

**Characterization of an antimicrobial peptide from *Enterococcus faecium* active against bacteria associated with sexually transmitted diseases**

by

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Thesis presented for the Masters Degree in Microbiology at the University of Stellenbosch

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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.

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Date: 13/02/06

## SUMMARY

Urogenital tract infections are a major health problem among women. These infections include sexually transmitted diseases such as gonorrhoeae, chlamydia, syphilis and acquired immunity deficiency syndrome (AIDS) (Parratt and Hay, 2003), as well as opportunistic urogenital infections such as vaginosis and abnormal urinary tract infections (Foxman, 2003; Sobel and Chaim, 1996; Wang, 2000). Vaginosis and urinary tract infections affect more than 1 billion women worldwide annually (Reid and Bruce, 2003). Antibiotic resistance and the public demand for natural products have shifted the focus towards the application of probiotic lactic acid bacteria to try and combat these infections.

Probiotics are marketed as microbial food supplements or capsules that may prevent diseases and clinical disorders (Schrezenmeir and de Vrese, 2001). Most of the well-known probiotics are lactic acid bacteria and include species such as *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 (Hoesl and Altwein, 2005; Stiles and Holzapel, 1997). Many of these bacteria are natural inhabitants of the urogenital tract which they colonize to form a protective barrier against intestinal pathogens (Hoesl and Altwein, 2005; Song *et al.*, 1999). Some probiotic strains produce antimicrobial peptides (bacteriocins), usually with a narrow spectrum of activity, i.e. inhibition of only a certain group of pathogens (Klaenhammer, 1993).

Bacteriocins are secreted either through ATP-binding cassettes (ABC) – transporters or via a general secretion pathway. Bacteriocins secreted by ABC-transporters have a double-glycine-type leader sequence in their N-terminal, which serves as a recognition signal for protein procession and secretion (Haverstein *et al.*, 1995). A *sec*-dependent leader signal peptide is characteristic of bacteriocins secreted through the general secretion pathway. The *sec*-dependent leader peptide is usually positively charged with a hydrophobic core and a specific cleavage region (von Heijne and Abrahmsen, 1989).

In this study, lactic acid bacteria were screened for the production of bacteriocins active against uropathogenic strains of *Enterococcus faecalis* and *Candida albicans*. *Enterococcus faecalis* is usually associated with secondary bacteraemia of the urogenital tract and is linked to a large number of hospital-acquired infections. Human immunodeficiency virus (HIV) patients are especially susceptible to *E. faecalis* infections. *Candida albicans*, on the other hand, is an opportunistic pathogen responsible for 80-90% of reported cases of candidal vaginosis, which is diagnosed in approximately 75% of women (Sobel, 1993). Approximately

half of the patients suffering from candidal vulvovaginitis (CVV) suffer from follow-up infections (Ferrer, 2000).

Strain T8, isolated from vaginal secretions of children infected with HIV, inhibited the growth of uropathogenic strains of *E. faecalis* and was identified as an *Enterococcus faecium* isolate. Two additional strains, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST15, isolated from sorghum beer and soy flour, respectively, also inhibited *E. faecalis* and were included in the study. None of the three strains produced hydrogen peroxide. All three strains produced bacteriocin-like peptides that inhibited *E. faecalis*.

Plantaricin 423, produced by *L. plantarum* 423, has been described previously (van Reenen *et al.*, 1998) and is similar to pentocin TV35b (Okkers *et al.*, 1999). Bacteriocin ST15, produced by *Enterococcus mundtii* ST15, is similar to mundticiin ST4SA produced by a different strain of *E. mundtii* (Knoetze, Department of Microbiology, University of Stellenbosch, personal communication). Bacteriocin T8, produced by *E. faecium* T8, is a class IIa *sec*-dependent bacteriocin and may be considered a novel peptide produced by enterococci. The operon containing the structural gene, a potential immunity gene and mobilization genes is located on a 7 kb-plasmid. The operon was sequenced and the mode of activity of bacteriocin T8 determined. Bacteriocin T8 is 69% homologous to bacteriocin 31, produced by *Enterococcus faecalis* Y1717 (Tomita *et al.*, 1996). The signal peptide of bacteriocin T8 is only 27% homologous to the signal peptide of bacteriocin 31. The immunity peptide of bacteriocin T8 displayed 50% homology to that of bacteriocin 31.

## OPSOMMING

Urogenitale infeksies is 'n groot gesondheidsprobleem onder vroue. Hierdie infeksies sluit in seksuele oordraagbare siektes soos gonorree, chlamydia, sifilis en verworwe immunitetsgebreks sindroom (VIGS) (Parratt en Hay, 2003), asook opportunistiese urogenitale infeksies soos vaginose en abnormale urinekanaal infeksies (Foxman, 2003; Sobel en Chaim, 1996; Wang, 2000). Vaginose en urinekanaal infeksies affekteer meer as 1 biljoen vroue jaarliks wêreldwyd (Reid en Bruce, 2003). Weerstand teen antibiotika en die publieke vraag na natuurlike produkte plaas die fokus op probiotika vir die bevegting van hierdie infeksies.

Probiotika word bemark as mikrobiële voedsel-supplemente of kapsules wat siektes en kliniese kondisies kan voorkom (Schrezenmeir en de Vrese, 2001). Meeste van die bekende probiotika is melksuurbakterieë en sluit spesies soos *Lactobacillus rhamnosus* GR-1 en *Lactobacillus fermentum* RC-14 in (Hoesl en Altwein, 2005; Stiles en Holzapfel, 1997). Baie van hierdie bakterieë is natuurlike inwoners van die urogenitale weg waar hulle koloniseer om 'n beskermende versperring te vorm (Hoesl en Altwein, 2005; Song *et al.*, 1999). Sommige probiotiese stamme produseer antimikrobiële peptiede (bakteriosiene), maar gewoonlik met 'n beperkte spektrum van aktiwiteit, m.a.w. slegs sekere patogene word gehinbeer (Klaenhammer, 1993).

Bakteriosiene word met behulp van ATP-bindende kaset (ABK)-vervoerders of 'n meer algemene sekresie-metode uitgeskei. Bakteriosiene wat deur ABK-vervoerders uitgeskei word besit 'n dubbel-glisien leiertype volgorde in hul N-terminaal wat as 'n herkenningsstel vir proteïen-prosessering en sekresie dien (Haverstein *et al.*, 1995). 'n Sec-afhanklike leierseineptied is kenmerkend van bakteriosiene wat deur die algemene sekresie-pad uitgeskei word. Die sec-afhanklike leier-peptied is gewoonlik positief gelaai en het 'n hidrofobiese kern en klewing area (von Heijne and Abrahmsen, 1989).

In hierdie studie is melksuurbakterieë vir die produksie van bakteriosiene aktief teen patogene stamme van *Enterococcus faecalis* en *Candida albicans* geselekteer. *Enterococcus faecalis* is gewoonlik geassosieer met sekondêre bakteraemia en is gekoppel aan 'n groot aantal hospitaal-verwante infeksies. Mens immunitetsgebrek virus (MIV)-pasiente is veral vatbaar vir *E. faecalis* infeksies. *C. albicans*, aan die anderkant, is 'n opportunistiese patogeen wat vir 80 tot 90% van gerapporteerde gevalle van kandidale vaginose verantwoordelik is (Sobel, 1993). Simptome hiervan is in soveel as 75% van

gevalle aangeteken. Omtrent helfte van die pasiënte wat aan kandidale vulvovaginitis lei ervaar verdere infeksies (Ferrer, 2000).

Stam T8, geïsoleer uit vaginale sekresies van kinders geïnfecteer met MIV, het die groei van patogene stamme van *E. faecalis* geïnhibeer. Stam T8 is as *Enterococcus faecium* geïdentifiseer. Twee addisionele stamme, *Lactobacillus plantarum* 423 en *Enterococcus mundtii* ST15, geïsoleer van sorgumbier en sojameel, onderskeidelik, het ook *E. faecalis* geïnhibeer en is in hierdie studie ingesluit. Nie een van hierdie stamme produseer waterstofperoksied nie. Die drie stamme produseer bakteriosien-verwante peptiede wat die groei van *E. Faecalis* inhibeer.

Plantaricin 423, geproduseer deur *L. plantarum* 423, is deur van Reenen *et al.* (1998) beskryf en is soortgelyk aan pentocin TV35b (Okkers *et al.*, 1999). Bakteriosien ST15, geproduseer deur *Enterococcus mundtii* ST15, is soortgelyk aan mundticiin ST4SA wat deur 'n ander *E. mundtii* stam geproduseer word (Knoetze, Departement Mikrobiologie, Universiteit van Stellenbosch, persoonlike kommunikasie). Bakteriosien T8, geproduseer deur *Enterococcus faecium* T8, is 'n nuwe klas Ila *sec*-afhanklike bakteriosien. Die operon wat die strukturele geen, 'n potensiële immuniteits-geen en mobiliseringsgene bevat, is op 'n 7 kb plasmied gelokaliseer. Die operon se DNA volgorde en die meganisme van werking van die bakteriosien is bepaal. Bakteriosien T8 is 69% homolog aan bakteriosien 31, geproduseer deur *Enterococcus faecalis* Y1717 (Tomita *et al.*, 1996). Die sein-peptied en immuniteitsproteïen van bakteriosien T8 is onderskeidelik slegs 27% en 50% homolog aan die ooreenstemmende peptiede van bakteriosien 31.

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## PREFACE

The literature study is an overview of infections occurring in the urogenital tract. Probiotics, with the emphasis on strains used in the urogenital tract, and their bacteriocins are also discussed.

The articles "Screening of lactic acid bacteria for antimicrobial activity against *Enterococcus faecalis* and *Candida albicans* isolated from patients diagnosed with vaginosis" and "Bacteriocin T8, a novel class IIa *sec*-dependent bacteriocin produced by *Enterococcus faecium* T8, isolated from vaginal secretions of children infected with HIV" are written according to the style of Applied and Environmental Microbiology.



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## INTRODUCTION

Non-sexually and sexually transmitted infections in the urogenital tract affect billions of women worldwide (Hoesl and Altwein, 2005; [www.avert.org/statisticsworldwide.htm](http://www.avert.org/statisticsworldwide.htm)). Failure to treat these infections may lead to pyelonephritis (kidney infection), premature delivery, infertility, fetal mortality, impaired renal function, pelvic inflammatory diseases, cervical cancer and even death (Foxman, 2003; Jung *et al.*, 2004; Martin *et al.*, 1999; Parratt and Hay, 2003; Rhoton-Vlasak, 2000; Romero *et al.*, 2004). Treatment of urogenital tract infections is complicated by the spread of antibiotic resistance amongst uropathogens. Resistance to antibiotics is developed through mutations and the transfer of resistance genes to other microorganisms. The use of broad-spectrum drugs, the high frequency of infected people in hospitals, the prolonged stay in hospitals and intensive care units, and the abuse of antibiotics in the treatment of farm animals are contributing to the spread of antibiotic resistance (Hancock, 2005). A few antibiotics are used in the treatment of vancomycin-resistant enterococci, including *Enterococcus faecalis* that causes urinary tract infections (Tenover and McDonald, 2005). Multi-drug resistant uropathogens such as *Candida albicans* (Prasad and Kapoor, 2005), *Neisseria gonorrhoeae*, *Escherichia coli* (Levy, 2005), *Gardnerella vaginalis* and *Chlamydia trachomatis* (Ruef, 2005) are also emerging. Most of the latter species form biofilms, which renders them inaccessible to antibiotics (Greiner *et al.*, 2005; Mukherjee *et al.*, 2005; Swidsinski *et al.*, 2005; Tendolkar *et al.*, 2005).

The trend to use natural products in the treatment of urogenital tract infections, as well as increased antibiotic resistance, has shifted the focus towards probiotics as a possible alternative means of therapy. Probiotic foods and supplements are worth an estimated US \$6 billion annually. Currently, *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 are marketed as oral supplements for the treatment of urogenital tract infections (Hoesl and Altwein, 2005). The latter two strains have undergone intensive tests for safety and effectiveness (Reid *et al.*, 2001a; Reid *et al.*, 2001b; Reid *et al.*, 2003). Lactic acid bacteria inhibit the growth of uropathogens by production of lactic acid, hydrogen peroxide and antimicrobial peptides (bacteriocins). For these bacteria to successfully compete with pathogens, they need to colonize the urogenital tract and form a barrier against invading microorganisms (Reid, 1999).

In this study, lactic acid bacteria were screened for the production of bacteriocins inhibitory towards uropathogens. Selected strains were also studied for probiotic properties. A novel class IIa *sec*-dependent bacteriocin, produced by *Enterococcus faecium* strain T8, is described.

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[www.avert.org/stdstatisticsworldwide.htm](http://www.avert.org/stdstatisticsworldwide.htm)

## 1. INFECTIONS OF THE UROGENITAL TRACT

### 1.1. Introduction

Infections of the urogenital tract affect millions of women. Some of the opportunistic pathogens, such as *Candida albicans*, *Gardenerella vaginalis* and *Escherichia coli* cause a variety of diseases, including candidal vulvovaginitis (CVV), bacterial vaginosis (BV) and urogenital infections (Foxman, 2003; Sobel and Chaim, 1996; Wang, 2000). Infections caused by sexually transmitted pathogens such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Treponema pallidum* and the human immunodeficiency virus (HIV) are classified as sexually transmitted diseases (Parrat and Hay, 2003).

Candidal vulvovaginitis is associated with antibiotic therapy, pregnancy, uncontrolled diabetes, estrogen therapy, and the use of oral contraceptives with high levels of estrogen. Candidal vulvovaginitis can develop into recurrent symptomatic episodes of vaginitis or even chronic CVV (Sobel and Chaim, 1996).

Bacterial vaginosis (BV) is the most common cause of vaginitis, affecting over three million women in the United States annually (Wang, 2000). Bacterial vaginosis is characterized by a decrease of the normal population of lactobacilli in the vagina, associated with an overgrowth of anaerobic and facultative anaerobic microorganisms (Eschenbach *et al.*, 1989; Hill, 1993; Kimerlin and Andrews, 1998). This disease has been reported in 15 to 20% of pregnant women (Eschenbach *et al.*, 1984; McGregor and French, 2000) and has been associated with spontaneous preterm delivery (Eschenbach *et al.*, 1984), premature rupture of membranes (Lamont, 2003), and postpartum endometritis (Eschenbach *et al.*, 1984).

Urinary tract infection (UTI) is the most common nosocomial bacterial infection and is caused by *Enterobacteriaceae* and *Enterococcus* spp. The infection increases the risk of pyelonephritis (kidney infection) and may lead to premature delivery, often fetal mortality. Other symptoms are impaired renal function and end-stage renal disease, especially in pediatric patients (Foxman, 2003).

Sexually transmitted disease (STD), also known as Sexually Transmitted Infection (STI), is associated with pelvic inflammatory disease, infertility, ectopic pregnancy, cervical cancer,

neonatal infections and even death (Parratt and Hay, 2003). Sexually transmitted diseases are divided into two groups based on teratogenicity. Infections caused by *N. gonorrhoeae*, *C. trachomatis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *T. pallidum* are curable and belongs to the first group. Viral infections, especially HIV, human papillomavirus (HPV), hepatitis B/C virus (HBV, HCV), cytomegalo virus (CMV) and herpes simplex virus (HSV) are incurable (Schneede *et al.*, 2003). Infections caused by *C. trachomatis*, *N. gonorrhoeae* and *T. pallidum* are discussed in the following sections.

## 1.2. Microbiology

*C. albicans* is a unicellular, eukaryotic yeast. The colonies are cream-colored to yellowish. The texture of the colonies varies, depending on the species and may be pasty, smooth, glistening or dry, wrinkled and dull. Variations in morphology are common (Eggimann *et al.*, 2003). *Candida* is a dimorphic fungus present in two states. Blastospores and spores are the phenotype for extension, dissemination and transmission. Mycelium is the phenotype for germination (Ferrer, 2000).

BV is caused by *G. vaginalis*; Gram-negative anaerobic rods such as *Prevotella* spp.; Gram-positive anaerobic cocci, such as *Peptostreptococcus* spp.; genital mycoplasmas (*M. hominis* and *Ureaplasma urealyticum*); and curved Gram-positive anaerobic rods, e.g. *Mobiluncus* spp. *G. vaginalis* is, however, considered the main causative agent of BV (Hill, 1993).

