-producing strains of carnobacteria are identified as *C. piscicola* and were isolated from meat or fish although bacteriocinogenic strains of *C. divergens* have been reported more recently. The bacteriocins produced by *C. piscicola* are mostly small hydrophobic peptides. Their antibacterial spectrum comprises different genera of LAB and generally includes *L. monocytogenes* (118).

Carnobacteriocin A and B

C. piscicola LV17, isolated from vacuum-packaged meat, produces three bacteriocins, nl. carnobacteriocin (Cbn) A, B and BM1. In contrast to Cbn A and B, which is encoded by a plasmid, Cbn BM1 is chromosomally encoded. However, the transport genes for Cbn BM1 are encoded *in trans* by the transport genes of Cbn B. The bacteriocins are active against other LAB (carnobacteria, lactobacilli, pediococci) and strains of *Enterococcus* and *Listeria* spp. The bacteriocins have a bactericidal mode of action (21, 118).

Pisicolin 61

C. piscicola LV61 produces pisicolin 61 which is active against other *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *L. monocytogenes* (21, 118). Other LAB such as lactobacilli, pediococci and leuconostocs were more or less resistant (21).

Carnocin UI49

C. piscicola UI49, isolated from fish, produces carnocin UI49 which is active against a wide range of LAB. It has a bactericdal mode of action and is classified as a lantibiotic (21).

Piscicocin V1a and V1b

C. piscicola V1, isolated from fish, produces piscicocin V1a and piscicocin V1b which inhibit various Gram-positive bacteria, including *L. monocytogenes* (21, 118).

Piscicolin 126

The bacteriocin is produced by a *C. pissicola* strain isolated from spoiled ham and inhibits several species of *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria* and *S. thermophilus* (21, 118).

Carnocin CP51 and CP52

C. piscicola CP5, isolated from French mold-ripened cheese, produce two anti-listerial bacteriocins, carnocin CP51 and CP52 (21, 118).

C. piscicola UAL26 produces a chromosomally mediated bacteriocin of which the activity spectrum extends beyond very closely related species, which include *Enterococcus* and *Listeria* spp. *C. piscicola* strains DX and GN, isolated from beef, produce bacteriocins that are active against *L. monocytogenes* and *A. hydrophila* (21, 118).

Divergicin 750

The bacteriocin produced by *C. divergens*, inhibits species of *Carnobacterium*, *Enterococcus* and *Listeria*, as well as *C. perfringens* (46).

Other bacteriocins produced by *C. divergens* include divergicin A produced by strain LV13 isolated from meat and divergicin V41 produced by strain V41 isolated from fish viscera (71, 89, 119).

Bacteriocins produced by *Leuconostoc* and *Weisella* spp.

The genus *Leuconostoc* comprises Gram-positive cocci that are claimed to be taxonomically and ecologically related to group N streptococci. However, leuconostocs are heterofermentative and produce rod-shaped cells in growth media, making them similar to heterofermentative lactobacilli (103). The genus currently comprises 10 species. Leuconostocs are found on plants, milk and dairy products, fermented food products and have been isolated from the lactic microflora of chill-stored vacuum-packaged meats (21, 118). Inhibition by *Leuconostoc* has been attributed primarily to production of lactic and acetic acid as well as diacetyl. However, there have been reports of bacteriocin-like compounds produced by the genus (100). Most of these compounds are small, heat stable peptides classified as Class IIa bacteriocins although several characterized bacteriocins in this genus cannot be classified in the currently described groups (25, 60). Bacteriocins produced by leuconostocs may or may not be active against other LAB, but all include *Listeria* in their activity spectra (103).

Leucocin A-UAL 187

Leuconostoc gelidum, isolated from chill-stored vacuum packaged meat, produces leucocin A-UAL 187. Leucocin A is classified as a Class IIa bacteriocin. The antibacterial spectrum of the bacteriocin includes other LAB, *L. monocytogenes* and *E. faecalis*; however the activity may be bacteriostatic rather than bactericidal (42).

Carnosin 44A

Leuconostoc carnosum, isolated from Vienna-type sausages, produces carnosin 44A (21, 103). It has a similar antibacterial spectrum to leucocin A, including *Enterococcus* and *Listeria* spp (21).

Leucocin B-Ta11a

L. carnosum Ta11a, isolated from vacuum packaged vienna sausages, produces leucocin B-Ta11a. Leucocin B is active against *L. monocytogenes* (21).

Leucocin D-La54a

The bacteriocin is produced by *L. carnosum* La54a isolated from spoiled vacuum-packaged vienna sausages. The characteristics of this bacteriocin are similar to leucocin A and B (21).

Mesentericin Y105

L. mesenteroides subsp. *mesenteroides*, isolated from goat's milk, produces mesentericin Y105 (21). Mesentericin Y105 is classified as a Class IIa bacteriocin (43). Another producing strain has also been isolated from cheddar cheese (25). The peptide is bactericidal to a wide range of *Listeria* spp., but not to a limited range of other bacteria, including strains of LAB (110).

Other bacteriocins produced by *L. mesenteroides* include mesenterecin 52A and B produced by *L. mesenteroides* subsp. *mesenteroides* FR52 and dextranicin 24, produced by *L. mesenteroides* subsp. *dextranicum* J24 (94, 95).

Leuconocin S

L. paramesenteroides produces leuconocin S. The bacteriocin is bacteriostatic and it has a broad antibacterial spectrum, including strains of *Yersinia enterocolitica, C. botulinum*, *L. monocytogenes* and *S. aureus* (21, 103).

L. gelidum, isolated from meat, produces a bacteriocin which inhibits lactobacilli, leuconostocs and *L. monocytogenes* (21, 118). *L. mesenteroides* produces a bacteriocin that has a bacteriostatic effect on strains of *Listeria*, *Brevibacterium linens, E. faecalis* and *P. pentosaceus* (118). Production of the bacteriocin is encoded by one of the two plasmids present in the organism (110).

Leuconocin J is produced by *Leuconostoc* sp. J2, isolated from Korean Kimchi. Leucocin H is a two-peptide bacteriocin produced by *Leuconostoc* MF215B. Leucocin F10 is produced by *L. carnosum* strain F10 isolated from meat. Leuconocin S and leucocin C-LA7a are produced by *Weissella paramesenteroides* (41, 65).

Bacteriocins produced by *Pediococcus* spp.

The genus is heterogeneous and includes organisms able to grow in beer and those active during soya sauce manufacture. They are also found in wine and ciders, soft drinks, cheese as well as meat and fish products, etc. (118).

Pediocin A

P. pentosaceus strains FBB-61 and L-7230, isolated from fermenting cucumber brine, produce pediocin A (21). The bacteriocin is also produced by *P. pentosaceus* FBB-63 (100). The bacteriocin inhibits the growth of selected strains of Gram-positive bacteria from the genera *Pediococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Micrococcus*, *Clostridium*, *Staphylococcus* and *Bacillus* (21, 100).

Pediocin AcH

P. acidilactici H, isolated from fermented sausage, produces pediocin AcH which is active against a variety of LAB and strains of *Listeria* (21, 100). Pediocin AcH binds to specific receptors on cell walls of sensitive strains and destabilizes membrane function, causing cell death (100). Other pediocin AcH-producing strains include strains H, E, F and M of *P. acidilactici* (21).

Pediocin PA-1

The bacteriocin is produced by *P. acidilactici* PAC 1.0. The bacteriocin inhibits other pediococci, some lactobacilli, strains of *L. mesenteroides* subsp. *dextranicum* and *L. monocytogenes* (100). The bacteriocin is also produced by two *Pediococcus parvulus* strains isolated from minimally processed vegetables (110).

Another bacteriocin produced by *P. acidilactici* is pediocin SJ-1 (110). Pediocin 5 and pediocin PD-1 is produced by *P. acidlactici* UL5 and *P. damnosus* NCFB 1832, respectively (39, 47). Pediocin production is often plasmid linked (118). Bacteriocins are also produced by *P. pentosaceus* strains MC-01 and MC-03 isolated from pepperoni as well as *P. acidilactici* strains PO_2, B5627, PC and HP and some isolated from human clinical sources (21).

Bacteriocins produced by *Streptococcus* spp.

Streptococci comprise a significant component of the commensal flora of man and animals, colonizing mucous membranes of the mouth, respiratory tract, alimentary tract and genitourinary tract. Some species are also found on the skin, and others may be isolated from milk and dairy products. Many of the known species are parasitic in man or other animals and some are important pathogens (118). The species in the genus can roughly be divided into the oral or viridans streptococci and the pyogenic streptococci. Pyogenic streptococci are of considerable medical importance. Although oral streptococci form an important component of the microflora of the mouth and the upper respiratory tracts of humans, they may be isolated from almost any type of clinical specimen, including blood cultures, pus, body fluids, biopsies, wounds, etc. or even from soil. Oral streptococci comprise at least three groups of species: the mutans group with seven species; the oralis group with at least nine species, and the salivarius group (21).

Bacteriocins STB 40 and STB 78

The bacteriocins are produced by strains of *S. thermophilus* isolated from milk. The bacteriocins are active to other *S. thermophilus* and *Enterococcus* strains (21).

Bacteriocin St10

Bacteriocin St10 is produced by *S. thermophilus* St10. It is only active towards *S. thermophilus* strains (21).

Other bacteriocins produced by *S. thermophilus* include thermophilin 13 produced by *S. thermophilus* Sfi13, thermophilin 347, produced by strain 347 isolated from yoghurt, and thermophilin T produced by strain ACA-DC0040, isolated from “feta” cheese (1, 69, 114).