Pathogens involved in UTIs are Enterobacteriaceae, *Proteus mirabilis*, *Klebsiella* spp., *Staphylococcus saprophyticus*, enterococci and *Pseudomonas* spp. *E. coli* is the predominant species, although *Enterococcus faecalis* is considered an important pathogen (Kahlmeter, 2003; Preston *et al.*, 1992; Reid and Seidenfeld, 1997).

*Chlamydia trachomatis* is the causative agent of chlamydia. These Gram-negative, obligate intracellular bacteria infect eukaryotic cells, but differ from other intracellular parasites in that they are not primarily parasites of invertebrates and have a biphasic cycle of replication (Cevenini *et al.*, 2002; Oehme *et al.*, 1991). *N. gonorrhoeae* causes gonorrhoea. This highly infectious, Gram-negative intracellular aerobic diplococcus, colonizes mucosal surfaces of the urogenital tract and the large intestine (Ghosn and Kibbi, 2004). Syphilis is caused by the spirochete *T. pallidum*. Humans are the only natural host of *T. pallidum*, although the species



can be propagated in the testis and skin of rabbits (Cox *et al.*, 2003; Deka *et al.*, 2002). The cell has an outer membrane similar to those of Gram-negative bacteria, but lacks lipopolysaccharides. The cell wall structure is more related to Gram-positive bacteria. Located in the periplasmic space is a contractible endoflagella (Cox *et al.*, 2003).

### 1.3. Epidemiology

*C. albicans* is one of approximately 200 *Candida* spp. and belongs to genus 4 in the family blastomycetes. *C. albicans* is the most frequently isolated yeast from colonized and infected sites in humans and is associated with 80 to 90% of reported cases of vaginal candidiasis (Sobel, 1993). *Candida glabrata* is the second most commonly isolated species (5 to 15% of reported cases), followed by *Candida tropicalis*, *Candida pseudotropicalis* and *Candida krusei* (Spinillo *et al.*, 1995). Candidal vulvovaginitis affects 75% of women during their life span, and approximately half of them suffer a second CVV event. Recurrent CVV develops in 5% of the cases, showing frequent and refractory episodes (Ferrer, 2000).

BV affects over three million women in The United States of America annually (Wang, 2000). BV has been reported in 10 to 25% of patients that visit general gynaecologic and obstetrics clinics. The statistics increases to 64% in sexually transmitted disease clinics (Sobel, 2000; Wang, 2000). BV has also been diagnosed in as many as 15 to 20% pregnant women (Eschenbach *et al.*, 1984; Sobel, 2000).

Women are more likely to suffer from UTIs than men. According to Foxman *et al.* (2000), one in three women will have at least one UTI diagnosed by a clinical physician before the age of 24. Fifty percent of the women diagnosed with UTI developed recurrent UTIs within the first year (Hooton, 2001). Catheter-associated UTIs are the most common nosocomial infections. A study conducted in 1992 indicated that 40% of all nosocomial infections were caused by catheters (Hashmi *et al.*, 2003). In non-institutionalized patients, UTIs accounts for nearly 25% of all infections (Raz, 2001).

*E. faecalis* is more frequently detected than *E. coli* amongst hospitalized patients (Hampel *et al.*, 2003). According to a study performed in intensive care units in Spain, enterococci are present in 10.2% of ICU-acquired infections. Enterococci, with *E. faecalis* being the predominant uropathogen, manifest as UTIs and secondary bacteraemia (Lerma *et al.*, 2003). *E. faecalis*

was also the most prevalent pathogen isolated from UTI patients over a period of 20 years (Shigemura *et al.*, 2005). *Enterococcus* spp. are the predominant bacteria isolated from HIV-patients suffering from urinary tract infections (Schonwald *et al.*, 1999).

According to the World Health Organisation (WHO), 340 million new cases of STDs have been reported in 1999. The largest number of new infections occurred in South and Southeast Asia, followed by Sub-Saharan Africa, Latin America and the Caribbean. Chlamydial infections increased between 1995 and 1999 with an estimated 2.5 million cases worldwide. In Sub-Saharan Africa 40.48 million infections have been reported in 1995, compared to 42.89 million in 1999. An estimated 62 million cases of gonorrhoea have been reported in 1999. During this period prevalence rates in Sub-Saharan Africa increased, while there was a decrease in other parts of the world. Cases of infection increased from 15.67 million in 1995 to 17.03 million in 1999. An estimated 12.22 million cases of syphilis have been reported worldwide in 1999, with a prevalence of 8.4 % in South Africa. The occurrence of all three infections is higher among women ([www.avert.org/statisticsworldwide.htm](http://www.avert.org/statisticsworldwide.htm)).

#### 1.4. Pathogenesis and the virulence factors involved

*C. albicans* follows a three-stage invasion mechanism. In each stage different virulence factors promote successful invasion. Adherence of *C. albicans* to vaginal epithelial cells is the first stage of infection and is necessary for blastospore survival and colonization. Adhesion of *C. albicans* is much higher compared to that of *C. glabrata*, *C. tropicalis*, *C. pseudotropicalis* and *C. krusei*. This explains the higher frequency of *C. albicans* infections in clinical settings (King *et al.*, 1980). *Candida* spp. recognize phospholipid- and fibronectin-containing receptors in the cell membranes of the vaginal epithelium. Blastospore adherence is dependent on mannoproteins in the fungal membrane. These proteins anchor itself to the receptors on the surface cells in the vaginal epithelium (Matthews and Burnie, 1998). The agglutinin-like sequences (ALS) are members of another family of adhesions that contribute to virulence in *C. albicans* (Hoyer, 2001).

Blastospores are not capable of penetrating epithelial tissue. However, once mycelium has developed, the yeast penetrates and invades the vaginal surface cells. A number of exogenous factors promote germination and mycelium or hyphae development (Ferrer, 2000). Penetration of epithelial cells is mediated by the production of several proteases (Ferrer, 2000). Proteases have also been implicated in phagocytosis. The phagolysosome produces proteinase after

germination of the ingested yeast. Proteinase attacks the proteins involved in the generation of microbicidal oxygen radicals, causing death of the phagocyte (Borg and Ruchel, 1990). Phospholipases have also been implicated in the virulence of *C. albicans*. Epithelial invasion causes the release of several substances, such as prostaglandins and bradykinins, inducing inflammatory changes in tissues. This process leads to edema, erythema, increased exudates, and cell shedding (Ferrer, 2000).

Phenotypic switching is a virulence factor enabling *C. albicans* to generate phenotypes that are beneficially adapted to different host environments. Phenotypic traits affected by this switching are cell morphology, lipid and sterol content, levels of protease secretion, hyphae formation, antigen expression and susceptibility to antifungal agents (Matthews and Burnie, 1998).

Anaerobic organisms such as *Bacteroides* species, *Prevotella bivia*, and *Peptostreptococcus* spp., as well as facultative anaerobic/aerobic organisms such as *G. vaginalis*, *Staphylococcus epidermis* and streptococci, constitutes 5% of the normal vaginal microflora (Kimerlin and Andrews, 1998). Lactobacilli account for the other 95%, as previously discussed. Reduction of the dominant lactobacilli (Eschenbach *et al.*, 1989; Hawes *et al.*, 1996) and overgrowth of anaerobic and facultative anaerobic organisms are implicated in BV (Hill, 1993; Kimerlin and Andrews, 1998).

Reduction of lactobacilli results in an increase in pH, which favours the growth of *G. vaginalis*. *G. vaginalis* decreases the oxygen level, which promotes growth of anaerobic bacteria and an increase in the production of proteolytic carboxylase enzymes (Wang, 2000). These enzymes break down vaginal peptides to a variety of amines such as trimethylamine, which at high pH, becomes volatile and malodorous. The amines are associated with increased vaginal transudation and squamous epithelial cell exfoliation, creating a typical discharge. Clue cells are created in conditions of elevated pH when *G. vaginalis* adheres to exfoliating epithelial cells. Amines provide a suitable substrate for the growth of *M. hominis* (Sobel, 2000). Although studies have shown that *G. vaginalis* and other BV-associated organisms may be transferred sexually, transmission alone is not sufficient for infection. The latter organisms are present in low numbers in a healthy vagina (Sobel, 2000).

Most uropathogens enter the bladder via the urethra and rectum. Vaginal colonization is a prerequisite to bladder infection. Uropathogens stimulate the release of cytokines in the bladder or kidneys, resulting in an inflammatory response and symptoms of UTI (Hooton, 2000).

Uropathogenic strains with certain virulence determinants have a selective advantage with regard to colonization and infection. Surface glycoproteins in the form of fimbriae or pili serve as ligands for glycoprotein and glycolipid receptors on uroepithelial cells. Type 1- and P-fimbriated strains of *E. coli* are strongly associated with cystitis and pyelonephritis (Johnson, 1991). Clinical evidence for the pathogenic role of P- and type 1-fimbriae in persistent bladder colonization and recruitment of inflammatory response have been published (Wullt, 2003). Potassium or capsular antigens, which inhibit phagocytosis, and the aerobactin system that chelates urinary iron, act as virulence factors by offering a growth advantage (Wullt, 2003).

*C. trachomatis* serovars D-K are responsible for genital chlamydia infections (Persson, 2002). During its unique developmental cycle, the bacteria alternate between two structurally and physiologically distinct forms. The one form, the elementary bodies (EB), is a stable structure, which remains viable extracellularly. The EB infect mainly epithelial cells and re-organizes to form metabolically active reticulate bodies (RB). DNA, RNA and protein are synthesized within the RB. This leads to binary fission and the transformation of RB into infectious EB. The EB are released within 48-72 hours and remain viable for a limited period before they infect other epithelial cells (Oehme *et al.*, 1991; Paavonen, 2004). The *C. trachomatis* heat shock protein (Hsp70) plays an important role in the interaction of EB to host cell ligands (Raulston *et al.*, 2002).

Syphilis is transmitted usually through sexual contact. *T. pallidum* infects the host through mucosal membranes and minor abrasions in the skin and replicates locally. A chancre appears at the primary site of inoculation. *T. pallidum* replicates very slowly with a generation time of approximately 33 hours. This explains the long-drawn-out nature of the illness and the relatively long incubation period (Zeltser and Kurban, 2004). *T. pallidum* induces endoarteritis by the binding of the spirochetes to endothelial cells and is facilitated by fibronectin molecules bound to the surface of spirochetes (Thomas *et al.*, 1986). No virulence factors have been described for *T. pallidum*. Weinstock *et al.* (1998) described 67 genes believed to be involved in interaction with the host.

Pili (thin, filamentous cell appendages) are essential for *N. gonorrhoeae* to infect the host. This type-4 pilus system attaches the organism to human epithelial cells (Fussenger *et al.*, 1997). The Opa-associated (class 5) proteins are outer membrane proteins with a molecular weight of approximately 28 kDa. These proteins are each encoded by an *opa* gene and are expressed by most strains of *N. gonorrhoeae*. It is speculated that these proteins play a role in invasion of epithelial cells following initial pili-mediated attachment. Once in the subepithelial space, they induce an inflammatory response (Nassif *et al.*, 1999).

Porins are another important virulence factor. *N. gonorrhoeae* produces two porins encoded by the genes *porA* and *porB*. Neisserial porins translocate as functional voltage-gated ion channels into the plasma membranes of eukaryotic cells. This causes a change in membrane potential and interferes with cell signalling (Ulmer *et al.*, 1992). Furthermore, *porB* plays a role in the invasion of epithelial cells (Bauer *et al.*, 1999). IgA1 protease also plays an important part in the pathogenesis of *N. gonorrhoeae*. IgA1 protease achieves this by cleaving the LAMP1 protein and therefore preventing phagolysosome fusion (Lin *et al.*, 1997).

#### 1.5. Host predisposing factors and risk factors

Pregnant women have a higher risk of developing CVV. During pregnancy there is an increased vaginal susceptibility to infection, with a high rate of colonization and symptomless vaginitis (Sobel, 1993). The high estrogen levels leads to increased glycogen concentrations in the epithelium, which is a nutritional source for yeast growth. Gestational hyperestrogenism promotes fungal adhesion and germination and, as a consequence, fungi are more capable to penetrate the vaginal wall (Zhang *et al.*, 2000). Oral contraceptives contain high levels of estrogen, which leads to increased candidal colonization. The mechanisms are the same as in pregnancy (Denning *et al.*, 1995). Metabolical disturbances in diabetic women predispose them to CVV. Appropriate diabetic control can minimize this factor (Reed, 1992). Candidal vulvovaginitis commonly occurs after systematic or vaginal antibiotic treatment. Antibiotic agents suppress lactobacilli, leading to the growth, adherence and germination of *Candida* spp. (Bluestein *et al.*, 1991).

Normal vaginal microflora, especially *Lactobacillus* spp., have been implicated in preventing vulvovaginitis (Hillier *et al.*, 1992). Lactobacilli compete with yeast for nutrients and block epithelial receptors for blastospore adhesion through co-aggregation (Klebanoff *et al.*, 1991).

However, the number of lactobacilli adhering to patients with CVV is higher than in the control group. This suggests that lactobacilli do not play a protective role against *C. albicans* (Demirezen, 2002). This suggestion is supported by a study in which the use of oral or vaginal lactobacilli preparations were unsuccessful in preventing post-antibiotic CVV (Pirodda *et al.*, 2004). Furthermore, in another study, lactobacilli were the predominant flora in both healthy asymptomatic women and women with acute recurrent CVV (Sobel and Chaim, 1996).

The frequency of BV is higher among women with multiple sexual partners (Hay *et al.*, 1997) and women visiting STD clinics (Cohen *et al.*, 1995). BV is also higher among lesbian women and women whose male partners have urethritis (Schmid, 1999). The treatment of partners to lower the risk of transmission has to be proven in therapy studies. Although this suggests that BV is associated with sexual activity, it is not a STD as stated before (Sobel, 2000). Women with black ethnicity have higher rates of BV than other racial groups (Goldenberg *et al.*, 1996). The use of intra-uterine device (IUD) is another risk factor for BV. Researchers have shown that IUD use is higher among women with BV than women without BV (Amsel *et al.*, 1983). The role douching plays in contracting BV is uncertain, although Hawes and co-workers have reported an association (Hawes *et al.*, 1996). The presence of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli has been associated with a decreased risk of developing BV (Eschenbach *et al.*, 1989; Hillier *et al.*, 1993; Klebanoff *et al.*, 1991; Nagy *et al.*, 1991; Wilks *et al.*, 2004).

Risk factors for UTIs in young women includes the following: Sexual intercourse, having a new sex partner during the past year, spermicidal use, a history of recurrent UTI, having a mother with a history of UTIs and having a first UTI at or before 15 years of age (Hooton *et al.*, 1996). The use of spermicides and antimicrobial agents are also important risk factors. Nonoxynol-9, the active ingredient in most spermicidal compounds, and antimicrobial agents disturb the natural microflora of lactobacilli in the urogenital tract. This in turn increases the colonization of uropathogens (Herthelius-Elman *et al.*, 1992; McGregor *et al.*, 1990; Winberg *et al.*, 1993). In a large case-control study of more than 450 women, the strongest risk factor for recurrent UTI in a multivariate analysis was the frequency of sexual intercourse. In the same study, 47% of the women had a maternal history of UTI and 22% had their first UTI episode before the age of 24. These variables were associated with a two-to four-fold increase in risk of recurrent UTIs and were the most strongly associated variables after sexual intercourse (Scholes *et al.*, 2000).

Inherited factors may play an important role in the occurrence of UTIs. Women with recurrent UTIs are three to four times more likely non-secretors of histo-blood group antigens than women without UTIs. Uropathogenic *E. coli* adheres better to epithelial cells of women who are non-secretors than to cells of secretors (Kinane *et al.*, 1982; Sheinfeld *et al.*, 1989). This could be due to the fact that epithelial cells of non-secretors selectively express globoseries glycolipid receptors to which *E. coli* binds. This is presumably due to sialylation of the gal-globoside precursor glycolipid, which in secretors is fucosylated and processed to ABH antigens (Stapleton, 1992). Another hereditary factor that may predispose women to UTI is the interleukin-8 receptor (IL-8R), CXCR1. Interleukin-8 is an inflammatory cytokine that promotes neutrophil migration across the infected uroepithelial cells (Godaly *et al.*, 1997). This was demonstrated in experiments where mice lacking the CXCR1 gene were unable to clear bacteria from the kidney and eventually developed bacteraemia. In addition, a preliminary analysis of interleukin-8 expression on the neutrophils of children with pyelonephritis has shown a defective version of CXCR1, which may explain their susceptibility to recurrent pyelonephritis (Finer and Landau, 2001).

The reduced levels of estrogen in postmenopausal women predispose them to UTIs. Topically applied intravaginal estrogen normalized the vaginal microflora and reduced the incidence of recurrent UTIs (Raz and Stamm, 1993). Mechanical and physiological factors that affect bladder emptying are strongly associated with recurrent UTIs in postmenopausal women, in contrast to the behavioural risk factors described for premenopausal women. Urinary incontinence, presence of a cystocele, and post-voiding residual urine are all urological factors associated with recurrent UTIs (Raz *et al.*, 2000). In postmenopausal women there is also a decrease in lactobacilli numbers. The presence of lactobacilli in postmenopausal women is associated with a lower frequency of vaginal colonization with Enterobacteriaceae (Altöparlak *et al.*, 2004). Treatment with lactobacilli to prevent the re-occurrence of UTIs yielded positive (Reid *et al.*, 1992; Asahara *et al.*, 2001) and negative results (Baerheim *et al.*, 1994). The role lactobacilli play in protecting the urogenital tract against UTIs is still not clear.

The prevalence of STDs is higher among adolescents. Adolescents are not only more susceptible to STDs because of their sexual behaviour, but biological and immunological characteristics also play a role. Women with a greater number of sexual partners are more likely to experience STDs. Lack of condom use, and the abuse of alcohol, tobacco and drugs are identified as risk factors for STDs (Manhart *et al.*, 2004; Norman, 2002; Taquette *et al.*, 2004).

The absence of vaginal lactobacilli has been associated with an increased risk of acquiring HIV-1 infection and gonorrhoea (Martin *et al.*, 1999; Mbizvo *et al.*, 2001; van de Wijgert *et al.*, 2000). BV has also been associated with a higher frequency of HIV-1 acquisition (Schwebke, 2001; Sewankambo *et al.*, 1997). Other researchers have found no association between BV and HIV-1 acquisition, contradicting previous research (Demba *et al.*, 2005).

#### 1.6. Clinical features, diagnosis and treatment

Symptoms of CVV include vaginal discharge, vulvar burning, dyspareunia, and external dysuria (Ward, 2002). CVV is diagnosed by microscopic examination of a saline wet-mount preparation and a potassium hydroxide preparation, and a litmus test for the pH of vaginal secretions (Egan and Lipsky, 2000). These tests are not very reliable due to poor sensitivity and lack of specific clinical signs (Sobel, 1997). Fluconazole is a triazole agent that is used as first-line management option for the treatment and prophylaxis of both localized and systematic *C. albicans* infections. Fluconazole is the drug of choice because of its well-known efficacy and safety profile, its relatively low costs, and suitability for children, the elderly and people with impaired immunity. Itraconazole is an antifungal with a wider spectrum and is used when treatment with fluconazole fails (Martin, 1999).

An unpleasant "fishy-smelling" discharge is the most common symptom of BV. The discharge is off-white, thin and homogenous. Erythema and inflammation are usually absent. Most women with BV are asymptomatic (Famularo *et al.*, 2001; Sobel, 2000). BV is diagnosed using the criteria established by Amsel and his co-workers. This requires identifying three of the following four signs: (1) The presence of clue cells, (2) a homogenous, non-inflammatory discharge that adheres to the vaginal walls, (3) an elevated pH of greater than 4.5, and (4) a positive whiff/amine test (Amsel *et al.*, 1983). The presence of clue cells is the most reliable predictor of BV. Vaginal gram stain is more reliable than wet mount and also serves as permanent record (Nugent *et al.*, 1991). Cultures are non-specific because *G. vaginalis* also resides in normal vaginal flora (Amsel *et al.*, 1983). The Centers for Disease Control and Prevention recommends the following three regimens as treatment guidelines for BV in nonpregnant women: 500 mg of metronidazole taken twice daily for 7 days, intravaginal 0.75% metronidazole gel (5 mg) for 5 days, and intravaginal 2% clindamycin cream (5 mg) for 7 days (Ward, 2002). Treating BV with oral metronidazole remains the most effective treatment with the lowest recurrence rates (20%



compared to 34-50% for other agents). Women should be treated if they are symptomatic, undergoing gynaecologic surgery, or are at risk of preterm labour (Alfonsi *et al.*, 2004).

An UTI is diagnosed if one or more of the following symptoms are present: Painful voiding, nocturnal urinary incontinence, frequency, urgency, burning and vomiting. A urine test and *in situ* urine reactive strip test improves the chances of detecting UTIs (Medina-Bombardo, 2003). The Infectious Disease Society of America published guidelines recommending trimethoprim-sulphamethoxazole in a 3-day regimen as the treatment for UTIs. Fluoroquinolone are as effective, but more expensive, and are used in cases where women have antimicrobial resistant organisms or are allergic to the conventional regimens (Warren *et al.*, 1999). The safety and efficiency of exogenous estrogen replacement must be confirmed with further research. The recommended dosage is 0.5 mg twice weekly after a loading dose, applied for 14 days (Raz, 2001).

Up to 70% of women with chlamydia infections are asymptomatic but highly contagious. The symptoms start 1 to 3 weeks after becoming infected and vary from vaginal discharge, intermenstrual or post-coital bleeding to pelvic pain. Gonorrhoea is also asymptomatic in many women. Symptoms include dysuria and vaginal discharge and appear 2 to 10 days after infection. The symptoms of syphilis are not very specific. During primary syphilis highly infectious sores appear anywhere on the body, usually at the site of infection. These sores resolve spontaneously in 2 to 6 weeks. Symptoms in secondary syphilis are highly variable, but may include a rash on the palms or soles. Tertiary syphilis occurs four or more years after primary infection. Syphilis will, if not detected, eventually cause symptoms in 40% of infected people. Symptoms are variable and include skin lesions and dementia. Complications may occur in the mucocutaneous tissue, heart, respiratory tract or nervous system (Parratt and Hay, 2003).

Enzyme-linked immunosorbent assays (ELISA) and direct fluorescent antibody (DFA) testing are readily available diagnostic methods for the detection of chlamydia infections. Specificity is high (>95%), but sensitivity may be low (0 to 100%). Nucleic acid amplification techniques (NAAT) have high sensitivity (60 to 100%) and specificity (>99%) but are expensive. The method of choice for diagnosing gonorrhoea is culture of an endocervical or urethral swab. Not only is the sensitivity high (>95%), but is also an inexpensive method. Syphilis is either diagnosed by demonstration of *T. pallidum* from lesions by dark field microscopy or DFA screening, or by serological testing. VDRL (venereal disease reference laboratory) or TPHA (treponema pallidum

haemagglutinin test) are the serological tests used. The disadvantage of these tests is that they cannot differentiate between other treponemal infections (Parratt and Hay, 2003).

The Centers for Disease Control and Prevention recommends the following treatments for STDs. The recommended regimens for chlamydial infections are 1 g of azithromycin taken orally or 100 mg of doxycycline taken orally twice a day for seven days. The following regimens are recommended for uncomplicated gonococcal infections: 400 mg cefixime taken in a single dose orally, 125 mg ceftriaxone taken intramuscularly in a single dose, 500 mg ciprofloxacin taken orally in a single dose, or 250 mg levofloxacin taken orally in a single dose. Benzathine penzillin is the recommended regimen for treating syphilis. The suggested dosage for adults is 2.4 million units and 50 000 units/kg up to 2.4 million units for children taken in a single dose intramuscularly (Ward, 2002).

## 2. PROBIOTICS

### 2.1. Introduction

There is a renewed interest in the use of probiotics to prevent and treat infections of the human gastrointestinal and urogenital tract. A probiotic is defined as a live microbial feed supplement, which beneficially affects the host by improving its intestinal microflora balance (Fuller, 1989). This definition has been revised and refined as "a preparation of, or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora in a compartment of the host and by that exert beneficial health effects in this host" (Schrezenmeir and de Vrese, 2001). Probiotics consist mainly of bacteria (especially lactic acid bacteria and bifidobacteria) and yeasts, especially *Saccharomyces cerevisiae* (Marteau and Shanahan, 2003).

The increased resistance of pathogens to antibiotics has created the need to produce new ways to combat these pathogens. This has brought the focus of using probiotics not only in animal feed but also as a biotherapeutic agent in humans. Over the last few years, research on the influence of probiotics on specific diseases and medical conditions have grown substantially (Mombelli and Gismondo, 2000; Ouwehand *et al.*, 1999). Table 1 lists a few areas in which the beneficial effects of probiotics have been studied.

Table 1: Health benefits of probiotics

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Diseases and conditions beneficially effected by probiotics

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Rotavirus diarrhoea <sup>(Guandalini *et al.*, 2000)</sup> and antibiotic-associated diarrhoea <sup>(Arvola *et al.*, 1999)</sup>

Treatment of urinary tract infections <sup>(de Ruiz *et al.*, 1996; Reid *et al.*, 1985)</sup>

Treatment of *Candida* and bacterial vaginosis <sup>(Hilton *et al.*, 1992; Mclean and Rosenstein, 2000)</sup>

Alleviation of symptoms of lactose intolerance <sup>(Burton and Tannock, 1997)</sup>

Reduction of serum cholestrol <sup>(Gilliland, 1990)</sup>

Bladder and colon cancer <sup>(Akaza, 1997; Reddy, 1999)</sup>

Food allergy <sup>(Sutas *et al.*, 1996)</sup>

Immunopotentialiation <sup>(Perdigon *et al.*, 1995)</sup>


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The criteria for bacteria to be consumed as a probiotic are the following: The bacteria have to be of human origin and nonpathogenic. They have to be able to grow *in vitro* and remain viable in various preparations for extended periods of time. They have to be resistant to stomach acids, bile salts and pancreatic enzymes. They have to be able to adhere to mucosal surfaces and maintain a colonized state in the host. In addition to this, they have to exert a beneficial effect by colonization, production of useful byproducts of bacterial metabolism, or other mechanisms (Marcon, 1997).

The increase in the number of probiotic products with unreliable content and no proven clinical benefits has led to the development of Operating Standards in 2002. The Food and Agricultural Organization of the United Nation and the World Health Organization assembled guidelines to ensure product safety and reliability. A product can only be termed a probiotic after it has fulfilled the following parameters: Guidelines for the use of probiotics must be implemented. Clinical trials consisting of three phases must be run to prove health benefits as good as, or better than, standard prevention or treatment methods for a particular condition or disease. The product must be of high quality and have informative and precise packaging. The mechanisms of action *in vivo* must be studied and identified (Reid *et al.*, 2005).

Further research is required to evaluate the safety of probiotics, especially in immunodeficient patients. Certain lactic acid bacteria currently used as probiotics, have been associated with infectious diseases in humans. Strains of *Leuconostoc pseudomesenteroides* have been recovered from patients diagnosed with UTIs (Cappelli *et al.*, 1999). Probiotic bacteria may transfer genes to commensal and pathogenic bacteria, thereby increasing the risk of developing

more antibiotic resistant bacteria. Intestinal bacteria have recently also been associated with inflammatory and auto-immune diseases in immunodeficient hosts. Similar problems may arise when probiotics are fed to immunodeficient patients (Wagner and Balish, 1998).

## 2.2. Lactic acid bacteria and probiotics

Lactic acid bacteria (LAB) are constituted of a heterogeneous group of Gram-positive, non-sporulating micro-aerophilic bacteria. These bacteria follow a strictly fermentative metabolism with lactic acid as main end product. LAB are either cocci- or rod-shaped and includes the following genera: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Oenococcus*, *Weisella* and *Melissococcus* (Cai and Collins, 1994; Stackebrandt and Teuber, 1988; Stiles and Holzapfel, 1997). LAB are commonly used in probiotic products and foods, since they are desirable members of the intestinal microflora. These bacteria have GRAS (generally regarded as safe) status and have been used in traditionally fermented foods since the Middle Ages (Dunne *et al.*, 2001).

## 2.3. Natural microflora of the urogenital tract

*Lactobacillus* spp. are the dominant organisms colonizing the vagina of healthy women. *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus jensenii*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus leichmanii*, *Lactobacillus delbrueckii* and *Lactobacillus salivarius* are associated with vaginal secretions (Nagy *et al.*, 1991; Rogosa and Sharpe, 1960). *L. acidophilus* is widely accepted as the predominant species (Magliano *et al.*, 1984; Nagy *et al.*, 1991; Rogosa and Sharpe, 1960). Classical identification is not very reliable and the development of genetic identification methods has provided new insight on the taxonomy of vaginal lactobacilli. DNA homology studies have led to the revision of the taxonomy of *L. acidophilus* and its reclassification into six DNA homology groups (*L. casei*, *Lactobacillus gasseri*, *Lactobacillus crispatus*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov.). *L. gasseri* and *L. crispatus* are frequently isolated from healthy women (Antonio *et al.*, 1999).

Vaginal lactobacilli have been isolated from different population groups. A range of genetic techniques is used to identify these isolates. Giorgi *et al.* (1987) identified *L. gasseri*, *L. jensenii*, *L. crispatus* and *L. fermentum* as the species colonizing the vagina of asymptomatic women. During this study a genetically homologous group of heterofermentative lactobacilli that could represent a new species was also isolated. Dicks *et al.* (1994) described a new species, *Lactobacillus fornicalis*, isolated from the posterior fornix of the human vagina. Protein fingerprinting suggested a close relatedness between *L. fornicalis*, *L. gasseri* and *L. jensenii*. The most frequently isolated species in this study was *L. plantarum*, followed by *L. fermentum*, *L. crispatus* and *L. jensenii* (Dicks *et al.*, 1994). According to API CH Rapid strips, the predominant vaginal lactobacilli in 100 premenopausal women are *L. jensenii* (Reid *et al.*, 1996). Antonio *et al.* (1999) isolated vaginal lactobacilli from 215 women and identified them through whole chromosomal DNA probes. *L. crispatus*, followed by *L. jensenii*, and a previously undescribed species designated *L. 1086V*, were the most frequently isolated. Vallor *et al.* (2001) studied the vaginal lactobacilli of 101 pregnant women and found that *L. crispatus* and *L. jensenii* were the predominant species. The species were identified by DNA homology. *L. crispatus* and *L. gasseri* were described as the dominant vaginal species from a group of pregnant Japanese women (Song *et al.*, 1999). Classical methods and polymerase chain reaction (PCR) were used to type vaginal lactobacilli species of 200 women who were admitted to a gynecology clinic. The most frequently isolated lactobacilli were found to be *L. gasseri*, followed by *L. delbrueckii* (Alpay *et al.*, 2002).

Vaginal lactobacilli were also identified through 16S rRNA and 16S rDNA gene sequencing in four separate studies. The most commonly isolated species were *L. crispatus*, *L. gasseri* and *L. jensenii*. *L. iners* were also identified in two studies and *L. vaginalis* in another (Pavlova *et al.*, 2002; Tarnberg *et al.*, 2002; Wilks *et al.*, 2004; Zhou *et al.*, 2004). *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* were the dominant species isolated from a group of Swedish women. The lactobacilli were typed by randomly amplified polymorphic DNA (RAPD) and identified by temporal temperature gradient gel electrophoresis, multiplex PCR and 16S rDNA sequencing (Vasquez *et al.*, 2002). *L. iners* and *L. crispatus* were the most commonly isolated vaginal lactobacilli when examined by PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing of the V2-V3 region of the 16S rRNA gene (Burton *et al.*, 2003).

It is evident that *L. crispatus*, *L. gasseri* and *L. jensenii* are the predominant vaginal lactobacilli (Antonio *et al.*, 1999; Giorgi *et al.*, 1987; Pavlova *et al.*, 2002; Reid *et al.*, 1996; Song *et al.*,

1999; Tarnberg *et al.*, 2002; Wilks *et al.*, 2004; Vasquez *et al.*, 2002; Zhou *et al.*, 2004). *L. iners* was only recently described (Burton *et al.*, 2003; Tarnberg *et al.*, 2002; Vasquez *et al.*, 2002; Zhou *et al.*, 2004). This may be due to the fact that *L. iners* does not grow on MRS and Rogosa-Sharpe medium, the major selective media used for the isolation of *Lactobacillus* spp. It could also be that the organisms have been confused with members of the *L. acidophilus* complex (Burton *et al.*, 2003).