Mutacins

This is another group of bacteriocins produced by *Streptococcus.* These peptides have bactericidal activity towards Gram-positive bacteria, but not Gram-negative bacteria except for *Neisseria sicca* (117).

PROBIOTIC TREATMENT OF MASTITIS

There has been speculation that the administration of probiotic therapy to infected mammary glands may be an effective treatment of clinical mastitis. It would be economically advantageous if an effective probiotic were to be identified, since this would not require any unnecessary withdrawal of milk following treatment. Treatment of subclinical mastitis was attempted using intramammary infusions of *Lactobacillus* spp. However, cattle receiving intramammary antibiotic agents had a greater reduction (73.7%) than cows infused with *Lactobacillus* spp. (21.7%). Cows treated with intramammary *Lactobacillus* also had higher somatic cell counts following treatment (40, 108).

In another study, where elimination of clinical mastitis was attempted, the cure rate for *Lactobacillus* was half that of cephapirin treatment (26% vs. 49%). None of the Gram-negative bacilli quarters treated with *Lactobacillus* recovered, while the majority of cephapirin-treated quarters were cured. Mean quarter SCC remained unchanged following the treatment with *Lactobacillus*, while it decreased with cephapirin treatment. These results do not support the speculation that this type of *Lactobacillus* product is effective in the treatment of clinical mastitis (108).

MASTITIS TREATMENT THROUGH BACTERIOCINS

The application of biotechnology to mastitis treatment is opening up new avenues of prevention and control. For mastitis treatment, bacteriocins can be either infused into the udder (in the same way as antibiotics), or used in solutions (such as teat dips). These proteins are larger molecules than antibiotics and are expected to persist in the udder longer. Unlike antibiotics, the rapid action of bacteriocins reduces the likelihood of an induced resistance in target and nontarget organisms (72).

Current research conducted so far seems to indicate that bacteriocins used for mastitis treatment are nontoxic to other organisms. This facilitates their inclusion into food products under FDA health regulations. This means that treated lactating or dry cows that

calve prematurely need not have their milk withheld, as would be the case with antibiotics in subclinical treatment. Although most work to date has been done on enzymatic proteins to control *S. aureus*, research with bactericidal proteins against other organisms like *S. agalactiae* suggests that the conclusions drawn can be generalized (72).

Nisin

Nisin has been used as a food presevative in certain countries since 1954 and has recently gained approval in the United States of America for use in certain dairy products. Nisin is nontoxic to humans and is readily broken down by digestive enzymes when consumed. Hypersensitivity to nisin has not been recorded. However, it has a low solubility in body fluids and is unstable at physiological pH (72, 109).

A purified preparation of nisin has been developed as a suitable sanitizer against mastitis pathogens on cows' teats. This product was able to kill *S. aureus* and *E. coli* after a 1-min exposure at a rate comparable to one percent iodophor (100). Nisin in combination with lysostaphin has also been shown to be an effective treatment for mastitis, with cure rates of 66, 95 and 100% reported for animals infected with *S. aureus*, *S. agalactiae* and *Streptococcus uberis* respectively. In addition, nisin is now the active ingredient in at least two products currently sold for the prevention of mastitis: Consept (Applied Microbiology, Inc., New York, N.Y.), which is applied as a teat dip, and Wipe-Out (Applied Microbiology), which is used as a teat wipe (98).

Lacticin 3147

Lacticin 3147 has also been evaluated as a possible means of mastitis control. This specific application involved the incorporation of the bacteriocin into a commercial teat seal. The teat seal is an oil-based formulation that forms a physical barrier against infection in the area of the teat canal and sinus. It consists of a heavy inorganic salt in a paraffin base and is antibiotic-free. The effect of this lacticin 3147-containing teat seal is two–fold; first, the seal itself provides an effective physical barrier against infection at the teat orifice, and secondly, the bacteriocin inhibits any infectious Gram-positive bacteria which have the potential to evade the teat seal plug. Its use is preceded by an antibiotic treatment at the drying-off stage, and the seal is then administered as an added barrier treatment over the antimicrobial protection afforded by the antibiotic. The incorporation

of bacteriocin into the teat seal is advantageous in that the bacteriocin is localized within the teat seal, which is in the teat duct and sinus (98).

In one study lacticin 3147 appeared to be more potent against streptococci than staphylococci. Lacticin 3147 caused rapid cell death when added to actively growing cells of *S. aureus* 10 and *S. dysgalactiae* M. The addition of lacticin 3147 to approximately 10^6 and 10^7 of actively growing cells of these organisms caused a rapid loss of viability in comparison to control cultures to which no bacteriocin was added. For both *S. aureus* 10 and *S. dysgalactiae* M, >99.9% of cells were killed within 2 hours (using 1,280 and 10,240 AU/ml, respectively), demonstrating the potency of this bacteriocin against mastitic pathogens (98).

By using higher concentrations of lacticin 3147, an increased rate of killing was observed, indicating that the residual populations were not resistant. The frequency of spontaneous resistance was less than 1 in 1 million cells after 24 hours of incubation for *S. dysgalactiae* M, while no resistance was observed after 24 hours of incubation for *S. aureus* 10 (98).

In addition, the somatic cell counts (SCC) were monitored during the morning and evening milkings to ensure that the bacteriocin-containing teat seal was non-irritating. No notable increase in SCC over that of the untreated controls was observed, suggesting that this product is a non-irritating agent. Hence, the teat seal appears to provide a safe and effective vehicle for localization of the bacteriocin in the teat canal. Moreover, the resultant seal retained its bacteriocin activity even after being infused in the teat of an animal (98).

Future prospects

Bacteriocins are comparable with antibiotic treatment and is twice as effective as penicillin for first time infections in heifers (72). Combining bacteriocins that produce a synergistic action may prove to be very significant. Treating cows with one of these combined products is likely to be more effective. Combinations appear to require lower concentrations, which may reduce expenses involved in treatment (72). The milk

exclusion for such a product would require shortened withholding periods of as little as 1 day compared with 6.6 days for antibiotic treatments. Milk would only need to be withheld because of abnormal secretion but not because of toxicity (72).

The specificity of bacteriocins means that specific bacteriocins will have to be prescribed by veterinarians, who are able to determine the organism causing the infection. This is in contrast to broad-spectrum antibiotics, which require no prescription for use to control mastitis. The cost of a veterinary diagnosis could make enzyme use not cost effective compared with antibiotics. However, a combination of several bacteriocins into a single medication could allow the products to be sold as non-prescription drugs. Farmers must perceive this treatment method as economically competitive with antibiotic alternatives. The adoption of enzymes is expected to be slow initially, until the treatment can be sold as a non-prescriptive drug (72).

The research done thus far does not indicate that milk production per cow will be changed. Treatment effectiveness is the same for older cows. The cost of discarded milk in the United States of America is reduced from $9.22 per cow to $1.40 per cow when milk is withheld for only 1 day during treatment (72). The cost of drugs is not anticipated to change. All current marketing investigations indicate that price competitiveness with antibiotics will be necessary for successful adoption (72). The result of bacteriocin administration should be similar to that of treatment with antibiotics. Labor requirements are decreased slightly, to the extent that first time infections can be treated effectively during lactation and eliminated. A monetary value cannot be assessed yet. Cow replacement costs are not anticipated to change (72).

Milk will not be adulterated because bacteriocins are compounds that enjoy GRAS status. There is therefore no need for a withholding period of the milk after treatment. Testing will be unnecessary. Enhanced fat production will be modest and noticeable only to the degree that first time infections are eliminated. Losses in cheese manufacturing should not occur if no antibiotics are used. Bacteriocins are organism-specific and should not inhibit starter cultures or cause casein or fat decomposition (72).

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PREVENTION AND TREATMENT OF *STAPHYLOCOCCUS AUREUS* MASTITIS IN DAIRY COWS BY USING THE CYCLIC PETIDE ANTIBIOTIC AS-48

Prevention and treatment of *Staphylococcus aureus* mastitis in dairy cows by using the cyclic peptide antibiotic AS-48

E. K. DAVIDSE,[1] E. BALLA,[1] W.H. HOLZAPFEL,[2] C.J.C. MULLER,[3] S.W.P. CLOETE,[3] AND L.M.T. DICKS[1]

[1]*Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa,*

[2]*Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Haid-und-Neu-Str. 9, 76131, Karslruhe, Germany,*

[3]*Department of Agriculture, Private Bag X1, Elsenburg 7607, South Africa*

Running headline: Mastitis prevention and treatment, peptide AS-48.

* Correspondence to: Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa. Tel: +27-21-808 5849, Fax: +27-21-8085846, e-mail: lmtd@sun.ac.za

Peptide AS-48, produced by *Enterococcus faecalis* FAIRE 92, inhibited the growth of a pathogenic strain of *Staphylococcus aureus* isolated from mastitic milk. *In vivo* activity tests were done by infusing infection-free udder quarters with 3.3 x 10^3 cfu/ml of a pathogenic strain of *S. aureus*, in the presence or absence of peptide AS-48. Relative to the control quarters, reduction in somatic cell (SCC) and *S. aureus* counts in quarters pre-treated with peptide AS-48 amounted to 50% and 90%, respectively. In a second experiment, already infected udder quarters of lactating cows were treated with peptide AS-48 or left untreated. The activity tests were done with purified peptide AS-48, obtained by using a combination of Triton X-114 phase partitioning and cation exchange chromatography. Reduction in SCC and *S. aureus* counts in quarters treated with peptide AS-48 amounted to 80% and 94%, respectively, when compared to the control quarters. Encapsulated peptide AS-48 was used in the *in situ* activity tests. The efficiency of encapsulation varied between 12 and 25%. Conjugation of the plasmid encoding peptide AS-48 (p92) to *E. faecalis* FA2/Ent, already producing enterocins 1071A and 1071B, was also performed. The presence of plasmid p92 was confirmed with Southern blot hybridization experiments. Other mastitic isolates that were also inhibited were *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, but not *Escherichia coli*.