Several factors influence the natural microflora of the urogenital tract and have been investigated. The microflora of pre- and postmenopausal women differ, with postmenopausal women having a lower number of lactobacilli. The lack of estrogen in postmenopausal women, which stimulates the proliferation of lactobacilli, contributes to their lower lactobacilli numbers (Raz, 2001). Postmenopausal women who underwent hormone replacement therapy have the same vaginal lactobacilli composition as premenopausal women (Devillard *et al.*, 2004). Antibiotics have little inhibitory effect on the lactobacilli population. Metronidazole in low concentrations even stimulated the growth of lactobacilli (Agnew and Hillier, 1995; Simoes *et al.*, 2001b). High concentrations of metronidazole only partially inhibited the growth of lactobacilli (Simoes *et al.*, 2001b). A randomized, double blinded, placebo-controlled crossover trial suggested that the spermicide, nonoxynol-9, had no adverse effects on H<sub>2</sub>O<sub>2</sub>-producing lactobacilli. However, the use of the spermicide resulted in a loss of non-H<sub>2</sub>O<sub>2</sub>-producing lactobacilli (Richardson *et al.*, 1998).

### 3. UROGENITAL PROBIOTICS

#### 3.1. Introduction

LAB have to comply with a list of specific characteristics to be considered a urogenital probiotic. They must be able to adhere to urogenital epithelial cells to colonize and persist in the urogenital tract. They must also be able to inhibit the growth and colonization of uropathogens in the urogenital tract by the production of lactic acid, hydrogen peroxide, bacteriocins and biosurfactants. LAB fulfilling these criteria must be tested further in *in vivo* studies. Clinical studies need to be conducted to verify if the bacteria will survive the urogenital tract. *In vivo* studies are also important to see whether probiotic cells have any adverse effects on the host. Urogenital probiotic characteristics will be discussed in detail below followed by results obtained from animal studies and clinical trials (Hoesl and Altwein, 2005; Reid, 1999).

### 3.2. Lactic acid production

The mild acidity of the human vagina, pH 4.5 (Paavonen, 1983; Andersch, 1986), is a key protective mechanism against pathogens. This low pH is microbicidal against organisms implicated in STD's (Hanna *et al.*, 1985) and UTIs (Stamey and Kaufman, 1975; Skarin and Sylwan, 1986; Dembele *et al.*, 1998). Bacterial vaginosis, the most common vaginal pathology worldwide, is also associated with an increase in vaginal pH (Eschenbach, 1993). The anaerobic fermentation of glycogen to lactic acid is responsible for the acidity of the vagina. During times of high estrogen availability glycogen is deposited into the vagina epithelial cells (Paavonen, 1983) and subsequently fermented by lactobacilli to lactic acid (Boskey *et al.*, 1999, 2001).

### 3.3. Hydrogen peroxide production

The ability of certain lactobacilli to generate hydrogen peroxide ( $H_2O_2$ ) is an important property to combat uropathogens, especially in the presence of peroxidase and a halide. Peroxidase utilizes  $H_2O_2$  to oxidize the halide, forming the corresponding hypohalous acid or halogen, which has toxic properties (Boris and Barbes, 2000). *L. delbruekii*, *L. acidophilus*, *L. crispatus*, *L. johnsonii* and *L. gasseri* are dominant  $H_2O_2$  producers (Strus *et al.*, 2004).

Only 6% of women with BV contained  $H_2O_2$ -producing lactobacilli in their vaginas, while 96% of women with uninfected vaginas contained  $H_2O_2$ -producing lactobacilli (Eschenbach *et al.*, 1989). Other researchers reported similar results (Hillier *et al.*, 1993; Klebanoff *et al.*, 1991; Nagy *et al.*, 1991). Wilks *et al.* (2004) associated the low risk of BV and subsequent preterm birth in high-risk women with the presence of  $H_2O_2$ -producing lactobacilli. Contradicting these results, Rosenstein *et al.* (1997) reported that pregnant women obtained BV despite the presence of  $H_2O_2$ -producing lactobacilli. In a study of 182 women, BV was associated with a lack of  $H_2O_2$ -producing lactobacilli, while the absence of lactobacilli did not increase the acquisition of CVV or vaginal trichomoniasis (Hawes *et al.*, 1996). *C. albicans* produces catalase that degrades the  $H_2O_2$  (Nakagawa *et al.*, 1999). In contrast to this evidence other researchers reported the inhibition of *C. albicans* by  $H_2O_2$ -producing *Lactobacillus acidophilus* (Fitzsimmons and Berry, 1994) and *Lactobacillus* strains isolated from diary products and the genital tract (Paraje *et al.*, 2000).

Women colonized by  $H_2O_2$ -producing lactobacilli have decreased acquisition of HIV, *N. gonorrhoeae* and *E. coli* (Gupta *et al.*, 1998; Martin *et al.*, 1999).  $H_2O_2$ -producing *L. acidophilus*

inhibited the growth of *N. gonorrhoeae* by the production of H<sub>2</sub>O<sub>2</sub>, acidification of the environment and production of protein inhibitors (Zheng *et al.*, 1994). *L. crispatus* and *L. jensenii* inhibited *N. gonorrhoeae* growth in both acidic and neutral conditions. The inhibitory effect was lost in the presence of bovine catalase. This also suggests that H<sub>2</sub>O<sub>2</sub> is the primary mediator of inhibition (Amant *et al.*, 2002). Not only is H<sub>2</sub>O<sub>2</sub> produced by lactobacilli inhibitory to uropathogens, but it has also been proposed to have an antitumor effect in the vagina (Bauer, 2001; Klebanoff *et al.*, 1991).

### 3.4. Bacteriocins and biosurfactants

LAB produce inhibitory metabolites other than H<sub>2</sub>O<sub>2</sub> to protect the urogenital tract against infection. Bacteriocins are ribosomally synthesized antimicrobial proteins or peptides that have shown to inhibit the growth of certain uropathogens (Tagg *et al.*, 1976). Bacteriocins and their importance in the urogenital tract will be discussed in detail in the following sections.

Biosurfactants are compounds released by microorganisms that accumulate at interfaces, most notably liquid-air interfaces. Certain lactobacilli produce biosurfactants that have anti-adhesive properties against uropathogens. Velraeds *et al.* (1996) investigated the anti-adhesive ability of biosurfactants produced by *L. casei* subsp. *rhamnosus* 36 and ATCC 7469, *L. fermentum* B54 and *L. acidophilus* RC14. The initial adhesion of *E. faecalis* 1131 to glass with biosurfactants produced by *L. fermentum* B54 and *L. acidophilus* RC14 decreased by 70% (Velraeds *et al.*, 1996). The anti-adhesive abilities of surlactin, produced by *L. acidophilus* RC14, on silicone rubber were also tested. Surlactin inhibited the initial adhesion of *E. faecalis*, *E. coli* and *Staphylococcus epidermis* (Velraeds *et al.*, 1997; Velraeds *et al.*, 1998). A 29 kDa collagen-binding protein was characterized from the biosurfactant produced by *L. fermentum* RC-14. This protein exerts anti-adhesive activity against uropathogenic bacteria (Heinemann *et al.*, 2000). The inhibition of uropathogenic biofilm growth by biosurfactants in the urine of men and women differ, being greater in the urine of men (Velraeds *et al.*, 2000).

Biosurfactants are of great interest to researchers because of their practical applications. This includes the use of biosurfactants to prevent biomaterial-related infections in the urinary tract. As earlier stated, catheter-associated UTIs are the most common nosocomial infection (Hashmi *et al.*, 2003). Catheters coated with biosurfactants can reduce the adhesion of uropathogens and hence reduce infection (Velraeds *et al.*, 1997). Biosurfactants can also be applied to prevent



biofilm formation on ureteral stents. Ureteral stents are implanted to improve kidney drainage. The attachment of uropathogens leads to infection and this can lead to serious kidney damage. Research showed that coating the stents with the protein (29 kDa) isolated from *L. fermentum* RC-14 reduced attachment of *E. coli* 67 and *Ent. faecalis* 1131 *in vitro*. However, the protein had no effect when the stents were coated with urine before the challenge (Cadieux *et al.*, 2003; Heinemann *et al.*, 2000).

### 3.5. Adhesion of lactic acid bacteria to the urogenital epithelial cells

Adhesion of LAB to uroepithelial and vaginal epithelial cells (VEC) is not only important for the colonization of the vagina, but also forms a protective barrier against pathogenic bacteria (Chan *et al.*, 1985). LAB differ in their ability to adhere to epithelial cells. Lactobacilli isolated from the vagina (Ocana and Nader-Macias, 2001; Wood *et al.*, 1985) and the urinary tract (Reid *et al.*, 1987; Chan *et al.*, 1984) have higher adherence than lactobacilli isolated from other sources.

The cell surface structure of LAB plays an important role in their adhesion abilities. Three vaginal lactobacilli that adhere strongly to VECs surfaces were studied. In the case of *L. acidophilus* and *L. gasseri*, adherence involve proteins and a carbohydrate, while in *L. jensenii* adherence depends exclusively on carbohydrates (Boris *et al.*, 1998). An extracellular, proteinaceous adhesin and a trypsin-insensitive cell wall adhesin were identified from lactobacilli species that adhere strongly to uroepithelial cells (Reid *et al.*, 1993). Lipoteichoic acid in the cell wall has also been suggested to be responsible for the adherence of *Lactobacillus* cells to uroepithelial cells (Chan *et al.*, 1985). *L. fermentum* RC-14, *L. rhamnosus* GR-1 and 36, and *L. casei* Shirota produce biosurfactants, which contain proteins that bind to both collagen types III and IV. These proteins may play an important role in the adhesion of the bacteria since collagen is a major component of the dermis (Howard *et al.*, 2000).

Fimbriation increases the adhesion of lactobacilli to VECs *in vitro* (McGroaty, 1994). Lactobacilli were grouped based on their ability to hemagglutinate red blood cells (RBCs) (Andreu *et al.*, 1995). Strains from group II (hemagglutinated sheep and human RBCs) and group III (hemagglutinated all the RBCs studied) adhered in large numbers to VECs. A direct correlation was found between the strains' adhesion ability and their hemagglutination patterns (Andreu *et al.*, 1995).

Lactobacilli inhibit uropathogens from adhering by competitive exclusion, auto-aggregation, and co-aggregation with uropathogens. Pre-incubation of uroepithelial cells with *Lactobacillus* strains completely or partially inhibited the adherence of several uropathogens (Chan *et al.*, 1984; Chan *et al.*, 1985; Reid *et al.*, 1987). Reid *et al.* (1988) was first to observe co-aggregation between lactobacilli and uropathogens. Surface proteins of lactobacilli have been identified that co-aggregate and aggregate with pathogens. An example of these proteins is the 19.9 kDa prepeptide produced by *L. coryniformis* DSM 20001<sup>T</sup>. This prepeptide is encoded by the *cpf* gene and is responsible for co-aggregation and aggregation with the human pathogens *E. coli* K88, *Campylobacter coli*, and *C. jejuni* (Schachtsiek *et al.*, 2004). An aggregation promoting factor, a 2 kDa hydrophilic peptide, was also isolated from vaginal *L. gasseri* 2459 (Boris *et al.*, 1997). Lactobacilli from hemagglutination group III showed a greater capacity to block uropathogens adherence than lactobacilli from hemagglutination groups II and I (Osset *et al.*, 2001). The ability of lactobacilli to closely interact with uropathogens constitutes an important host defense mechanism against infection.

### 3.6. *In vivo* probiotic studies of the urogenital tract

Reid and co-workers studied the urogenital properties of *L. rhamnosus* GR-1 and GG, and *L. fermentum* RC-14 extensively. The three strains were selected for clinical studies based on their adherence to epithelial cells (Reid, 1999), although *L. rhamnosus* GG was not able to colonize the human vagina (Colodner *et al.*, 2003; Gardiner *et al.*, 2002). Furthermore, they have the ability to inhibit uropathogens (Reid, 1999). *L. rhamnosus* GR-1 and *L. fermentum* RC-14 have been detected in the vagina following oral intake. In the same study six cases of asymptomatic or intermediate bacterial vaginosis were resolved within one week (Reid *et al.*, 2001a). The efficacy of orally administered lactobacilli to restore and maintain a healthy vagina was further investigated in a trial consisting of 42 women. Women received encapsulated *L. rhamnosus* GR-1 plus *L. fermentum* RC-14 or *L. rhamnosus* GG orally. A healthy flora was reported in 90% of women treated with *L. rhamnosus* GR-1 and *L. fermentum* RC-14. Symptomatic BV was resolved or converted to asymptomatic BV in 7 of 11 people. Treatment with *L. rhamnosus* GG had no effect (Reid *et al.*, 2001b). The safety of the daily intake of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 was tested in a randomized, placebo-controlled trial consisting of 64 women. The women showed no adverse effects after taking the capsules for 60 days. Asymptomatic BV microflora returned to normal lactobacilli microflora in 37% of the women who received the lactobacilli treatment compared to the 13% of the placebo group (Reid *et al.*, 2003).

*L. fermentum* CRL1058 was inoculated intraurethrally into 2 month-old female mice. The mice were then challenged by the same method with *E. coli* and *K. pneumoniae*. A double dose of lactobacilli ( $10^7$  CFU per dose) were adequate to protect the mice from *E. coli*. The pathogen disappeared completely by the third day. *L. fermentum* was ineffective against *K. pneumoniae* (Nader de Macias *et al.*, 1996). The effect of antibiotics and estrogen were tested on the colonization and efficiency of *L. fermentum* CRL1058. *L. fermentum* CRL1058 was shown to be effective with low doses of orally applied ampicillin (de Ruiz *et al.*, 1996) and one dose of beta-estradiol (de Ruiz *et al.*, 2001). Lastly, histological and ultrastructural techniques were used to evaluate the effect of *L. fermentum* CRL1058 on the organs of the urinary tract. *L. fermentum* CRL1058 had no adverse effect on the urinary tract organs ensuring that the *Lactobacillus* is safe to be used as a probiotic for UTIs (de Ruiz *et al.*, 2003).

*L. acidophilus* is present in a wide range of dairy products and the *L. acidophilus* NCFM™ strain is a known commercial probiotic for the intestine. Since *L. acidophilus* is widely consumed, clinical studies were undertaken to see if the lactobacilli had a beneficial effect in the urogenital tract (Reid, 1999; Sieber and Dietz; 1998). *L. acidophilus* inhibited the growth of *C. albicans*. Vaginal implantations of nonfermented *L. acidophilus* milk products for preventing recurrence of vaginitis after Nystatin treatment were studied on 30 women. The ingestion of *L. acidophilus* containing milk products was shown to decrease the number of *Candida* infections in two separate studies (Collins and Hardt, 1980; Hilton *et al.*, 1992).

In another study, 28 women with recurrent non-specific vaginitis were treated with a vaccine containing inactive strains of *L. acidophilus*. The vaccine clinically cured or at least markedly improved the vaginal microflora of 85.6% of the cases (Muller and Salzer, 1983). Lyophilized, H<sub>2</sub>O<sub>2</sub>-producing *L. acidophilus* was also used to treat 60 women with BV. After treatment, 16 of the 28 women who were treated with lactobacilli had normal vaginal smear results, compared to none of the 29 women in the placebo group (Hallen *et al.*, 1992). Ingestion of yogurt containing live *L. acidophilus* was compared with pasteurized milk. The study concluded that the ingestion of yogurt enriched with *L. acidophilus* might reduce episodes of BV (Shalev *et al.*, 1996). The effect of *L. acidophilus* in combination with estriol was also tested in a multicentric, randomized, placebo-controlled clinical trial consisting of 32 women experiencing BV. The treatment not only increased the cure rate but the lactobacilli were also able to recolonise the vagina (Parent *et al.*, 1996).

The *in vitro* characteristics of *L. acidophilus* NCFM™ were investigated by Reid (2000) to examine its suitability as an urogenital probiotic. *L. acidophilus* NCFM™ is able to adhere to urogenital cells and produce biosurfactants that inhibits uropathogenic enterococci adhesion. *L. acidophilus* NCFM™ probiotic properties are not as high as those of *L. rhamnosus* GR-1 and *L. fermentum* RC-14. Hence Reid (2000) suggested that although *L. acidophilus* applied directly to the vagina may be feasible, it would not be the optimal choice for a urogenital probiotic (Reid, 2000).