S. aureus is one of the most common etiological agents of bovine mastitis in lactating dairy cows and contributes to significant economic losses in the dairy industry (9, 20). Despite mastitis management programs to reduce the incidence of intramammary udder infections (IMI), *S. aureus* remains a major problem (20). In the Western Cape of South Africa, *S. aureus* is responsible for 40% of all mastitis cases reported. After entering the mammary gland through the teat canal, the bacteria multiply rapidly, leading to inflammation and tissue damage (9). Apart from the infection, *S. aureus* secretes various toxins and enzymes, which may lead to food poisoning when the milk is consumed (3).

Staphylococci found in infected tissues are mainly located extracellularly (14). However, virulent staphylococci such as *S. aureus* can survive within leukocytes following phagocytosis. Studies have shown that cells of *S. aureus* can penetrate into developing phagocytic cells (2, 9, 14). The adhesion of *S. aureus* to the epithelium cells is considered the first and most crucial event of infection (24). Antibiotics are routinely administered at drying-off to assist in the elimination of subclinical cases and prevent new IMIs from becoming established (20). However, animals infected with *S. aureus* respond poorly to antibiotic treatment (25), probably due to the intracellular location of the bacterial cells in the alveoli and/or macrophages (2, 9). Although many of the antibiotics that are used penetrate neutrophils, *S. aureus* may survive (5) and cause chronic intraphagocytic infections (14).

Improved efficacy may be achieved by incorporating antimicrobial compounds in liposomes, which in turn deliver the compounds to phagocytic cells and accumulate intracelluarly (5, 14). Upon phagocytosis of the liposomes, the antimicrobial compound is released into the phagolysosome (5).

The emergence of antibiotic resistance in bacteria has lead to a considerable debate in the use of antibiotics for prophylactic treatment. The World Health Organization issued recommendations on global programs to try and reduce the use of antibiotics. Such a limitation requires the consideration of alternative treatments (20).

Immunization against *S. aureus* does not prevent IMI (13), as evident from the lack of high concentrations of antibody and phagocytic cells in the milk. Furthermore, inadequate knowledge of the pathogenesis of staphylococcal mastitis and the immune mechanisms protecting the mammary gland from infection has limited the scope for novel approaches to vaccination (24).

Attractive alternatives may include the use of lactic acid bacteria, and specifically bacteriocins, to achieve a low cost-effective method of preventing and treating *S. aureus* infections. In a previous study (15) Nisin was used in combination with lysostaphin to treat mastitis caused by *S. aureus*. Only 66% of the infections could be cured. Lacticin 3147, incorporated into a teat seal (1280 AU/ml), killed 99.9% of *S. aureus* (16).

We performed *in vitro* and *in situ* tests to determine the inhibitory activity of peptide AS-48 (classified as a bacteriocin) against a pathogenic strain of *S. aureus* isolated from mastitic milk. *In situ* activity tests were done with peptide AS-48 encapsulated in liposomes. Conjugation of the plasmid p92 encoding peptide AS-48 to *E. faecalis* FA2/Ent, which produces enterocins 1071A and 1071B, was also performed. The transformant, *E. faecalis* FA2/Ent/AS-48 displayed a broader spectrum of antimicrobial activity and may even be more effective as an anti-mastitic treatment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. faecalis* FAIRE 92, the producer of peptide AS-48, was obtained from prof. W. H. Holzapfel, BFE, Karslruhe, Germany. *E. faecalis* FA2/Ent was constructed in a previous study (Balla *et al*., 2000) and harbours plasmid pEF1071, which contains the genes encoding enterocins 1071A and 1071B. All enterococci were cultured in brain heart infusion (BHI) broth (Biolab Diagnostics, Midrand, South Africa). The indicator strains used in this study (Table 1) were from the Laboratorium voor Microbiologie, University of Ghent (LMG), Ghent, Belgium and our own culture collection. Strains of *S. aureus, S. agalactiae*, *S. dysgalactiae* and *E. coli* were isolated from mastitic milk and obtained from the Diagnostic Veterinary Laboratory, Stellenbosch, South Africa. All lactic acid bacteria were grown in MRS broth (Biolab). Other indicator bacteria were cultured in BHI broth (Biolab).

Production and inhibitory activity of peptide AS-48. An overnight culture (10 ml) of *E. faecalis* FAIRE 92 was inoculated into 1 liter of BHI-G broth (BHI broth supplemented with 1% (wt/vol) glucose and 0.15 M $NaH_2PO_4 \cdot H_2O$) and incubated at 37^{o}C without aeration until stationary growth. On an hourly basis 1 ml was sampled, serially diluted and plated onto BHI Agar (Biolab) and the number of viable cells (cfu/ml) determined. At the same time 1 ml of the cell-free supernatant was sampled,

adjusted to a pH between 6.0 and 7.0 with 1 N sterile NaOH, concentrated ten-fold and tested for activity against *S. aureus* by using the spot-on-lawn method (23). One arbitrary unit (AU) of peptide AS-48 is defined as the reciprocal of the highest dilution of the bacteriocin that produced an inhibition zone of at least 2 mm in diameter. From the antimicrobial activity, expressed as AU/ml, and the cell count (log_{10} cfu/ml) the specific activity ([AU/ml]/ [log_{10} cfu/ml]) was determined.

Isolation, purification and concentration. Peptide AS-48 was isolated and purified according to the method described by Métivier *et al*. (12). One liter of BHI-G broth was inoculated with 10 ml of an actively growing culture of *E. faecalis* FAIRE 92 and incubated for 7 h at 37°C. Cells were harvested by centrifugation (9000 x g, 4°C) and Triton TX-114 added to the supernatant to obtain a final concentration of 2% (wt/vol). The sample was adjusted to pH 5.5 with concentrated HCl, heated to 25-30°C, and incubated at this temperature for 1-2 h, after which the upper-phase was removed and replaced with the same volume of cold Millipore water (Ω18.2) containing Triton TX-114 (0.2%, wt/vol). The Triton TX-114 was dissolved by careful stirring. The mixture was heated to 25-30°C and left to separate into phases. The lower detergent-rich phase was recovered, diluted five-fold with cold Millipore water, and loaded onto a 15 ml SP-Sepharose Fast flow column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was washed with cold Millipore water until a constant baseline absorbance at 280 nm was reached. The loading and washing was done at 8°C to avoid phase partitioning. The bacteriocin was eluted with an ammonium acetate step-gradient of 0.1- 1.6 mol/liter^{-1} (pH 6.0). Fractions of 4 ml were collected, concentrated by freeze-drying, dissolved in 100 μl Millipore water and tested for activity against *S. aureus*, as described before. The active samples were pooled and stored at -80°C. Purified peptide AS-48 was also tested against *S. agalactiae*, *S. dysgalactiae* and *E. coli*.

Sensitivity to proteolytic enzymes. Purified peptide AS-48 (3200 AU/ml) was used in these tests. Resistance of peptide AS-48 to proteolytic enzymes was determined by incubation in the presence of proteinase K (20 U/mg of peptide AS-48), pronase (7 U/mg of peptide AS-48), pepsin (2500 U/mg of peptide AS-48), papain (30 U/mg of peptide AS-48), α-chymotrypsin (90 U/mg of peptide AS-48) and trypsin (110 U/mg of peptide AS-48) at 37°C for 1 h. All enzymes were from Boehringer-Mannheim (Howard Place, South Africa) and tested at their optimum activity pH. After incubation, the enzymes were heat-inactivated for 3 min at 100°C and peptide AS-48 tested for antimicrobial

activity against *S. aureus*. The activity of the treated samples was compared to that of a control sample, i.e. peptide AS-48 that has not been treated with proteolytic enzymes.

Molecular mass determination. A sample collected after separation in SP-Sepharose was subjected to tricine-SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described by Schägger and Von Jagow (19). A protein marker with sizes ranging from 2.35 to 46 kDa (Rainbow Marker; Amersham Pharmacia Biotech, Uppsala, Sweden) was used. One half of the gel was stained with Coomassie Brilliant Blue R250. The position of the active peptide AS-48 in the gel was determined by overlaying the other half of the gel, prewashed as described by Van Belkum *et al*. (22), with cells of an overnight grown culture of *S. aureus*, embedded in BHI agar (0.75% agar, wt/vol). In another experiment approximately 100 pmol of purified sample containing the bacteriocin was diluted in 10 µl of 10:90 acetonitrile-water containing 0.01% formic acid and injected via the Rheodyne injection port of a Quattro triple quadropole mass spectrometer (Micromass, Manchester, United Kingdom). The carrier solvent was 10:90 acetonitrile-water at a flow rate of 20 µl/min, delivered by a Pharmacia-LKB 2249 high-pressure liquid chromatography pump. The capillary voltage and the cone voltage were set at 3.5 kV and 60 V, respectively. Data were collected by scanning from 400 to 1500 *m/z* at 2 s/scan. The multiple charged spectra were deconvoluted to obtain the accurate mass of the peptides. Calibration was done by using horse heart myoglobin (Sigma, St. Louis, Mo).

Preparation of liposomes and determination of encapsulation efficiency. The method of Degnan and Luchansky (4) was used. Multilamellar vesicles were prepared using phosphatidyl choline from Sigma Chemical Co., St. Louis, MO (100 mg/ml chloroform). Four ml methanol/chloroform (1:1) and 2 ml phosphatidylcholine/chloroform mixture was placed into a 500 ml round-bottom flask and the solvent removed through rotary evaporation (37^{o}C, 60 rpm, 30 min, under vacuum). After evaporation, a thin, opaque lipid film was formed on the inner surface of the flask. An aliquot of peptide AS-48 (5 ml, 6400 AU/ml) and about 8 glass beads (4 mm in diameter; Fisher Scientific, Pittsburgh, PA) were added to the contents of the flask and rotated for a further 1 h at atmospheric pressure. The suspension (5 ml) was then incubated at 37^{o}C for 2 h to complete swelling of the liposomes. The peptide AS-48/liposome preparation was then transferred to a sterile 15 ml polypropylene test tube and stored at 4^{o}C for 7 days. The encapsulation efficiency (E%) was determined by adding 45 µl of the peptide AS-

48/liposome suspension to each of two microcentrifuge tubes. Five µl proteinase K (10 mg/ml) was added to one of the tubes to inactivate free, i.e. non-encapsulated, peptide AS-48. Sterile distilled water was added to a second tube. The samples were incubated at 37^{o}C for 1-2 h and then heated (100^{o}C, 3 min) to inactivate proteinase K and at the same time release encapsulated peptide AS-48 from the intact liposomes. The antimicrobial activity of released peptide AS-48 was determined by using the spot-on-lawn method, as described before. The E% was calculated as follows: E% = activity (AU/ml) of peptide AS-48 in the liposome mixture treated with proteinase K divided by the activity (AU/ml) of peptide AS-48 in the liposome mixture to which sterile distilled water was added (x100).

Antimicrobial activity assays. One ml of encapsulated peptide AS-48 (3200 AU/ml) was added to a 100 ml culture of *S. aureus* at the beginning of the lag and mid-exponential growth phases, respectively. Sterile demineralized water (1 ml) was added to the control flask. Changes in the turbidity of the cultures were recorded at 600 nm, and the number of viable cells (cfu/ml) was determined by plating the samples onto BHI Agar (Biolab).

***In situ* activity tests:**

Prevention experiment: Ten teats of five cows with somatic cell counts (SCC) < 500 000 cells/ml were selected. This threshold value gives a good indication of mastitis and milk with a SCC higher than it usually contains pathogenic organisms. Milk from these teats contained no viable cells of *S. aureus*, as determined by plating onto Baird-Parker Agar. Five teats were individually infused with 1 ml encapsulated peptide AS-48 (6400 AU/ml), followed by 2 ml of *S. aureus* (3.3 x 10^3 cells/ml). The other five teats that acted as controls were treated the same as above, except that 1 ml saline solution (0.75% NaCl) was used instead of peptide AS-48. Infusion was done immediately after milking, thus on day 1 of milk collection. The AS-48 peptide and *S. aureus* cells were massaged upwards into the teats. Milk samples were collected daily (up to day 7) from which viable cells of *S. aureus* and SCC counts were determined. It is conceded that the number of replication was relatively low. It was, however, dependant on the number of available cows in a specific stage of lactation. Since the microbial challenge with *S. aureus* was expected to result in marked changes in SCC and *S. aureus* counts, the number of replicates was regarded as sufficient for the purposes of this study. Moreover, since only a marked

response would be of value for therapeutic purposes, it would be argued that a very high number of replicates is not advisable for an experiment of this nature.

Statistical methods

The SCC and *S. aureus* counts were variable, ranging from 1.7 x 10^4 to 9.6 x 10^6 cells/ml and from to low to be detected with the methods used to 1.92 x 10^5 cfu/ml, respectively. The counts were expressed as $\log_{10}$ values. Where no *S. aureus* was detected 100 was added to each count prior to analysis. The analyses were also complicated by the fact that ten different quarters of five individual cows were sampled as experimental units. The data could thus not be described as uncorrelated, as assumed for analysis of variance. This complication was accounted for by the estimation of the intraclass correlation depicting all possible correlations between repeated samples obtained from the cows (8). Covariation arising from the repeated samples from specific cows was not only accounted for by this procedure, but it was also possible to estimate the repeatability of SCC and *S. aureus* counts (21). Repeatability (t) was estimated as:

$t = \sigma_b^2 / \sigma_b^2 + \sigma_e^2$

$= \sigma_b^2$ the intraclass correlation between cows

$= \sigma_e^2$ the residual variation.

Apart from the random effect of cows in the models used to analyze the data, fixed effects of treatment and days post treatment were included. The interaction between treatment and days post treatment was also estimated.

Treatment experiment: Six teats of three cows with a SCC > 500 000 cells/ml were selected. Milk collected from these teats tested positive for *S. aureus.* This field strain of *S. aureus* was also tested as sensitive towards peptide AS-48. Three of these teats were infused with 1 ml encapsulated peptide AS-48 (6400 AU/ml). The other three teats acted as controls and were infused with 1 ml saline solution (0.75% NaCl). The infusion was done immediately after milking, thus on day 1 of milk collection. The samples were massaged into the teats. Milk samples were collected daily (up to day 7) from which viable *S. aureus* and SCC counts were determined. For this experiment the number of replication was also relatively low. Again it was dependant on the number of available cows in a specific stage of lactation and therefore the number of replicates was regarded as sufficient for the purposes of the study. Thus a high number of replicates is again not advisable for this type of experiment.

Statistical Methods

The SCC and *S. aureus* counts were variable, ranging from 1.8 x 10^5 to 1 x 10^7 cells/ml and from to low to be detected with the methods used to 1.5 x 10^5 cfu/ml respectively. Data were analyzed as a 2 (treatments) x 7 (day) factorial design. As for the prevention experiment the analysis was complicated by the fact that the same quarter was sampled repeatedly. The same basic procedure was followed to account for repeated sampling. The difference was that individual quarters were confounded with treatments in this instance. The appropriate analysis was thus to nest individual quarters within treatments. Repeatability was estimated from between quarter variance components as described previously.

Conjugative transfer experiments. Filter mating experiments were done as described by Reichelt *et al*. (15). An overnight culture of *E. faecalis* FAIRE 92 (500 µl) and *E. faecalis* FA2/Ent (500 µl) was added to 10 ml of MRS, mixed and filtered through a 0.45 µm pore-size sterile membrane (HAWP, Millipore). The membrane was placed onto MRS agar and incubated overnight at 37^{o}C. The cells were washed from the membrane and suspended in 2 ml MRS, serially diluted, and placed onto MRS agar plates containing 25 µg fucidic acid, 25 µg rifampicin, 2133.3 AU/ml purified peptide AS-48 and 533.3 AU/ml crude enterocin 1071A and 1071B. The antibiotics used were inhibitory towards *E. faecalis* FAIRE 92 but not against *E. faecalis* FA2/Ent, while both bacteriocins were inhibitory towards opposite producing strains. Colonies were selected at random and screened for plasmid content by using standard techniques (Sambrook *et al*., 1989) A colony of conjugated cells of *E. faecalis* FA2/Ent that contained plasmid p92 (conjugant FA2/Ent/92) was cultured, and the cell-free supernatant was subjected to peptide AS-48 purification and mass spectrometry analysis, as described above.

Southern blot hybridization. Southern blot hybridizations were performed as described by Sambrook *et al*. (17). The plasmid DNA of *E. faecalis* FA2/Ent/92 was hybridized with a 200 bp probe made from a fragment containing part of the peptide AS-48 structural gene, from plasmid pAM 401-61 (11). The same DNA was also hybridized with the 8 kb plasmid, pBEco2, which contains a 2 kb fragment of the enterocin 1071A and B structural gene, as well as part of the abc transporter gene (3). Detection was by DNA probes radioactively marked with ^{32}P[dATP] (Amersham Pharmacia Biotech, Uppsala, Sweden).

RESULTS

Production and inhibitory activity of peptide AS-48. The highest activity levels of peptide AS-48 (148.15 AU/$\log_{10}$cfu) was recorded during late exponential growth, after 7 h of fermentation (Fig. 1). Slight antimicrobial activity was recorded against *S. aureus* in a ten-fold concentrated, cell-free supernatant. Increased activity was only recorded when peptide AS-48 was isolated and purified. The titer of purified peptide AS-48 varied between 3200 and 12800 AU/ml. This corresponded to a two to eight-fold increase in specific antimicrobial activity after purification on SP-Sepharose. *S. agalactiae* and *S. dysgalactiae* were also inhibited, but not *E. coli*.

Sensitivity to proteolytic enzymes. Peptide AS-48 was sensitive towards proteinase K, pronase, pepsin, papain, α-chymotrypsin and trypsin.

Molecular mass determination. Mass spectrometry analysis indicated that the active samples, collected from the SP-Sepharose column and pooled together, contained a single peptide with a molecular mass of 7.150 kDa (Fig. 2). Separation on tricine-SDS-PAGE yielded only one active peptide band in the range of 6.4 kDa (Fig. 3).

Liposome encapsulation. Encapsulation efficiencies varied between 10 and 25%. For the prevention experiment the titer of peptide AS-48 was 400 AU/ml after proteinase K treatment, compared to 3200 AU/ml for the control sample. This resulted in an encapsulation efficiency (E%) of 12.5%. In the case of the treatment experiment the titer of peptide AS-48 was 1600 AU/ml after proteinase K treatment, compared to 6400 AU/ml of the control sample. This resulted in an E% of 25%.