## 4. BACTERIOCINS

### 4.1. Introduction

LAB are well known for their production of ribosomally synthesized antimicrobial proteins or peptides, collectively known as bacteriocins. Bacteriocins exhibit antimicrobial properties against other bacterial species that are usually closely related to the producer strain. LAB bacteriocins are, in general, small cationic proteins with high isoelectric points and amphiphilic characteristics (Tagg *et al.*, 1976). LAB that produce bacteriocins have been used for extending the shelf life of foods. Nisin is the only bacteriocin licensed as a food preservative and has been produced commercially since 1953 (Parente and Ricciardi, 1999). The application of bacteriocins to treat and prevent infectious diseases has received a lot of attention over the last few years and the field of research is still growing.

### 4.2. Classification of bacteriocins

Bacteriocins produced by Gram-positive bacteria are divided into two main groups: Group I consists of modified bacteriocins (the lantibiotics), and group II of the unmodified peptide bacteriocins (nonlantibiotics) (Nissen-Meyer and Nes, 1997). Klaenhammer classified lactic acid bacterial bacteriocins further into four distinct classes based on their biochemical and genetic characteristics, structures and mechanisms of action. Class I consists out of lantibiotics, small membrane-active peptides (< 5 kDa) containing the unusual amino acids lanthionine,  $\beta$ -methyl lanthionine and dehydrated residues. Nisin, lacticin 481, carnocin U149 and lactocin S are a few lantibiotics (Klaenhammer *et al.*, 1993). Class II is the small heat-stable, non-lanthionine containing, nonmodified peptides (< 10 kDa). The group is further divided into three subgroups, namely class IIa; the antilisterial bacteriocins with the YGNG motif; class IIb the two-peptide

bacteriocins; and class IIc the other small heat stable nonantibiotics which do not fall into the first two groups (Eijsink *et al.*, 2002). Previously, *sec*-dependent secreted bacteriocins were classified as class IIc bacteriocins. Pediocin PA-1, lactococcin A, leucocin A, sakacin A, curvacin A and lactacin F are all class II bacteriocins. Class III is the large heat-labile proteins (< 30 kDa) and is characterized by helveticin J, helveticin V-1829, acidophilucin A and lactacins A and B. Class IV consists out of complex bacteriocins composed of a protein plus one or more chemical moieties required for activity. Plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1 are examples of class IV bacteriocins (Klaenhammer, 1993).

#### 4.3. Class IIa bacteriocins

Class IIa bacteriocins are characterized on their antilisterial activity and the presence of the YGNGX<sub>2</sub>CX<sub>4</sub>CXV motif (X represents any amino acid) in their N-termini. The two cysteines form a disulfide bridge and the motif has been suggested to be part of a recognition sequence. Class IIa bacteriocins are produced by a variety of lactic acid bacteria, including *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Carnobacterium*, *Leuconostoc* and *Weisella*. The class IIa bacteriocins characterized so far contains between 37 and 48 residues. Other characteristics shared among these proteins are their net positive charge with iso-electric points varying from 8.3 to 10, their high content of non-polar amino acid residues and small amino acids (Eijsink *et al.*, 2002; Ennahar *et al.*, 2000).

#### 4.4. Biosynthesis of bacteriocins and the genes involved

Bacteriocins of LAB are produced as pre-peptides with short N-terminal leader sequences cleaved during maturation. Cleavage of the leader peptides is conducted by specific peptidases, either proline or glycine, or by a proteolytic domain of the dedicated ABC transporter. The leader peptides contain highly conserved sequences that are recognized by the peptidases or proteolytic domain of the ABC transporters (Klaenhammer, 1993; Nes *et al.*, 1996).

The genes required for the production of non-modified bacteriocins are in close proximity and are as follows: (1) the structural gene (two structural genes for the two-peptide bacteriocins) encoding for the pre-peptide, (2) an immunity gene that protects the producer against its own bacteriocin, (3) a gene encoding a membrane-associated ABC transporter that transfers the bacteriocin across the membrane, and (4) a gene encoding an accessory protein also needed

for secretion of the bacteriocin (Nes *et al.*, 1996). The gene structure of *sec*-dependent bacteriocins is different and will be discussed later. Similarities exist between genes involved in the production of modified and unmodified bacteriocins. This is because both modified and unmodified bacteriocins are synthesized from prepeptides with characteristic N-terminal leader peptides. The genes involved in the production of lantibiotics are as follows: (1) the structural gene encoding the lantibiotic pre-peptide, (2) one or more immunity proteins, (3) a serine protease that presumably functions to cleave off the leader sequence, (4) one or two proteins catalyzing dehydration and lanthionine ring formation, and (5) the two-component regulatory proteins that transmit an extracellular signal and thereby induce bacteriocin expression (Jack and Sahl, 1995).

#### 4.5. Mode of action

The common mode of action is dissipation of the proton motive force (PMF). Bruno and Montville demonstrated this by investigating the influence of bacteriocins from the four major classes, pediocin PA-1, leuconocin S, lactacin F, and nisin, on the PMF of a sensitive cell. They mediated the total or major dissipation of the PMF in sensitive cells in a concentration-dependent manner (Bruno and Montville, 1993). PMF plays a fundamental role in bacterial energy metabolism. PMF dissipation leads to low intracellular levels of ATP, the inability to conduct active transport of nutrients, and the inability to maintain sufficient concentrations of co-factors. This ultimately leads to growth inhibition and cell death (Konisky, 1982). Treatment of sensitive cells with lantibiotics leads to ATP leakage (Moll *et al.*, 1996), compared to no leakage when sensitive cells are treated with class II bacteriocins. This could be due to the fact that the pores formed with class II bacteriocins are smaller in size. ATP depletion is suggested to be the result of the cell's attempt to regenerate the decreased PMF (Ennahar *et al.*, 2000).

The secondary structure of bacteriocins plays an important role in the pore formation in sensitive cells. Barrel-stave poration complexes have been proposed for a number of bacteriocins in which  $\alpha$ -helices or  $\beta$ -sheets form two faces, the one hydrophobic and the other one hydrophilic. Lateral oligomerization of peptide monomers occurs in the membrane with the hydrophobic part facing the membrane and the hydrophilic part forming the pore (Nissen-Meyer and Nes, 1997).

#### 4.6. Sec-dependent bacteriocins

The majority of bacteriocins produced by lactic acid bacteria contain a double-glycine motif in their N-terminal leader sequence that serves as recognition signal for protein processing and secretion. Dedicated ATP-binding cassette (ABC) transporters and their accessory proteins remove the Gly-Gly double motif leader sequence and translocate the bacteriocins across the cytoplasmic membrane (Haverstein *et al.*, 1995). There is, however, a small group of bacteriocins that does not contain the double-glycine motif in their N-terminal. These bacteriocins are *sec*-dependent and are secreted through the general secretory pathway (Cintas *et al.*, 1997).

*Sec*-dependent signal peptides are divided into three distinct domains, namely the N, C, and H regions according to von Heijne's rules for signal peptides. The first 6 to 8 amino acids contain at least one lysine and makes up the N region. The H region is the middle part of the signal peptide and has a strong hydrophobicity. The C region contains the cleavage site, which consists of a neutral amino acid flanked by small side chains at position -3 and -1 (von Heijne and Abrahmsen, 1989).

Divergicin A (Worobo *et al.*, 1995), acidocin B (Leer *et al.*, 1995), enterocin P (Cintas *et al.*, 1997), bacteriocin 31 (Tomita *et al.*, 1996), lactococcin 972 (Martinez *et al.*, 1999) and enterolysin A (Nilsen *et al.*, 2003) are *sec*-dependent bacteriocins. The class IIa *sec*-dependent bacteriocins enterocin P and bacteriocin 31, produced by *Enterococcus faecium* and *Enterococcus faecalis*, respectively, have 44 and 43 amino acids (Cintas *et al.*, 1997; Tomita *et al.*, 1996). Divergicin A and acidocin B, produced by *Carnobacterium divergens* LV13 and *Lactobacillus acidophilus* M46, are heat stable hydrophobic bacteriocins and does not contain the conserved YGNG motif (Leer *et al.*, 1995; Worobo *et al.*, 1995). *Lactococcus lactis* subsp. *lactis* IPLA 972 produces lactococcin 972, which is composed of two copies of a single 7.5 kDa peptide. Unlike other two-peptide class IIb bacteriocins, lactococcin 972 has only one structural gene. Another interesting observation is that the monomer is hydrophilic in its active form, indicating that the membrane is not its primary target (Martinez *et al.*, 1999). Enterolysin A produced by *Enterococcus faecalis* LMG 2333 is a large heat-labile class III bacteriocin. The mature bacteriocin consists of 316 amino acids and has a weight of 34.5 kDa (Nilsen *et al.*, 2003). It's clear that *sec*-dependent bacteriocins are present in all the different classes of bacteriocins and that they have diverse characteristics.

The gene encoding enterocin P (*entP*) was cloned in *Pichia pastoris*. The yeast successfully produced the protein in high quantities with high antimicrobial activity. *P. pastoris* X-33t<sub>1</sub> grown in BMMY medium increased enterocin P protein production by 3.7-fold and 16-fold greater antimicrobial activity (Gutierrez *et al.*, 2005c). Previous heterologous bacteriocin expression of pediocin PA-1 (Beaulieu *et al.*, 2005; Schoeman *et al.*, 1999) and plantaricin 423 (van Reenen *et al.*, 2002) in *P. pastoris* and *Saccharomyces cerevisiae* were less successful. In the latter three studies the supernatants had either no antimicrobial activity or low activity after the supernatant was concentrated. *EntP* was also successfully cloned into *E. coli* (Gutierrez *et al.*, 2005b) and *Methylobacterium extorquens* (Gutierrez *et al.*, 2005a) and the active enterocin P detected in the supernatants.

#### 4.7 *Enterococcus* spp. and their bacteriocins

*Enterococcus* spp. were previously classified as *Streptococcus sensu lato*. DNA:DNA and DNA:rRNA hybridization studies showed that *Streptococcus faecalis* and *Streptococcus faecium* are phylogenetically distinct from other streptococci. They were reclassified as *Enterococcus* (Schleifer and Kilpper-Balz, 1984;1987). Since then 28 species have been added to the genus *Enterococcus* based on phylogenetic evidence strengthened by 16S rRNA DNA sequencing and/or DNA DNA hybridization studies (Moreno *et al.*, 2005).

Enterococci are widely distributed in the environment and are present in foods, the gastrointestinal tract of humans and animals, water and soil (Franz *et al.*, 1999; Moreno *et al.*, 2005). Enterococci are used in the fermentation of certain dairy products (Giraffa, 2003), meat products (Hugas *et al.*, 2003), and olives (Franz *et al.*, 1996), and in probiotic preparations (Benyacoub *et al.*, 2003; Pollmann *et al.*, 2005). Enterococci enhance the flavour and taste of cheese during ripening through proteolysis, lipolysis and citrate breakdown (Litopoulou-Tzanetaki and Tzanetakis, 1992; Manolopoulou *et al.*, 2003) and serves as a natural preservative in food fermentations (Hugas *et al.*, 2003; Laukova and Czikkova, 2001; Laukova *et al.*, 1999).

*E. faecalis* and *E. faecium* produce bacteriocins generally referred to as enterocins. The different bacteriocins produced by enterococci are listed in Table 2. Enterocins, as most other bacteriocins, target the cytoplasmic membrane of sensitive cells and dissipates the transmembrane potential and/or pH gradient, leading to cell inhibition and death (Moreno *et al.*,



2005). Production of enterocins and the inhibitory spectrum of 122 enterococci were tested by de Vuyst *et al.* (2002). The inhibitory spectra of the enterocins varied considerably, inhibiting closely related enterococci, other LAB, food spoilage and pathogenic bacteria. Furthermore no correlation could be found between the origin of the strain, the type of inhibitory spectrum and/or the presence of enterocin genes.

TABLE 2: Bacteriocins produced by *Enterococcus* spp.

Bacteriocins	Producer strain	Isolated from	Size (kDa)	Reference
<b>Class I:</b>				
Cyl <sub>L</sub> and Cyl <sub>S</sub>	<i>E. faecalis</i> DS16			Gilmore <i>et al.</i> (1994)
<b>Class IIa:</b>				
Enterocin A	<i>E. faecium</i> P21, <i>E. faecium</i> WHE 81	Dry-fermented saugages; cheese	4.8	Herranz <i>et al.</i> (2001), Ennahar <i>et al.</i> (2001)
Enterocin P	<i>E. faecium</i> P13	Dry-fermented saugage	4.5	Cintas <i>et al.</i> (1997)
Bacteriocin 31	<i>E. faecalis</i> YI717	Clinical isolate	5.0	Tomita <i>et al.</i> (1996)
Mundticin	<i>E. mundtii</i> ATO6, <i>E. mundtii</i> KS	Vegetables and grass silage	4.3	Bennik <i>et al.</i> (1998), Kawamoto <i>et al.</i> (2002)
Enterocin CRL35	<i>E. faecium</i> CRL35	Cheese	3.5	Wachsmann <i>et al.</i> (2003)
Enterocin SE-K4	<i>E. faecalis</i> SE-K4	Grass silage	4.9	Eguchi <i>et al.</i> (2001)
Bacteriocin RC714	<i>E. faecium</i> RC714	Human fecal sample	4.9	Del Campo <i>et al.</i> (2001)
<b>Class IIc:</b>				
Enterocin AS-48	<i>E. faecalis</i> subsp. <i>liquefaciens</i> S-48	Intestinal tract of porcine	7.1	Galvez <i>et al.</i> (1986),

				Samyn <i>et al.</i> (1994)
Enterocin B	<i>E. faecium</i> P21; <i>E. faecium</i> WHE 81	Dry-fermented sausages; cheese	5.5	Herranz <i>et al.</i> (2001), Ennahar <i>et al.</i> (2001)
Enterocin 1071A and 1071B	<i>E. faecalis</i> BFE 1071	Feces of porcine	4.3 and 3.9	Balla <i>et al.</i> (2000)
Enterocin EJ97	<i>E. faecalis</i> EJ97	Municipal water	5.3	Sanchez- Hidalgo <i>et al.</i> (2003)
Enterocin RJ-11	<i>E. faecalis</i> RJ-11	Rice bran	5.0	Yamamoto <i>et al.</i> (2003)
Enterocins L50A, L50B and Q	<i>E. faecium</i> L50	Spanish dry- fermented sausages	5.1, 5.2 and 3.9	Cintas <i>et al.</i> (1998), Cintas <i>et al.</i> (2000)
<b>Class III:</b>				
Enterolysin A	<i>E. faecalis</i> LMG 2333	LMG panel	34,5	Nilsen <i>et al.</i> (2003)

The presence of enterococci in food products and probiotics presents a potential health risk. This is a result of the emergence of enterococci that is resistant to glycopeptides and antibiotics, the production of biogenic amines, and the finding of virulence traits in both clinical and food borne isolates. The safety of enterococci is also in question because of the role they play in nosocomial infections including bacteraemia, endocarditis, urinary tract, neonatal, central nervous system (CNS), intraabdominal, and pelvic infections (Franz *et al.*, 1999; Moreno *et al.*, 2005).

#### 4.8. Bacteriocins and the urogenital tract

Bacteriocins and bacteriocin-like inhibitory substances active against uropathogens have been identified and described. *L. acidophilus* 160 produces a bacteriocin active against 9 different

isolates of *G. vaginalis* (Aroutcheva *et al.*, 2001). Not all bacteriocins produced are active against *G. vaginalis*. Aroutcheva (2001) reported that about 80% of vaginal lactobacilli tested produced bacteriocins active against *G. vaginalis*. In another study, 8 out of 36 strains of *G. vaginalis* tested were susceptible to a bacteriocin produced by *L. acidophilus* (Simoes *et al.*, 2001a). A bacteriocin-like inhibitory substance was isolated from a vaginal strain of *Lactobacillus salivarius* subsp. *salivarius* CRL 1328 with activity against *E. faecalis*, *E. faecium*, and *N. gonorrhoeae* (Ocana *et al.*, 1999). Mutacins A, B, I, J and T produced by *Streptococcus mutans*, nisins A and Z produced by *Lactococcus lactis* subsp. *lactis*, and epidermin produced by *S. epidermis*, inhibited two antibiotic-resistant strains of *N. gonorrhoeae* (Morency *et al.* 2001). Enterocin CRL35 (Wachsman *et al.*, 2003) and Enterocin ST4V (Todorov *et al.*, 2004), produced by *E. faecium* CRL35 and *E. mundtii* ST4V, even inhibited replication of the HSV.

Bacteriocins produced by bacteria other than LAB have activity against uropathogens (Barberis *et al.*, 1997; Sharma *et al.*, 2002; Smith and Stiles, 1980). Strains of *Streptococcus faecalis* produce inhibitors resembling enterococcal bacteriocins that inhibit the growth of *G. vaginalis* and *N. gonorrhoeae* (Smith and Stiles, 1980). *Eubacterium limosum* produces more than one low molecular weight amino compound that inhibited the growth of *N. gonorrhoeae* (Morin *et al.*, 1984). Several *Pseudomonas aeruginosa* strains produce bacteriocins called pyocins. Pyocins whose sensitivity is subject to environmental changes inhibits the growth of *N. gonorrhoeae* by interfering with its energy metabolism (Barberis *et al.*, 1997; Morse *et al.*, 1980; Stein *et al.*, 1980; Stein *et al.*, 1983). *Photobacterium luminescens* is an insect pathogenic bacterium that produces a bacteriocin, luminescens, active against uropathogenic *E. coli* (Sharma *et al.*, 2002).

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## SHORT NOTE

SCREENING OF LACTIC ACID BACTERIA FOR ANTIMICROBIAL PEPTIDES  
INHIBITORY TO ISOLATES OF *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS*  
FROM PATIENTS DIAGNOSED WITH VAGINOSIS

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A total of 125 lactic acid bacteria (LAB) were screened for antimicrobial activity against five isolates of *Enterococcus faecalis* and a *Candida albicans* strain, respectively, isolated from patients diagnosed with vaginosis. Three LAB strains, viz. *Lactobacillus plantarum* 423 isolated from sorghum beer, *Enterococcus mundtii* ST15 from soy flour, and a catalase-negative coccus (strain T8) isolated from the faeces of HIV-infected children, inhibited the growth of the *Enterococcus faecalis* and *Candida albicans* isolates on plates. None of the three strains produced hydrogen peroxide. The antimicrobial peptide produced by strain 423 is similar to peptide TV35b, an anti-*Candida* peptide produced by *Lactobacillus pentosus* TV35b described in one of our previous papers. The bacteriocin-like peptides produced by strains ST15 and T8 are 3.94 and 5.07 kb in size, respectively.

Lactic acid bacteria (LAB), including *Enterococcus* spp., are natural inhabitants of the urogenital tract (Song *et al.*, 1999). *Enterococcus faecalis* has, however, been associated with urinary infections (Preston *et al.*, 1992; Reid and Seidenfeld, 1997), especially in HIV-infected patients (Schonwald *et al.*, 1999). The species has also been linked to secondary bacteraemia (Lerma *et al.*, 2003). Untreated infections may lead to pyelonephritis (kidney infection), premature delivery, infertility, fetal mortality, impaired renal function, pelvic inflammatory diseases, cervical cancer and even increased transmission of HIV (Foxman, 2003; Jung *et al.*, 2004; Martin *et al.*, 1999; Parratt and Hay, 2003; Rhoton-Vlasak, 2000; Romero *et al.*, 2004). *Candida albicans* is associated with 80-90% of reported cases of candidal vaginosis (Sobel, 1993) and affects more than 1 billion women worldwide annually (Reid and Bruce, 2003). Approximately half of those infected experience recurrent infections (Ferrer, 2000).

Many of the uropathogens form biofilms, rendering them inaccessible to antibiotic treatment (Greiner *et al.*, 2005; Mukherjee *et al.*, 2005; Swidsinski *et al.*, 2005; Tendolkar *et al.*, 2005). Furthermore, extensive antibiotic treatment decreases the natural population of lactic acid bacteria in the vagina, which favours colonization of pathogens. This may lead to an increase in the acquisition of sexually transmitted diseases (Martin *et al.*, 1999; van der Wijkert *et al.*, 2000; Mbizvo *et al.*, 2001), bacterial vaginosis (Eschenbach *et al.*, 1989; Klebanoff *et al.*, 1991; Nagy *et al.*, 1991) and urinary tract infections (Gupta *et al.*, 1998).

Vaginal infections may be treated by colonizing the urogenital tract with LAB (Collins and Hardt, 1980; Hilton *et al.*, 1992; Hoesl and Altwein, 2005; Nader de Macias *et al.*, 1996; Reid *et al.*, 2001a,b; 2003). If carefully selected and correctly administered, these bacteria adhere to the mucus or epithelial cells and form a physical barrier against invasive pathogens (Song *et al.*, 1999). Many of these probiotic strains produce hydrogen peroxide and antimicrobial peptides (bacteriocins) active against pathogens (Hoesl and Altwein, 2005).

The purpose of this study was to screen LAB from plant, animal and human origin for the production of antimicrobial peptides active against uropathogenic strains of *E. faecalis* and *C. albicans*.

## MATERIALS AND METHODS

**Strains and growth conditions.** Lactic acid bacteria were cultured in Mann Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa). Strains of *E. faecalis* were propagated in Brain Heart Infusion (BHI) broth (Biolab) and *C. albicans* in YPD broth (yeast extract, 10 g l<sup>-1</sup>; peptone, 20 g l<sup>-1</sup>; glucose, 20 g l<sup>-1</sup>). Incubation of all strains was at 37°C. *Escherichia coli* Bluescript and DH5 $\alpha$  were cultured in Luria Bertani (LB) broth (Biolab), at 37°C with aeration. Bacteria were stored at -80°C in the presence of 40% (v/v) glycerol and *C. albicans* in the presence of 30% (v/v) glycerol.

**Isolation of *E. faecalis* and *C. albicans* and identification to species level.** Swabs collected from the vagina of patients diagnosed with vaginosis were suspended in BHI broth (Biolab) and YPD broth, respectively; incubated at 37°C for 24 h, streaked onto corresponding agar media and incubated for 24 - 48 h. Individual colonies were selected from plates displaying between 50 and 100 colonies and streaked to purity.

Bacteria isolated from BHI agar plates were Gram-stained and tested for catalase activity. Gram-positive, catalase-negative cocci were identified to genus level by phenotypic characteristics (Holt, 1994) and to species level by 16S rDNA sequencing. Total DNA was isolated using the High Pure PCR Preparation Kit (Roche Diagnostics, Indianapolis, USA). Amplification was by PCR using the 16S rDNA primers and conditions described by Felske *et al.* (1997). Amplified fragments were cloned into pGEM<sup>®</sup>-T Easy Vector Systems (Promega, Madison, USA). Constructs were transformed into *E. coli* DH5 $\alpha$ , according to the method of Ausubel (1994). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen<sup>®</sup>, Valencia, California, USA) and sequenced with an ABI Prism<sup>™</sup>377 DNA Sequencer (PE Biosystems SA, Pty, Ltd). All ligations and transformations were performed according to standard procedures (Ausubel, 1994). Homology with sequences in GenBank was determined by using the BLAST program (Altschul *et al.*, 1997).

Yeast-like colonies collected from YPD plates were examined morphologically and identified to species level by using the API 20 C AUX system (Biomérieux, France).

**Screening of LAB for antimicrobial activity.** A total of 125 LAB, isolated from plants, animals and humans (from our own culture collection) were screened for antimicrobial activity against five *E. faecalis* isolates by using the agar-spot test and the well-diffusion methods (Schillinger and Lucke, 1989; Tagg and McGiven, 1971). The same strains were

screened for antimicrobial activity against the *C. albicans* isolate by spotting cell-free supernatants onto YPD-agar plates seeded with *C. albicans* ( $10^6$  cfu/ml). Activity was expressed in arbitrary units (AU) per ml. One AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition (van Reenen *et al.*, 1998).

H<sub>2</sub>O<sub>2</sub> production was determined by growing the bacteria on MRS agar plates containing 0.25 mg/ml 3',3'-5',5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, St. Louis, USA) and 0.01 mg/ml horseradish peroxidase (Roche Diagnostics). If H<sub>2</sub>O<sub>2</sub> is produced, peroxidase releases oxygen that produces blue colonies in the presence of oxygen (Eschenbach *et al.*, 1989; Song *et al.*, 1999).

**Isolation of antimicrobial proteins.** LAB with antimicrobial activity against *E. faecalis* and *C. albicans* were cultured in 500 ml MRS broth at 37°C for 24 h. Cells were harvested (10 000 x g, 10 min, 4°C) and proteins precipitated from cell-free supernatants with 80% saturated ammonium sulphate (Sambrook *et al.*, 1989). Precipitates were collected by centrifugation (9 000 x g, 1h, 4°C) and the pellets suspended in 10 ml ammonium acetate (pH 6.5). The concentrated proteins were dialysed against 4 l sterile distilled water using a Spectra-Por membrane with a 1000 Da cut-off (Spectrum Inc., CA, USA).

**Cell aggregation.** Cell aggregation (clumping) was studied by using a modified method of Lee *et al.* (2005). *Lactobacillus plantarum* 423, *E. mundtii* ST15 and strain T8 were grown in MRS broth (Biolab) for 24 h, washed twice (10 min, 10 000 x g, 4°C) with sterile physiological water, and resuspended in Keratinocyte-SFM medium (GIBCO, Invitrogen Corporation, Auckland, N.Z). After 4 h at 37°C, the cells were examined for aggregation under a light microscope (600 x magnification). Co-aggregation between these three strains and *E. faecalis* and *C. albicans*, respectively, was studied using the same method. Once added together, the cells were incubated for 4 h at 37°C on a rotating wheel. The cells were Gram-stained and examined for aggregation as described before.

## RESULTS

**Identification of *E. faecalis* and *C. albicans*.** Five colonies selected from BHI plates were Gram-positive cocci, catalase negative and homofermentative (no CO<sub>2</sub> production from the fermentation of glucose). The 16S rDNA of the five isolates shared 84% homology with the 16S rDNA sequence of *E. faecalis* strain D13 listed in GenBank (accession number gi|78499506|gb|DQ23964.1|). A yeast colony was selected from YPD plates and classified as *C. albicans* according to reactions obtained by API 20 C AUX.

**LAB strains with antimicrobial activity against *E. faecalis* and *C. albicans*.** From a total of 125 lactic acid bacteria, only three strains, viz. *L. plantarum* 423, *E. mundtii* ST15 and a catalase-negative coccus (strain T8) inhibited the growth of the five *E. faecalis* strains isolated from patients diagnosed with vaginosis (Table 1). Antimicrobial activity levels recorded in cell-free supernatants of *L. plantarum* 423 and *E. mundtii* ST15 were low (400 and 1 600 AU ml<sup>-1</sup>, respectively), but increased to 51 200 and 102 400 AU/ml, respectively, after ammonium sulphate precipitation. Low antimicrobial activity was recorded for strain T8 (400 AU ml<sup>-1</sup> in cell-free supernatant and 1 600 AU ml<sup>-1</sup> after ammonium sulphate precipitation).

Cell-free supernatants of *L. plantarum* 423, *E. mundtii* ST15 and strain T8 caused only slight growth inhibition of *C. albicans* on plates. However, the morphology of *C. albicans* was clearly impaired when treated with the cell-free supernatants. The cells treated with the cell-free supernatant grew in small clumps and not as evenly as the untreated cells.

*L. plantarum* 423 decreased the medium pH from 6.4 to 3.5 within 24 h of growth. *E. mundtii* ST15 and strain T8, on the other hand, produced less acid and lowered the pH to 4.3. None of the three strains tested produced H<sub>2</sub>O<sub>2</sub> when grown on modified MRS agar.

**Cell aggregation.** The cells of *L. plantarum* 423, *E. mundtii* ST15 and strain T8 were arranged separately and did not form aggregates. No cell aggregation was detected between the *E. faecalis* isolates and *L. plantarum* 423, *E. mundtii* ST15 or strain T8, respectively (not shown). However, *E. mundtii* ST15 and strain T8 aggregated with cells of *C. albicans* (Fig. 1). No aggregation was recorded between *L. plantarum* 423 and *C. albicans* (Fig. 1).

## DISCUSSION

Strains of *E. faecalis* and *C. albicans* isolated from patients diagnosed with vaginosis were positively identified by 16S rDNA sequencing and API 20 C AUX fermentation reactions, respectively. The bacteriocin-like peptide produced by *E. faecium* ST15 was the most effective, followed by plantaricin 423 and the peptide produced by strain T8. Plantaricin 423 is similar to pentocin TV35b produced by *Lactobacillus pentosus* TV35b (Okkers *et al.*, 1999), as deduced from plasmid DNA sequences (Dr C.A. van Reenen, Department of Microbiology, Stellenbosch University, personal communication). Pentocin TV35b is also active against *C. albicans* (Okkers *et al.*, 1999). Plantaricin 423, mundtacin ST15 and bacteriocin T8 are 3.93, 3.94 and 5.07 kDa in size, respectively (de Kwaadsteniet *et al.*, 2005; van Reenen *et al.*, 1998).

*L. plantarum* 423, *E. mundtii* ST15 and strain T8 do not produce H<sub>2</sub>O<sub>2</sub>. Hydrogen peroxide producing LAB are killed by spermicides containing nonoxynol-9 (McGregor *et al.*, 1990). Spermicides serve as contraception and protect the urogenital tract of women against sexually transmitted pathogens such as *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and HIV (Richardson, 2002). Strains 423, ST15 and T8 could be resistant to spermicides (McLean and McGroarty, 1996) however, this resistance must be tested in *in vivo* experiments.

Orally administered *Lactobacillus rhamnosus* GR-1, in combination with *Lactobacillus fermentum* RC-14, was effective in the treatment of bacterial vaginosis and converted the microflora in the urogenital tract to normal levels (Reid *et al.* 2001a; 2001b; 2003). Strains GR-1 and RC-14 are adherent to uro-epithelial and vaginal cells and inhibit the growth and adhesion of urogenital pathogens. *L. rhamnosus* GR-1, a non-hydrogen peroxide producer, is resistant to spermicide. *L. fermentum* RC-14 produces H<sub>2</sub>O<sub>2</sub> and is not resistant to spermicide (Reid *et al.* 1994, 1995). The potential application of *L. plantarum* 423, *E. mundtii* ST15 or strain T8 as urogenital probiotics will possibly be more efficient if taken in combination with H<sub>2</sub>O<sub>2</sub>-producing lactic acid bacteria.

The safety of *E. mundtii* ST15 and strain T8 must be thoroughly tested in *in vivo* studies, since their use as probiotics for human consumption is questionable. Some strains of *Enterococcus* spp. (such as *E. faecalis*) have been associated in nosocomial infections. Furthermore, some enterococci are resistant to glycopeptides and antibiotics, produce biogenic amines and exhibit virulence factors. *In vivo* studies will not only evaluate the safety of the three lactic acid bacteria, but also their efficiency and their ability to attach to uro-epithelial cells (Franz *et al.*, 1999; Moreno *et al.*, 2005).

Aggregation of *E. mundtii* ST15 and *E. faecium* T8 to *C. albicans* may also inhibit *C. albicans* from adhering to epithelial cells. The fact that clinical strains of *E. faecalis* and *C. albicans* co-infect the urogenital tract of patients, (Bayo *et al.*, 2002; Lifshitz *et al.*, 1999; Reid *et al.*, 1992) renders the application of *E. mundtii* ST15, *L. plantarum* 423 and strain T8, or their bacteriocin-like peptides, even more attractive.