Antimicrobial activity. Addition of peptide AS-48 to *S. aureus* during the lag phase completely inhibited cell growth (Fig. 4). However, when peptide AS-48 was added during mid-exponential growth a slight decrease in viable cells for a very short period (30 min) was recorded. Thereafter cell growth continued as before. During this period the specific antimicrobial activity increased from 307.69 AU/$\log_{10}$ cfu (150 min.) to 354,37 AU/$\log_{10}$ cfu (180 min.). After this the specific antimicrobial activity again decreased to stabilize at values of 277,3 AU/$\log_{10}$ cfu in the stationary phase.

In situ activity tests:

Prevention experiment. The variation accounted for by the repeated sampling of different cows was significant in the analysis involving SCC ($P < 0.001$) and *S. aureus* counts ($P < 0.05$). Repeatability estimates (±SE) derived from these analyses were 0.26 ± 0.19 and

0.13 ± 0.13, respectively. Although these estimates could not be proven as significant ($P < 0.05$) from zero, the variation between cows controlled significant ($P < 0.05$) portions of the overall variation in both analyses. It was thus decided to retain this effect in the models used.

The interaction between treatment and days post treatment was not significant ($P = 0.79$) as far as SCC were concerned. This interaction is presented in Fig. 5a. A sharp increase ($P < 0.05$) in SCC was recorded on day 2. Afterwards, SCC stabilized at the same approximate level. In quarters treated with peptide AS-48 the SCC were generally lower than in the control quarters, with a significant ($P < 0.05$) difference on day 6. When the overall mean values for treated and untreated quarters were compared, there was a significant ($P < 0.01$) difference in favor of the treated quarters ($\log_{10}$ transformed means 5.710 ± 0.143 vs. 6.006 ± 0.143). When these mean values were transformed back to the normal scale they corresponded to a SCC of 512 625 for treated quarters and 1 013 911 for control quarters. Expressed relative to the control treatment, the reduction in SCC in the treated quarters amounted to approximately 50%.

In control quarters where no peptide AS-48 was included, *S. aureus* numbers increased ($P < 0.05$) linearly to reach a maximum of 3.4 ± 0.4 $\log_{10}$ cfu/ml on day 4. *S. aureus* counts decreased after day 4 and subsequently stabilized until day 7. The increase in *S. aureus* cell numbers in treated quarters was slower. Mean values for treated quarters were lower than for control quarters on day 4, 6 and 7 (Fig. 5b). In view of the fact that no significant ($P < 0.05$) interaction was found between treatment and days post treatment, it is appropriate to give overall mean values for treated and control quarters. These were (on the $\log_{10}$ scale) 2.785 ± 0.181 for control quarters and 2.181 ± 0.181 for treated quarters. Transformed back to the normal scale (with 100 subtracted) these means values correspond to respective *S. aureus* counts of 509 and 51, i.e. a near to 90% reduction in treated quarters.

Treatment experiment. The between quarter variance was significant ($P < 0.01$) in analyses on SCC and *S. aureus* counts. The respective repeatability estimates derived from the variance components were 0.40 ± 0.25 and 0.41 ± 0.25.

For SCC the interaction between treatment and days post treatment did not reach significance in this case ($P = 0.06$). Overall, SCC was reduced ($P < 0.05$) in treated quarters relative to control quarters (Fig. 6a). Overall mean values (±SE) for SCC were 6.70 ± 0.12 for control quarters, compared to 6.01 ± 0.12 for treated quarters.

Corresponding antilogs were 5.03 x 10^6 and 1.03 x 10^6. Expressed relative to the control treatment, the reduction in treated quarters amounted to nearly 80%.

In the case of *S. aureus* counts treatment interacted ($P < 0.01$) with the days of post treatment in this instance. In the case of untreated quarters, *S. aureus* counts basically remained on the same level throughout the monitoring period (Fig. 6b). In treated quarters there were a significant ($P < 0.01$) decline in *S. aureus* counts, to reach levels not significantly different ($P < 0.05$) from two, which corresponds to zero for backtransformed values. This decline was observed from day 4. Differences between mean values for treated and control quarters were significant ($P < 0.05$) from day 2 onwards. Overall means (±SE) were 4.1 ± 0.3 for control quarters, compared to 2.91 ± 0.26 for treated quarters. Transformed back to the normal scale (with 100 subtracted) these means correspond to respective *S. aureus* counts of 12 530 and 715, i.e. a reduction of up to 94% in treated quarters.

Conjugative transfer experiments. The *E. faecalis* FA2/Ent transconjugants contained a plasmid of approx. 21 kb and produced peptide AS-48. Purification of peptide AS-48 by a combination of Triton X-114 phase partitioning and SP-Sepharose chromatography, followed by mass spectrometry, indicated the presence of a peptide with a molecular mass of 7.150 kDa (data not shown). The spectrum of antimicrobial activity for the peptides isolated from the transconjugant (*E. faecalis* FA2/Ent/92) is broader than that recorded for *E. faecalis* BFE 1071 (Table 1).

Southern blot hybridization. Conjugation of plasmid p92 from *E. faecalis* FAIRE 92 to *E. faecalis* FA2/Ent did take place without the loss of pEF1071 (Fig. 7).

DISCUSSION

We have described the isolation and purification of the cyclic peptide AS-48 from *E. faecalis* FAIRE 92. The *in situ* antimicrobial activity of peptide AS-48 was evaluated as a means of reducing the number of viable cells of *S. aureus* in deliberately challenged as well as already infected quarters of lactating cows. In contrast to previous reports suggesting the broad inhibitory spectrum of peptide AS-48, which included the inhibition of *E. coli*, the spectrum of antimicrobial activity was narrower. Gram-negative organisms need higher concentrations because of the different concentrations of the active form of

peptide AS-48 and because of the presence of an outer membrane (6, 7, 10). However, the other major causative organisms of mastitis in the Western Cape, which include *S. aureus*, *S. agalactiae* and *S. dysgalactiae*, were inhibited.

The molecular mass of the peptide isolated was 7.150 kDa and confirmed that *E. faecalis* strain FAIRE 92 produced peptide AS-48. This was further supported by the fact that only one active band, corresponding to the size of peptide AS-48, was detected on the tricine-SDS-PAGE gel (Fig. 3). Martínez-Bueno *et al*. (11) was unable to determine the protein sequence of peptide AS-48 by Edman degradation. In this study we ascribe this phenomenon to the cyclic nature of peptide AS-48.

The E% compared good to the efficiencies previously achieved by the specific method used (4). On the other hand non-encapsulated peptide AS-48 holds the benefit of eliminating extracellularly located *S. aureus*, which is where the organism is mainly situated in infected tissue (14).

Peptide AS-48 exerts its biological action by incorporating the peptide into the cytoplasmic membrane, forming pores that leak potassium and inorganic phosphate (6, 7, 18). The fact that only a small decrease in the cell count was observed when encapsulated peptide AS-48 was added at a cell count of approximately 2.51×10^{10} may indicate that less AS-48 peptides were available per sensitive cell. The result was that the bacteriocin was present at sublethal concentrations for *S. aureus* (20).

According to the results gained from the *in situ* tests it seems that peptide AS-48 had a better effect in the treatment experiment in comparison to the prevention experiment, when treated quarters were compared to untreated quarters. It must however be remembered that the mean SCC and *S. aureus* counts were lower for the prevention experiment in comparison to the treatment trial. Although *S. aureus* was not eliminated totally in either of the two experiments, the reduction of *S. aureus* counts in the treatment experiment was better than in the prevention experiment. The mean SCC count in the prevention experiment (512 625) is much closer to the upper limit of 500 000 for healthy cows. In the treatment experiment the mean SCC was 1.03×10^{6}. The results can partly be explained through the fact that initial SCC and *S. aureus* counts in the treatment experiment were much higher than recorded in the prevention experiment.

The purpose of conjugating plasmid p92 from *E. faecalis* strain FAIRE 92 to *E. faecalis* strain FA2/Ent, already containing plasmid pEF1071, was to develop a strain with a broader antimicrobial spectrum and antimicrobial activity than *E. faecalis* FA2/Ent. This

strain would then be able to produce enterocins 1071A and 1071B, as well as peptide AS-48. Initially, it was feared the plasmids might have the same origin of replication. This might have lead to the random selection of only one of the plasmids being replicated, since the amplification of only one of the plasmids would lead to the elimination of the other plasmid. However, southern blot hybridizations confirmed the presence of both plasmids in the recipient strain (Fig. 7).

This study thus indicates the potential use of peptide AS-48 in the prevention and treatment of *S. aureus* mastitis. The only problem encountered was the low specific antimicrobial activity (296,30 to 1185,18 AU/$\log_{10}$ cfu). Further studies should thus be aimed at this problem before the potential use of peptide AS-48 in mastitis control can be exploited.