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TABLE 1. Antimicrobial activity (Arbitrary units/ml; AU/ML) of *L. plantarum* 423, *E. mundtii* ST15 and strain T8 against uropathogenic strains of *Enterococcus faecalis*

	<i>E. faecalis</i> MDK1	<i>E. faecalis</i> MDK2	<i>E. faecalis</i> MDK3	<i>E. faecalis</i> MDK4	<i>E. faecalis</i> MDK5
<b>Supernatant</b>					
<i>Lb. plantarum</i> 423	400	400	800	400	400
<i>E. mundtii</i> ST15	1600	1600	3200	1600	1600
Strain T8	400	400	400	400	400
<b>Partial purification</b>					
<i>Lb. plantarum</i> 423	3200	3200	3200	12800	12800
<i>E. mundtii</i> ST15	6400	6400	12800	12800	6400
Strain T8	1600	1600	800	1600	1600
<b>Concentrated</b>					
<i>Lb. plantarum</i> 423	51200	51200	51200	102400	102400
<i>E. mundtii</i> ST15	204800	102400	102400	102400	51200
Strain T8	6400	3200	6400	1600	1600

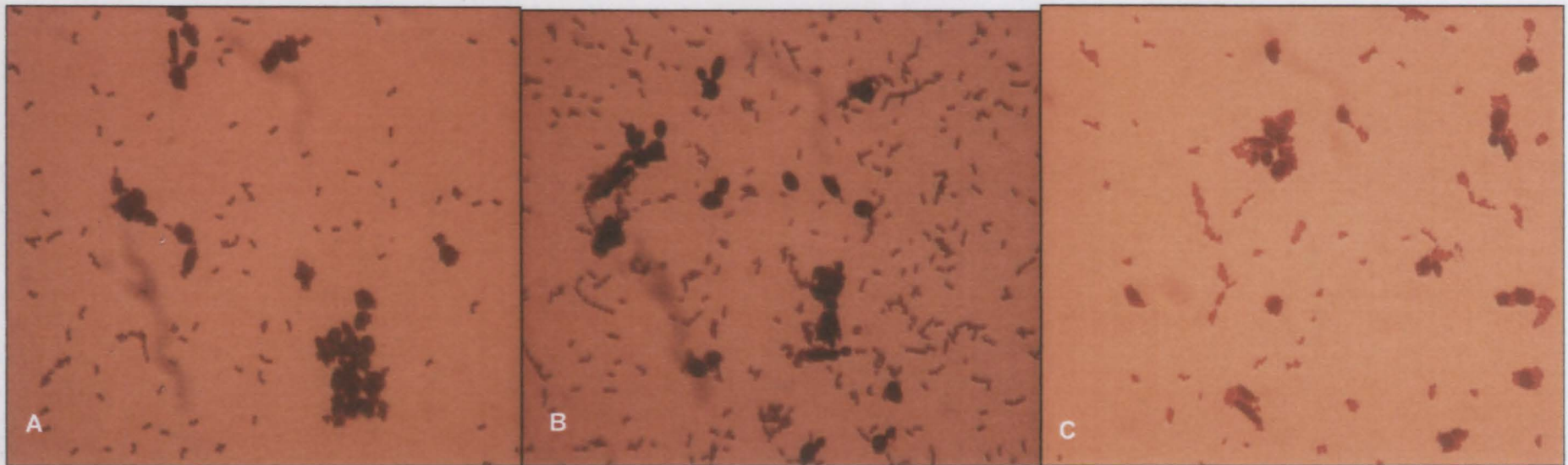


FIG 1. The two lactic acid bacteria strains (A: *E. mundtii* ST15 and C: strain T8) aggregated with *Candida albicans*. No aggregation took place between *Lb. plantarum* 423 and *Candida albicans* (B).

**BACTERIOCIN T8, A NOVEL CLASS IIa SEC-DEPENDENT BACTERIOCIN  
PRODUCED BY *ENTEROCOCCUS FAECIUM* T8, ISOLATED FROM VAGINAL  
SECRETIONS OF CHILDREN INFECTED WITH HIV**

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*Enterococcus faecium* T8, isolated from vaginal secretions of children with HIV, produces a class IIa *sec*-dependent bacteriocin different from other *sec*-dependent bacteriocins. Bacteriocin T8 is 5.07 kDa in size and active against strains of *Enterococcus faecalis* isolated from patients diagnosed with vaginosis, *Lactobacillus sakei* and a *Propionibacterium* sp. The bacteriocin is heat stable (withstands 100°C for 60 min) and remains active in phosphate buffer from pH 4 to 10. The mode of activity is bactericidal, as determined against *E. faecalis*. The gene encoding the *sec*-dependent leader peptide and mature peptide with the conserved YGNG-motif, characteristic of class IIa bacteriocins, is located on a 7 kb plasmid. A second ORF (ORF2) encodes a potential immunity gene. A further two ORFs (ORF3 and 4) encoding a mobilization protein and a relaxase nuclease, respectively, were identified.

Bacteriocins are ribosomally transcribed peptides antimicrobial against closely related bacteria (Klaenhammer, 1993). Four classes of bacteriocins have been described based on their structural, chemical and functional properties (Klaenhammer, 1993). Peptides from classes I and II are heat stable. Small membrane-active, post-translational modified peptides containing lanthionine and  $\beta$ -methyl lanthionine are classified as lantibiotics and are grouped into class I (Klaenhammer, 1993). Class II bacteriocins differ from class I in that they do not undergo post-translational modification and they have an YGNGX<sub>2</sub>CX<sub>4</sub>CXV-sequence at the N-terminal (Klaenhammer, 1993). Single-peptides (one-peptide bacteriocins) are grouped into class IIa. Typical examples of the latter are pediocin PA-1, produced by *Pediococcus acidilactici* (Marugg *et al.*, 1992); sakacin P, produced by *Lactobacillus sakei* (Tichaczek *et al.*, 1994); plantaricin 423, produced by *Lactobacillus plantarum* 423 (van Reenen *et al.*, 1998); and enterocin A, produced by *Enterococcus faecium* (Aymerich *et al.*, 1996). Two-peptide bacteriocins are grouped into class IIb. Large heat-labile bacteriocins are grouped into class III and complex bacteriocins into class IV (Klaenhammer, 1993).

The majority of class IIa bacteriocins have a double-glycine sequence in the N-terminal, which serves as recognition signal for peptide procession and secretion (Aymerich *et al.*, 1996; Marugg *et al.*, 1992; Tichaczek *et al.*, 1994; van Reenen *et al.*, 1998). ATP-binding cassette (ABC)-transporters translocate the bacteriocin across the cell membrane (Haverstein *et al.*, 1995). A few class IIa bacteriocins make use of a signal peptide instead of a double-glycine leader sequence (Cintas *et al.*, 1997; Tomita *et al.*, 1996). The leader peptide is usually positively charged and has a hydrophobic core and cleavage region. The peptide is processed by a signal peptidase during translocation across the cell membrane (von Heijne and Abrahmsen, 1989). Enterocin P (Cintas *et al.*, 1997) and bacteriocin 31 (Tomita *et al.*, 1996) produced by *Enterococcus faecium* P13 and *Enterococcus faecalis* Y1717, respectively, are examples of *sec*-dependent class IIa bacteriocins. Other examples of *sec*-dependent peptides are enterolysin A, produced by *Enterococcus faecalis* LMG 2333, classified as a large heat-labile class III peptide (Nilsen *et al.*, 2003) and lactococcin 972, a two-peptide bacteriocin grouped into class IIb (Martinez *et al.*, 1999). Two nonlantibiotics, divergicin A (Worobo *et al.*, 1995) and acidocin B (Leer *et al.*, 1995) are also secreted by a signal peptide.

\* The purpose of this study was to identify strain T8 and characterize bacteriocin its bacteriocin.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** LAB were propagated in Mann Rogosa Sharpe (MRS) broth (Biolab, Diagnostics, Midrand, South Africa) at 37°C. The uropathogenic strains of *Enterococcus faecalis* were cultured in Brain Heart Infusion (BHI) broth (Biolab) at 37°C. *Escherichia coli* Bluescript and DH5 $\alpha$  were cultured in Luria Bertani (LB) broth (Biolab) on a rotating wheel at 37°C. Other strains included in the test panel were from LMG (Laboratorium voor Microbiologie, University of Ghent, Belgium) and our own culture collection. Bacteria were stored at -80°C in 40% (v/v) glycerol.

**Identification of strain T8.** Strain T8 was identified to genus level by phenotypic characteristics (Holt, 1994) and to species level by 16S rDNA sequencing. DNA was isolated using the High Pure PCR Preparation Kit (Roche Diagnostics, Indianapolis, USA). DNA was amplified by PCR using the 16S rDNA primers and conditions described by Felske *et al.* (1997). Amplified fragments were cloned into pGEM<sup>®</sup>-T Easy Vector Systems (Promega, Madison, USA). Constructs were transformed to *E. coli* DH5 $\alpha$ . Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen<sup>®</sup>, Valencia, California, USA) and sequenced with an ABI Prism<sup>™</sup>377 DNA Sequencer (PE Biosystems SA, Pty, Ltd). All ligations and transformations were performed according to standard procedures (Ausubel, 1994). Homology with sequences in GenBank was determined by using the BLAST program (Altschul *et al.*, 1997).

**Antimicrobial activity spectrum of bacteriocin T8.** *Enterococcus faecium* T8 was cultured in 500 ml MRS broth (Biolab) for 24 h at 37°C. Cells were harvested (10 000 x g, 10 min, 4°C) and proteins precipitated from cell-free supernatants with 80% saturated ammonium sulphate (Sambrook *et al.*, 1989). The precipitate was collected by centrifugation (10 000 x g, 1 h, 4°C) and the pellet suspended in 10 ml ammonium acetate (pH 6.5). The concentrated bacteriocin was dialysed against 4 l sterile distilled water using a Spectra-Por membrane with a 1000 Da cut-off (Spectrum Inc., CA, USA). Antimicrobial activity was determined by using the agar-spot test method (Schillinger and Lucke, 1989) and the well-diffusion method (Tagg and McGiven, 1971). Activity of the crude extract was expressed in arbitrary units per ml (AU/ml). One AU is defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of inhibition of the indicator strain (van Reenen *et al.*, 1998).

The crude extract was used to determine the spectrum of antimicrobial activity and to characterize bacteriocin T8.

**Characterization of bacteriocin T8.** One ml of the crude-extract of bacteriocin T8 was incubated for 2 h at 37°C in the presence of 0.5 mg/ml (final concentration) amylase (Sigma Diagnostics, St. Louis, USA), Proteinase K (Roche Diagnostics Corporation, Mannheim, Germany), pronase (Boehringer Mannheim, GmbH, W-Germany) and pepsin (Boehringer Mannheim), respectively. The enzymes were inactivated (10 min at 80 °C) and antimicrobial activity determined as described previously. Bacteriocin T8 crude extract not treated with enzymes was used as control (Ivanova *et al.*, 1998).

In a separate experiment, the crude extract of bacteriocin T8 was adjusted to pH values ranging from 2.0 to 12.0 (increments of two pH units) with sterile 1M NaOH or 1M HCl and incubated for 2 h at 37°C. The pH was then neutralized by slowly adding 1M NaOH or 1M HCl (Ivanova *et al.*, 1998) and tested for antimicrobial activity as described before.

The cell-free supernatant of *E. faecium* T8 was incubated at 60°C and 100°C for 10, 30 and 90 min, respectively, and tested for antimicrobial activity as described previously. The control was cell-free supernatant of *E. faecium* T8 not subjected to heat treatment and kept at 8°C (Ivanova *et al.*, 1998).

*E. faecalis* MDK2, isolated from patients diagnosed with vaginosis, was used as sensitive strain in all bacteriocin activity tests.

The molecular size of bacteriocin T8 was determined by tricine-SDS-polyacrylamide gel electrophoresis (Schägger and von Jagow, 1987). One half of the gel was stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa). The other half was pre-washed with sterile distilled water and overlaid with *E. faecalis* MDK2 ( $10^6$  CFU/ml) to determine the position of bacteriocin T8 (van Reenen *et al.*, 1998). A Rainbow™ protein molecular weight marker, with fragments from 2.5 to 45.0 kDa (Amersham International, UK) was used.

**Mode of action studies.** Active growing cells of *E. faecalis* MDK2 were inoculated (0.2%, v/v) into BHI broth and incubated for 5 h at 37°C to mid-exponential growth.



Bacteriocin T8 crude-extract (6400 AU ml<sup>-1</sup>) was added to the culture and cell density readings recorded at 600 nm, hourly for 8 h (Ivanova *et al.*, 1998). The control was autoclaved (15 min at 121°C).

**Plasmid curing.** Active-growing cells of *E. faecium* T8 were inoculated into MRS broth, supplemented with acridine orange (Merck), novobiocin (Sigma) and SDS (Sigma), respectively (Ruiz-Barba *et al.*, 1991). The components were added at 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (m/v) increments, respectively. After 18 h of incubation at 37°C, cells from each treatment were plated onto MRS agar and incubated for a further 18 h. Colonies were replica-plated onto MRS agar and one set overlaid with *E. faecalis* MDK2 embedded into 1% (m/v) soft agar (10<sup>6</sup> cfu/ml). The plates were incubated for 18 h at 37°C. Colonies with no inhibition zones were identified. Corresponding colonies were selected from replica plates, inoculated into MRS broth and incubated for 18 h at 37°C. The cells were harvested (10 000 x g, 10 min, 4°C) and plasmid DNA was isolated using the Qiagen<sup>®</sup> Plasmid Midi Kit. Bacteriocin-producing cells of *E. faecium* T8 were used as control.

**Sequencing of plasmid T8.** Plasmid DNA isolated from *E. faecium* T8 was digested with *Hind*III (Roche) and ligated with T4 Ligase (Roche) into pBluescript SK(+/-) (Invitrogen, Paisley, UK). Constructs were transformed to *E. coli* DH5 $\alpha$ , according to the method of Ausubel (1994). DNA was isolated from the transformants by using the QIAprep Spin Miniprep Kit (Qiagen). Primers designed from M13 (pBluescript) were used to sequence the first DNA fragment of pT8. Primers designed from the latter sequence (Table 1, MDK1a and MDK1b) were used to amplify the DNA fragment and was then cloned into pGEM<sup>®</sup>-T Easy Vector Systems (Promega). The constructs were transformed to *E. coli* DH5 $\alpha$ . The DNA was isolated as described previously. Subsequent primers (MDK2a and MDK2b, Table 1,) were designed from amplified DNA fragments and the plasmid sequenced by primer-walking.

## RESULTS

**Antimicrobial activity spectrum of bacteriocin T8.** Bacteriocin T8 inhibited uropathogenic *Enterococcus faecalis* strains, a *Lactobacillus* sp., two *Enterococcus* spp. and a *Propionibacterium* sp. (Table 2).

**Identification of *E. faecium* T8.** *E. faecium* T8 was morphologically related to *Enterococcus* sp. and revealed 94% 16S rDNA homology with *Enterococcus faecium*

strain SF3 (gi|55442388|gb|AY735408.1|). The ability of the isolate to ferment glycerol and melibiose classified the isolate as *E. faecium* (Holt, 1994).

**Characterization of bacteriocin T8 and mode of action studies.** Bacteriocin T8 was sensitive to Proteinase K, pepsin and pronase (Table 3). Treatment with amylase had no effect on bacteriocin activity. No activity was recorded at pH 2 and 12, and after 90 min at 100°C. The mode of action is bactericidal as observed in Fig. 1. According to tricine-SDS-PAGE, the molecular size of bacteriocin T8 is between 3.5 and 6.5 kDa (Fig. 2).

**Plasmid curing and sequencing.** No loss in bacteriocin activity was recorded when *E. faecium* T8 was incubated in the presence of acridine orange or novobiocin. However, growth was impaired in the presence of 3% (m/v) SDS, accompanied by a complete loss in bacteriocin T8 activity.

Two plasmids were isolated from *E. faecium* T8. Growth in the presence of 3% (m/v) SDS resulted in the loss of the smaller 7 kb plasmid (Fig. 3). Sequencing of plasmid T8 revealed four open reading frames (ORFs) (Fig. 4). The first ORF encodes a 74-amino acid peptide, the bacteriocin precursor. The start codon (ATG) is preceded 9 bp upstream by a potential Shine-Dalgarno ribosome-binding site (AAAGGA; underlined) and 83 bp upstream by a potential -10 consensus promoter region (Pribnow box; TATAAT; underlined), as indicated in Fig. 5. The first ORF (ORF 1) of 222 bp encodes the pre-bacteriocin (leader peptide and pro-bacteriocin). The leader peptide contains a potential signal peptidase-processing site (VDA) from position 82 to 90 (double underlined). A conserved YGNG-sequence is located at position 100 to 111 (boxed). Two cysteine residues (positions 118 to 120 and 133 to 135) and a valine residue (position 49 to 51) are also conserved (boxed). The center of the signal peptide is hydrophobic (Fig. 6A) conforming to the rules of signal peptides (von Heijne and Abrahmsen, 1989). The mature peptide is overall hydrophilic with minor hydrophobic peaks (Fig. 6B).

The second ORF (ORF 2) is preceded 10 bp upstream by a potential Shine-Dalgarno ribosome-binding site (AGGGAG; underlined), as indicated in Fig. 5. ORF 2 encodes a 95-amino acid peptide, which resembles an immunity protein. ORF3 encodes a mobilization protein that has 96% homology to the mobilization protein of *E. faecium* DO (gi|68194353|gb|EAN08864.1|). ORF4 encodes a relaxase mobilization nuclease domain that has 75% homology with *E. faecium* DO

(gi|68194349|gb|EAN08860.1|). ORF4 is located immediately downstream of the mobilization gene.