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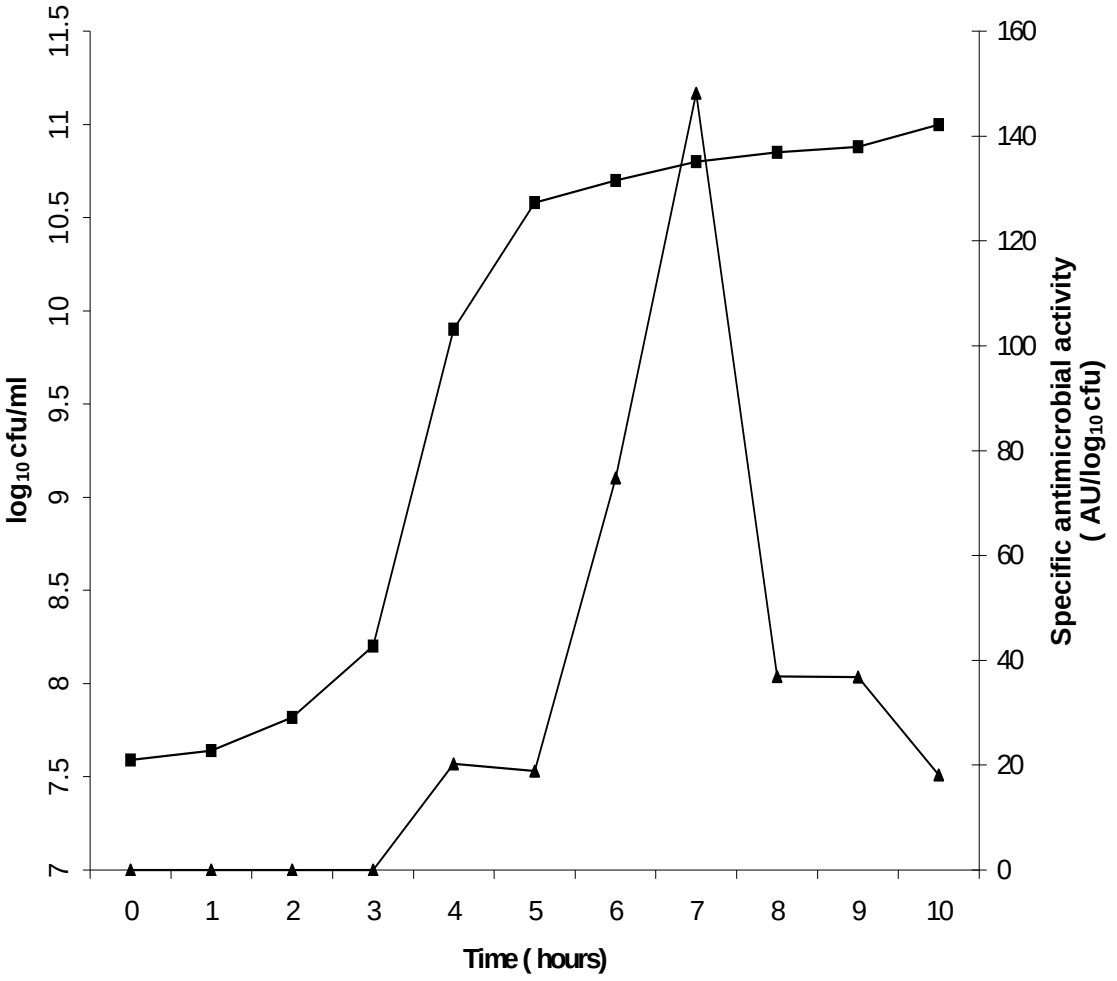

FIG. 1. Production of peptide AS-48 during the growth of *E. faecalis* FAIRE 92. ■ Growth of *E. faecalis* FAIRE 92. ▲ Specific antimicrobial activity of peptide AS-48 during the growth of *E. faecalis* FAIRE 92.

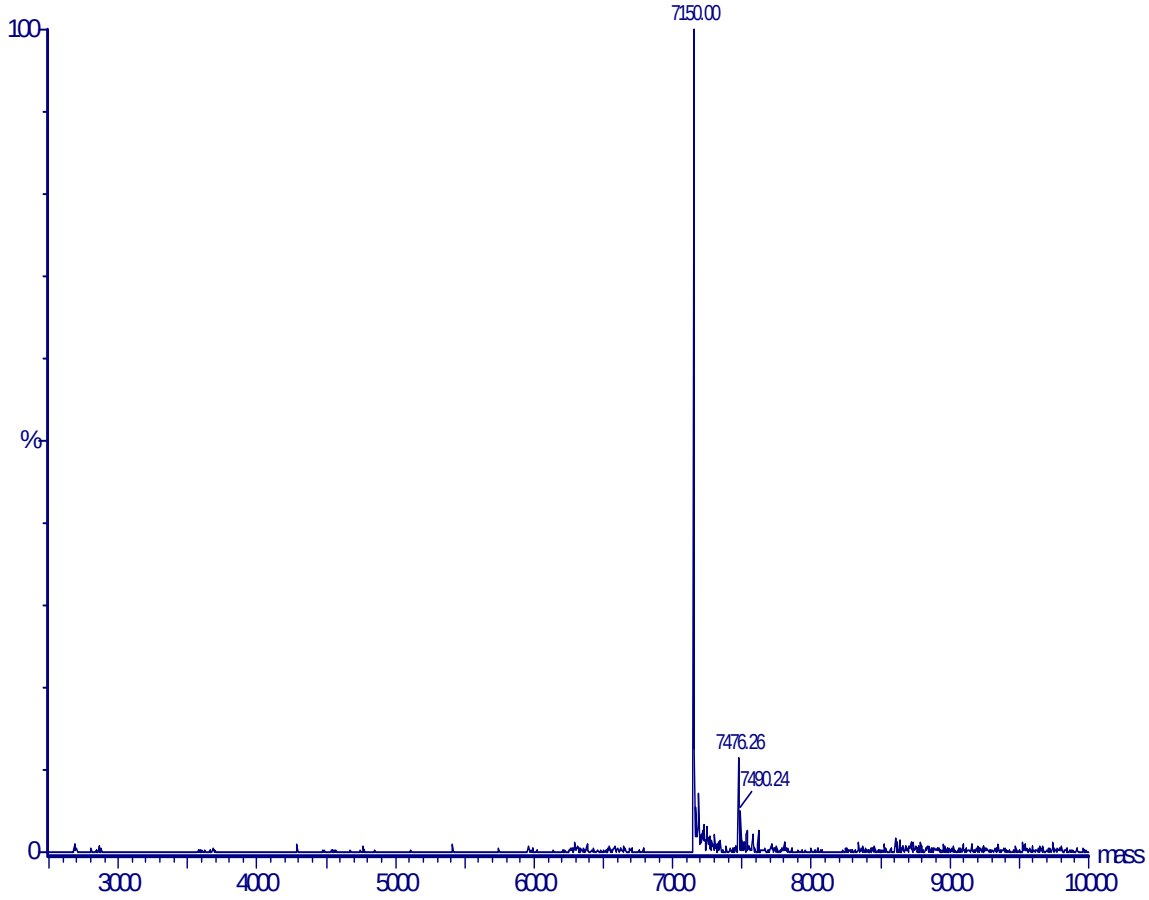

FIG. 2. Molecular mass of peptide AS-48 calculated from the electrospray ionization-mass spectrometry multiple charged spectra.

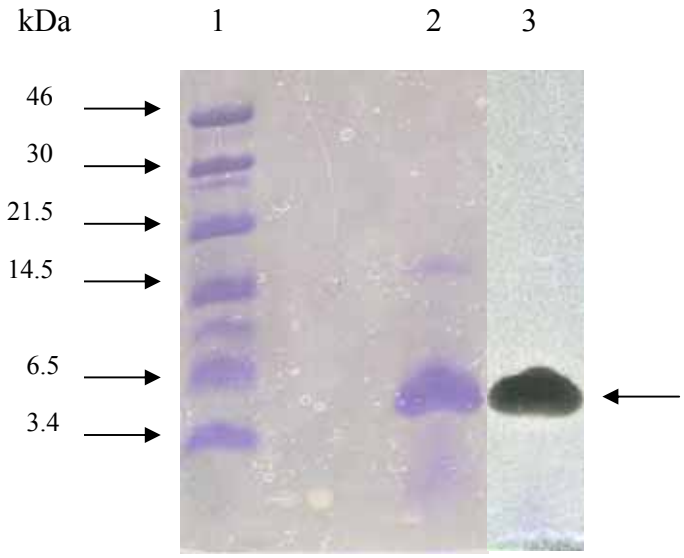

FIG. 3. Seperation of peptide AS-48 by tricine-SDS-PAGE. Lane 1 , Rainbow protein size markers; lane 2, peptide AS-48 stained with Coomassie brilliant blue R250; lane3, peptide AS-48 overlaid with cells of mastitic *S. aureus* embedded in BHI Agar (0.75% agar, wt/vol). The active peptide band is indicated by an arrow.

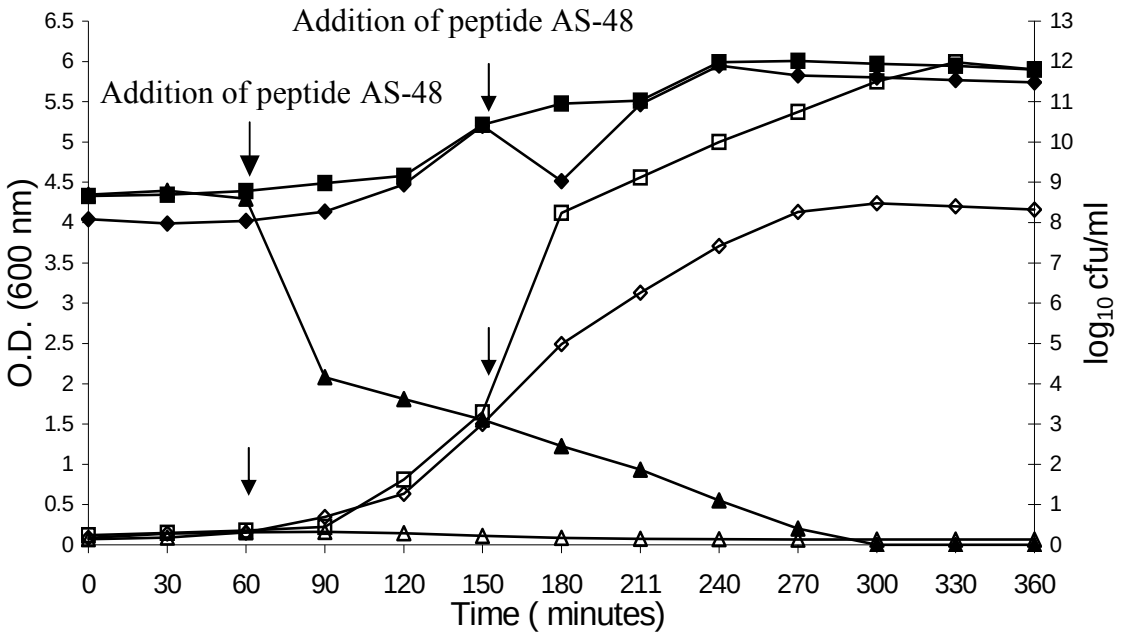

FIG. 4. Effect of peptide AS-48 on the growth of *S. aureus*. Counts were made in the absence of peptide AS-48 (■) and in the presence of peptide AS-48 added at the beginning of the lag phase (▲) and during mid-exponential growth (◆). Turbidity was determined for cells growing in the absence of peptide AS-48 (□) and in the presence of peptide AS-48 at the beginning of the lag phase (△) and during mid-exponential growth (◇).

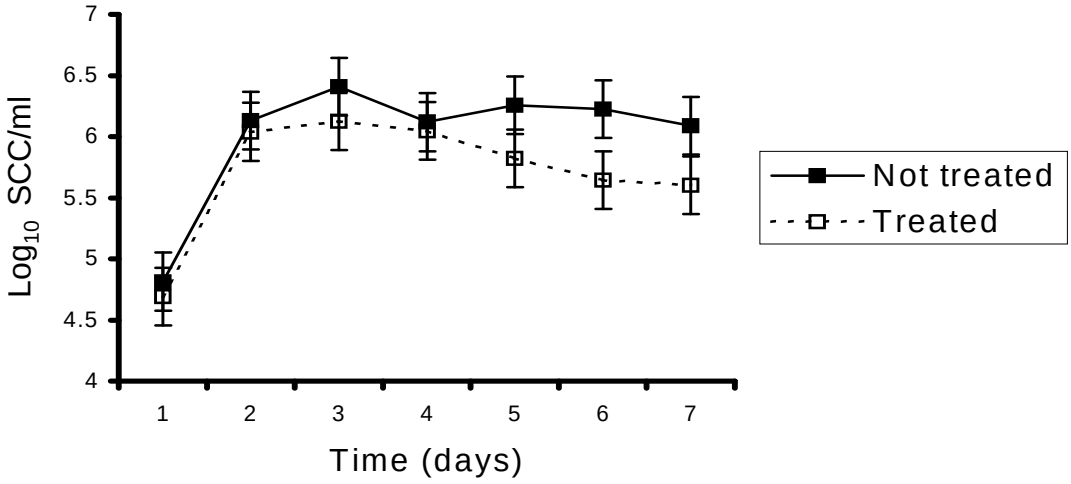

FIG. 5(a). The influence of treatment with peptide AS-48 on SCC relative to an untreated control. Each mean is based on five replicates. The experimental design involved five cows with on teat randomly assigned to the treated and control groups respectively. Vertical bars denote standard errors.

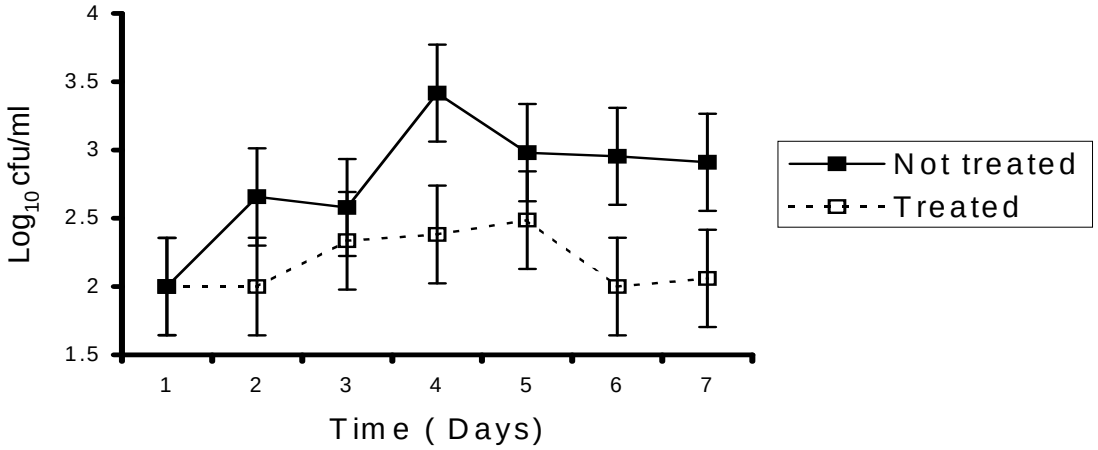

FIG. 5(b). The influence of treatment with peptide AS-48 on cell numbers of *S. aureus* relative to an untreated control. Each mean is based on five replicates. The experimental design involved five cows with on teat randomly assigned to the treated and control groups respectively. Vertical bars denote standard errors.

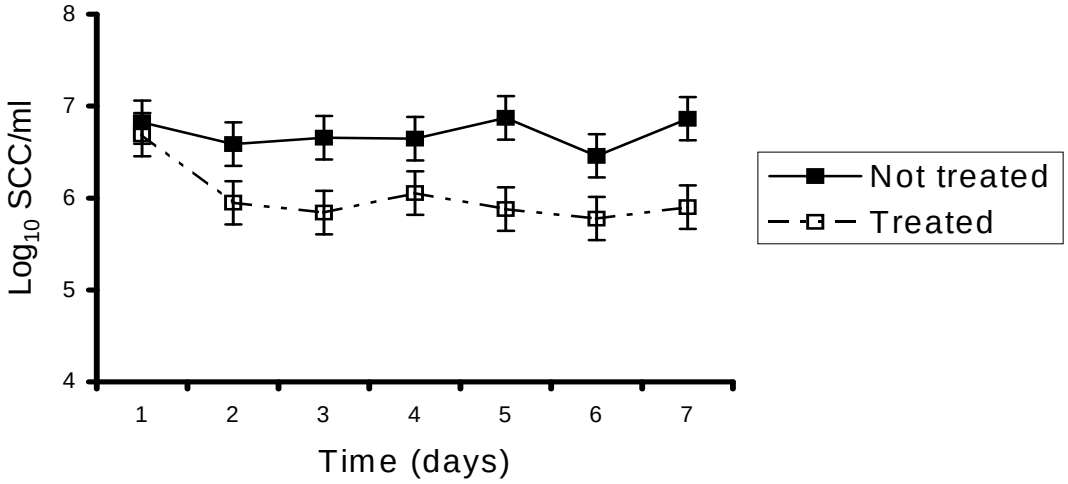

FIG. 6(a). The influence of treatment of infected quarters with peptide AS-48 on SCC relative to untreated control quarters. Each mean is based on three replicates. The experimental design involved three cows with on teat randomly assigned to the treated and control groups respectively. Vertical bars denote standard errors.

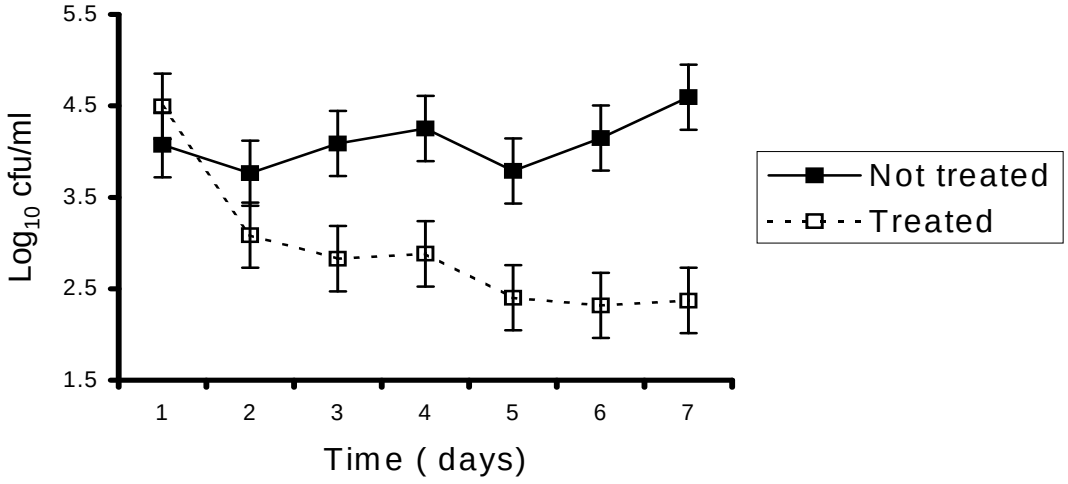

FIG. 6(b). The influence of treatment of infected quarters with peptide AS-48 on *S. aureus* counts relative to untreated control quarters. Each mean is based on three replicates. The experimental design involved three cows with on teat randomly assigned to the treated and control groups respectively. Vertical bars denote standard errors.

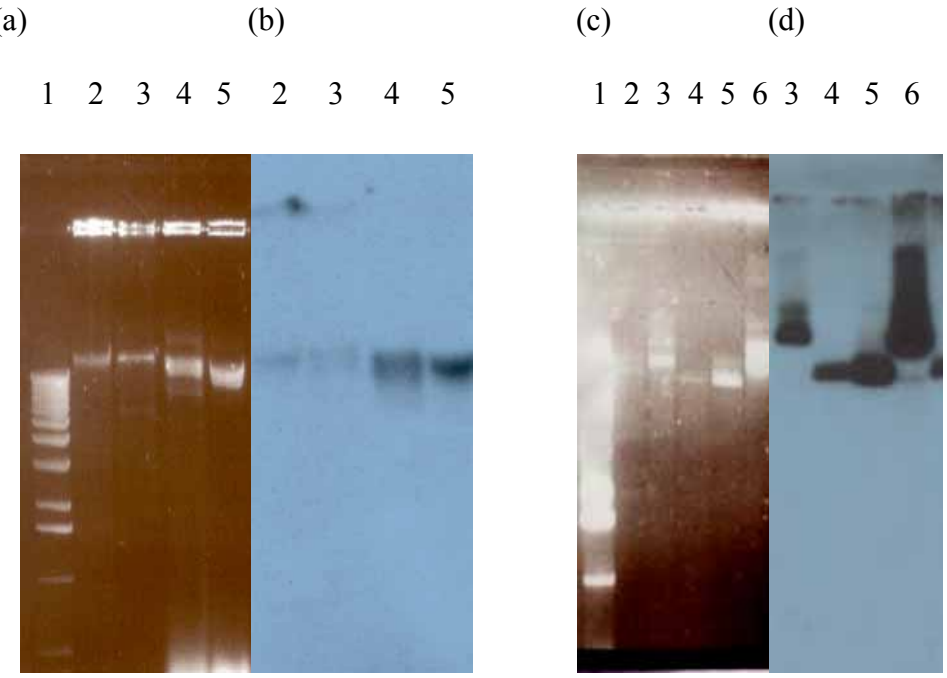

FIG. 7. Plasmid profiles of *E. faecalis* FA2/Ent/92 after conjugation of plasmid p92. (a) Comparison of the plasmid profiles of *E. faecalis* FAIRE 92 and the transconjugant. Lane 1, 1 kb marker; lane 2, plasmid p92 (uncut); lane 3, plasmid p92 (digested with Sal I); lane 4, transconjugant (digested with Sal I); lane 5, transconjugant (uncut). (b) The same gel after Southern blotting onto a MagnaGraph nylon transfer membrane (MSI, Westboro, Mass.) and hybridized with a radioactively labeled ^{32}P[dATP] 200 bp EcoRI and NdeI isolated fragment of plasmid pAM 401-61 containing part of the peptide AS-48 structural gene. (c) Comparison of the plasmid profiles of *E. faecalis* FA2/Ent and the transconjugant. Lane 1, 1 kb marker; lane 2, plasmid p92 (digested with Eco RV); lane 3, FA2/Ent (uncut); lane 4, FA2/Ent (digested with Eco RV); lane 5, transconjugant (digested with Eco RV); lane 6, transconjugant (uncut). (d) The same gel after Southern blotting onto a MagnaGraph nylon transfer membrane (MSI, Westboro, Mass.) and hybridized with a radioactively labeled ^{32}P[dATP] plasmid pBEco2 containing a fragment of the enterocins 1071A and 1071B structural gene.

TABLE 1. Spectrum of antimicrobial activity of ammonium sulfate precipitated supernatant of *Enterococcus faecalis* FA2/Ent/92 (transconjugant).

Organism[a]	Strain(s)[b]	Sensitivity[c]
Bacillus cereus	LMG 13569	++
Enterococcus faecalis	BFE 1071[d]	+++
Lactobacillus acidophilus	LMG 13550	++
Lactobacillus casei	LMG 13552	++
Lactobacillus curvatus	LMG 13553	++++
Lactobacillus fermentum	LMG 13554	++
Lactobacillus plantarum	LMG 13556	+
Lactobacillus reuteri	LMG 13557	+
Lactobacillus sakei	LMG 13558	++
Leuconostoc cremoris	LMG 13562, 13563	++
Leuconostoc pentosaceus	LMG 13560, 13561	+++
Staphylococcus aureus	MKB 38	+
Staphylococcus carnosus	LMG 13567	++

[a] Organisms resistant to the antimicrobial effect of enterocins 1071A and 1071B.

[b] Abbreviations: LMG, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; MKB, Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa.

[c] +, sensitive to peptide AS-48 (+, ++, +++, ++++ reflect the degree of sensitivity).

[d] Enterocins 1071A and 1071B producer strain.

GENERAL DISCUSSION AND CONCLUSIONS

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The dairy industry provides a valuable feed source to any nation. In South Africa, milk is produced under conditions which are regarded as sub-optimal for effective dairy farming. A low milk yield is mainly due to poor feeding, management, hygiene, and general health practices. Determined efforts should be made to rectify this and also to improve udder health by controlling and preventing mastitis, which is the most expensive disease confronting dairy farmers (3).

Mastitis causes a decrease in the milk yield of dairy cows, whilst it also changes the milk composition. Expenditures and adverse effects include an increase in the replacement heifer costs because of a shorter productive life, drug and veterinary costs, extra labour and loss of genetic material from the herd. Treatment of mastitis in South Africa amounted to R380 million in 1994 and is estimated to increase to R480 million in 2003. In South Africa only 40% of all cows have a healthy udder. The other 60% have some degree of mastitis (3).

Bacterial infection is the most common cause of mastitis and most of the cases can be attributed to staphylococci, streptococci and coliform bacteria. In the Western Cape of South Africa, *Staphylococcus aureus* is responsible for 40% of all mastitis cases reported. The organism is relatively resistant to disinfectants and many of the commonly used antibiotics. Apart from the infection, *S. aureus* secretes various toxins and enzymes and consumption of contaminated milk may result in food poisoning (2).

Prevention of staphylococcal infections by immunisation has been abandoned as it was found not to be a practical method. The reasons for this are the existence of so many different staphylococcal strains and inadequate knowledge of the pathogenesis of staphylococcal mastitis and the immune mechanisms protecting the mammary gland from infection (1, 5).

Attractive alternatives may include the use of lactic acid bacteria, and specifically bacteriocins, to achieve a low cost-effective method of preventing and treating *S. aureus* infections. In a previous study nisin, in combination with lysostaphin, could only cure

66% of infections caused by *S. aureus* (15). Lacticin 3147, incorporated into a teat seal, killed > 99.9% of *S. aureus* cells when used at a concentration of 1280 AU/ml (4).

In this study, we determined, *in vitro* and *in vivo*, the inhibitory activity of the cyclic peptide AS-48, classified as a bacteriocin, against a strain of *S. aureus* isolated from mastitic milk. Peptide AS-48, produced by *Enterococcus faecalis* strain FAIRE 92, is produced maximally during late exponential growth, i.e. after 7 h of fermentation. At this time the specific antimicrobial activity corresponds to 148.15 AU/ml in a ten-fold concentrated, cell-free supernatant. Purification of this crude extract of peptide AS-48 yielded a two to eight-fold increase in specific antimicrobial activity (from 148.15 to 296.29-1185.18 AU/ml). Peptide AS-48 was also active against *Streptococcus agalatiae* and *Streptococcus dysgalactiae* but not *Escherichia coli*. The proteinaceous nature of peptide AS-48 was confirmed by its sensitivity to proteolytic enzymes. The molecular mass of peptide AS-48 was determined at 7 150 Da and is in agreement with the size of the peptide isolated from other strains of *E. faecalis*. Peptide AS-48 added to lag-phase cells of *S. aureus* completely inhibited cell growth. However, when peptide AS-48 was added during mid-exponential growth a slight decrease in viable cells for a very short period (30 min) was recorded. Thereafter cell growth continued as before.

An *in vivo* study was conducted by administering peptide AS-48 (6400 AU/ml) to different quarters of the udders of cows, deliberately or already infected with *S. aureus*. *In situ* activity tests were done with peptide AS-48 encapsulated in liposomes. This was done to protect peptide AS-48 against proteolytic breakdown in the udder. Encapsulation would also lead to a slower release of the peptide in the udder and a more sufficient localization at the site of infection. Encapsulation efficiencies (E%) varied between 12 and 25%, which are good in comparison to the encapsulation of other peptides done according to the method used.

In the prevention experiment the reduction in SCC and *S. aureus* cell counts in the treated quarters amounted to 50% and 90%, respectively. The reduction in SCC and *S. aureus* cell counts in the treatment experiment amounted to 80% and 94%, respectively.

Conjugation of plasmid p92, which harbours the genes encoding peptide AS-48, to *E. faecalis* FA2-2, which contains plasmid pEF1071 encoding enterocins 1071A and 1071B, was done to construct a strain with a broader antimicrobial spectrum and antimicrobial activity than *E. faecalis* FA2/Ent. In addition, this may well prevent resistances of the target strains from occurring. Conjugation of plasmid p92 to strain FA2-2 was successful without the loss of plasmid pEF1071, although plasmid loss was not tested over many generations. The spectrum of antimicrobial activity recorded for the transconjugant *E. faecalis* FA2/Ent/92 was broader than that recorded for *E. faecalis* BFE 1071. Organisms inhibited after conjugation of plasmid p92 include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus sakei*, *Leuconostoc cremoris*, *Leuconostoc pentosaceus*, *Bacillus cereus*, *Staphylococcus carnosus*, *S. aureus* and *E. faecalis*. Southern blot hybridisations confirmed the presence of both plasmids in the recipient strain.

Results obtained in this study indicated that peptide AS-48 could be used in the prevention and treatment of *S. aureus* mastitis. Future research might be aimed at enhancing the specific antimicrobial activity of peptide AS-48 through fermentation studies. Future studies with the transconjugant strain may include using enterocins 1071A and 1071B as well as peptide AS-48, encapsulated in liposomes, in other *in situ* studies.

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