## DISCUSSION

Bacteriocin T8 is inactivated by proteolytic enzymes, but not by amylase. This suggests that the peptide is either not glycosylated or that its activity is not influenced by glycosylation. According to its size (5.07 kDa) and stability to heat (100°C for 60 min), bacteriocin T8 belongs to class IIa group of bacteriocins.

Bacteriocin T8 is different from the normal class IIa bacteriocins in having a *sec*-dependent leader signal peptide (Fig. 5 and 7). As far as we could determine, this is the fourth description of a class IIa *sec*-dependent bacteriocin described and the fifth description of a *sec*-dependent bacteriocin produced by a species of the genus *Enterococcus*. The other class IIa *sec*-dependent bacteriocins are enterocin P, produced by *Enterococcus faecium* P (Cintas *et al.*, 1997), bacteriocin 31, produced by *Enterococcus faecalis* Y1717 (Tomita *et al.*, 1996) and an enterocin P-like bacteriocin produced by *E. faecium* GM-1. The latter bacteriocin differs from enterocin P and bacteriocin 31 in being active against Gram-positive and Gram-negative bacteria (Kang and Lee, 2005). *E. faecalis* LMG 2333 produces a *sec*-dependent class III bacteriocin (Nilsen *et al.*, 2003).

The narrow spectrum of activity recorded for bacteriocin T8 is typical for many other class IIa bacteriocins. Bacteriocin 31 also has a narrow spectrum of activity and inhibits only *Listeria monocytogenes*, *E. faecalis* and *Enterococcus hirae* (Tomita *et al.*, 1996). Enterocin P and the enterocin P-like substance have a broader spectrum of activity. Enterocin P inhibits *L. monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus* spp. and *Propionibacterium* spp. (Cintas *et al.*, 1997). The mode of activity of bacteriocin T8 is bactericidal, similar to that recorded for enterocin P (Cintas *et al.*, 1997).

The genes encoding bacteriocin T8 are located on one plasmid. The structural gene encodes a *sec*-dependent leader peptide with three positively charged amino acids (Figs 5 - 7), a number of hydrophobic amino acids (Figs 5 - 7) and a cleavage region (Figs 5 - 7). Two small hydrophobic amino acids are positioned adjacent to the cleavage site (VDA). Conforming to the definition of a typical signal peptide (von Heijne and Abrahamsen, 1989), similar to other *sec*-dependent bacteriocins. Typical to

class IIa bacteriocins, bacteriocin T8 has a conserved YGNGX<sub>2</sub>CX<sub>4</sub>CXV-motif (Fig. 5 and 7). Deduced from the DNA sequence, the molecular weight of bacteriocin T8 is 5.1 kDa and similar to the 5.0 kDa recorded for bacteriocin 31 (Cintas *et al.*, 1997) but larger than the 4.6 kDa reported for enterocin P (Tomita *et al.*, 1996). On amino acid level, bacteriocin T8 is 69% homologous to bacteriocin 31 and 47% to enterocin P (Fig. 7)

The second ORF encodes a potential immunity protein that has a calculated molecular weight of 10.89 kDa (Fig 5). The gene is located immediately downstream of the structural gene. This is a common feature of LAB bacteriocins (Nes *et al.*, 1996). The protein is only 50% homologous to the immunity protein of bacteriocin 31. The immunity protein of bacteriocin 31 consists of 94 amino acids and has a molecular weight of 11.0 kDa (Cintas *et al.*, 1996). No homology has been detected with any other immunity proteins listed in GenBank.

The mobilization protein and relaxase mobilization nuclease domain are presumably involved in the transfer of the plasmid during conjugation. Mobilization genes in enterococci are often located on the same gene cluster as the structural genes of their bacteriocin, as observed for enterocins 1071A and 1071B. (Balla and Dicks, 2005). The genes encoding bacteriocins of enterococci are known to be associated with pheromone-responsive conjugative plasmids (Masqueda *et al.*, 2004; Nakayama *et al.*, 1995; Sanchez-Hidalgo *et al.*, 2003). Secretion of specific peptide sex-pheromones by recipients initiates cell aggregation and conjugation (Clewell, 1993; Clewell *et al.*, 2002). The genes encoding bacteriocin 31 are also located on a pheromone-responsive conjugative plasmid (Tomita *et al.*, 1996).

Although bacteriocin T8 and bacteriocin 31 share certain characteristics, the two peptides are structurally different (69% homology on amino acid level). The low homology recorded between the signal peptides and immunity proteins of the two strains is further proof that bacteriocin T8 is different from bacteriocin 31 and all other bacteriocins thus far described for enterococci.

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TABLE 1. Primers used in this study

Primer	Sequence
M13F	GTTTTCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
MDK1(a)	GTGTGTATTGCACCACTTAG
MDK1(b)	GAGGTTCAAGACAACACTATGAG
MDK2(a)	GGATAGGCGACAAGTTATTA
MDK2(b)	TCGGTCAAATTGTTGCAAT

TABLE 2. Spectrum of antimicrobial activity of bacteriocin T8

Microorganism	Strain*	Growth conditions	Growth medium	Bacteriocin activity
<i>Enterococcus faecalis</i>	MDK1	37 °C aerobic	BHI	+
<i>Enterococcus faecalis</i>	MDK2	37 °C aerobic	BHI	+
<i>Enterococcus faecalis</i>	MDK3	37 °C aerobic	BHI	+
<i>Enterococcus faecalis</i>	MDK4	37 °C aerobic	BHI	+
<i>Enterococcus faecalis</i>	MDK5	37 °C aerobic	BHI	+
<i>Lactobacillus acidophilus</i>	LMG 13550	37°C anaerobic	MRS	-
<i>Lactobacillus bulgaricus</i>	LMG 13551	37°C anaerobic	MRS	-
<i>Lactobacillus casei</i>	LMG 13552	37°C anaerobic	MRS	-
	LHS <sub>3</sub>	37 °C aerobic	MRS	-
<i>Lactobacillus curvatus</i>	LMG 13553	30°C anaerobic	MRS	-
	DF38	37 °C aerobic	MRS	-
<i>Lactobacillus fermentum</i>	LMG 13554	37°C anaerobic	MRS	-
<i>Lactobacillus helveticus</i>	LMG 13555	42°C anaerobic	MRS	-
<i>Lactobacillus plantarum</i>	LMG 13556	37°C anaerobic	MRS	-
<i>Lactobacillus reuteri</i>	LMG 13557	37°C anaerobic	MRS	-
<i>Lactobacillus sake</i>	LMG 13558	30°C anaerobic	MRS	+
<i>Lactobacillus salivarius</i>	241	37 °C aerobic	MRS	-
<i>Pediococcus pentosaceus</i>	LMG 13560	30°C anaerobic	MRS	-
	LMG 13561	30°C anaerobic	MRS	-
<i>Leuconostoc cremoris</i>	LMG 13562	30°C anaerobic	MRS	-
	LMG 13563	30°C anaerobic	MRS	-
<i>Streptococcus thermophilus</i>	LMG 13564	42°C anaerobic	MRS	-
	LMG 13565	42°C anaerobic	MRS	-
<i>Enterococcus faecalis</i>	LMG 13566	37°C aerobic	BHI	+
	BEF 1071	37 °C aerobic	MRS	+
<i>Staphylococcus carnosus</i>	LMG 13567	37°C aerobic	BHI	-
<i>Listeria innocua</i> LMG	LMG 13568	30°C aerobic	BHI	-
<i>Bacillus cereus</i>	LMG 13569	37°C aerobic	BHI	-
<i>Clostridium sporogenes</i>	LMG 13570	37°C anaerobic	RCM	-
<i>Clostridium tyrobutyricum</i>	LMG 13571	30 °C anaerobic	RCM	-
<i>Propionibacterium</i> sp.	LMG 13574	32 °C anaerobic	GYP	+

\* LMG codes depicts isolates from Laboratorium voor Microbiologie, University of Ghent, Belgium; remaining those of our own culture collection



TABLE 3. The effect of enzymes, pH and temperature on the activity of bacteriocin T8

Treatment	Bacteriocin activity
Enzymes (0.5mg/ml)	
amylase	+
pepsin	-
pronase	-
Proteinase K	-
control (not treated with enzymes, at 30 °C)	+
pH	
2	-
4; 6; 8; 10	+
12	-
control (pH not adjusted, at 30 °C)	+
Temperature	
10, 30 and 90 min at 30° C	+
10, 30 and 90 min at 60° C	+
10 and 30min at 100°C	+
90 min at 100°C	-
control (bacteriocin at 4 °C)	+

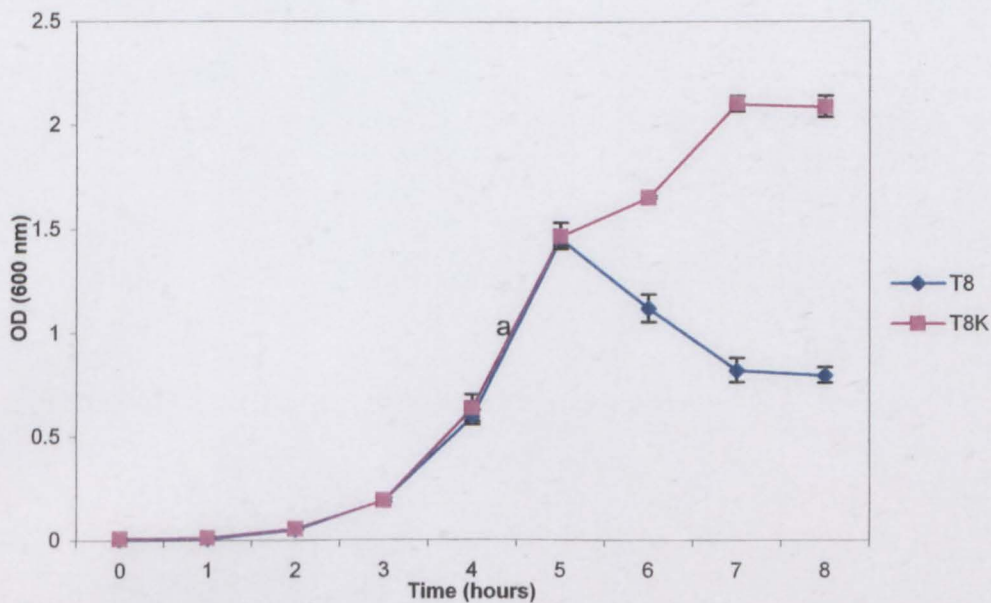


FIG. 1. Cell lysis of *E. faecalis* MDK2 following addition of bacteriocin T8 at mid-exponential phase (5 hours).

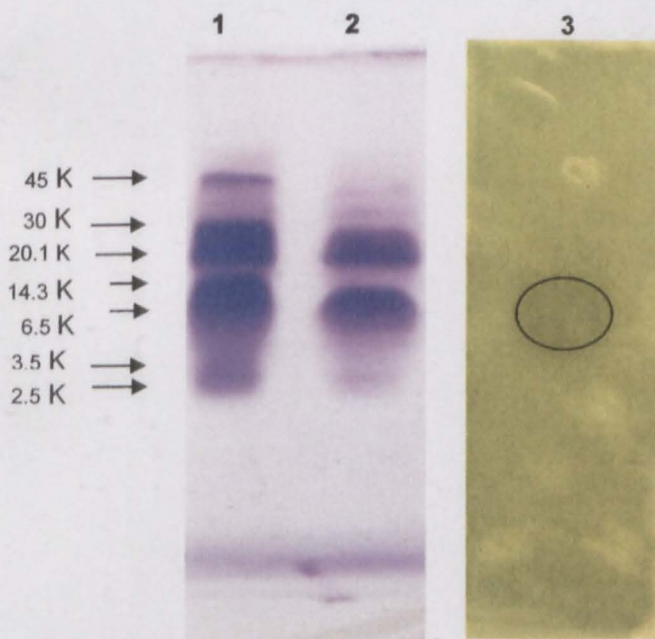


FIG. 2. Separation of partially purified bacteriocin T8 by SDS-PAGE. Lane 1, Rainbow protein size marker (Amersham); lane 2, bacteriocin T8 stained with Coomassie blue R-250; lane 3, bacteriocin T8 overlaid with cells of *E. faecalis* MDK2 embedded in BHI soft agar (1%). The circle indicates the inhibition zone.

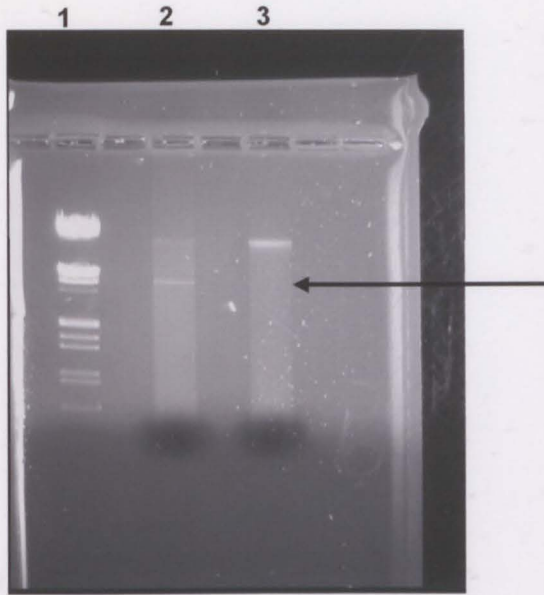


FIG. 3. Agarose gel showing curing of the plasmid of *E. faecium* T8. Lane 1:  $\lambda$  marker, lane 2: plasmid DNA isolated from strain T8 (not cured), and lane 3: Loss of plasmid DNA after curing of strain T8. The arrow indicates plasmid loss.

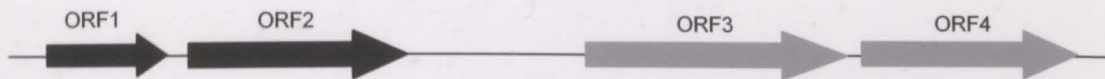


FIG. 4. Gene cluster of the bacteriocin determinants located on plasmid T8 (pT8). ORF1 encodes a bacteriocin precursor; ORF2 a potential immunity protein; ORF3 a mobilization protein and ORF4 a relaxase nuclease.

-10

TGTTATATAATAAAAATTACTAAGATTATTTAATATAACAAGCAATTTTAAAAAACGAAA

S.D.                    ORF1

AGGAAAAACAATCATGAAAAAGAAAGTATTA AACATTGTGTTATTCTAGGAATATTAGG

M K K K V L K H C V I L G I L G

AACTTGTCTAGCTGGCATCGGTACAGGAATAAAAGTTGATGCAGCTACTTACTATGGAAA

T C L A G I G T G I K V D A A T Y Y G N

TGGTCTTTATTGTAACAAAGAAAAATGTTGGGTAGATTGGAATCAAGCTAAAGGAGAAAT

G L Y C N K E K C W V D W N Q A K G E I

TGAAAAATTATTGTTAATGGTTGGGTTAATCATGGTCCATGGGCACCTAGAAGGTAGTG

G K I I V N G W V N H G P W A P R R

S.D.                    ORF2

GTAGGGAGGTTATTATAATGGATTTTACTAAAGAAGAAAACTTTTAAATGCAATTAGTA

M D F T K E E K L L N A I S

AAGTATACAATGAAGCAACTATAGATGACTATCCTGACTTAAAAGAAAAGCTCTTCTTT

K V Y N E A T I D D Y P D L K E K L F L

ATTCTAAAGAAATCAGTGAGGGAAAAAGTGTGGTGAAGTTAGTATGAAATTAAGTAGTT

Y S K E I S E G K S V G E V S M K L S S

TTCTTGAAGATATATTTTAAACATAAATTTGGATTACCTAAATCTTTAATAGAATTAC

F L G R Y I L K H K F G L P K S L I E L

AAGAAATTGTTAGTAAGGAATCTCAAGTATATAGAGGATGGGCTTCTATTGGTATTTGGA

Q E I V S K E S Q V Y R G W A S I G I W

GTAAATCTTTGGAATAAAATGAAAATGGGACTGAAGAATCAATTCTGATTCATCAGTCC

S

FIG. 5. Nucleotide sequence of the bacteriocin region and the deduced amino acid sequence. The -10 promoter box and the Shine-Dalgarno (S.D.) ribosome binding sequences are underlined. ORF1 encodes the structural gene of bacteriocin T8 and ORF2 the potential immunity gene.

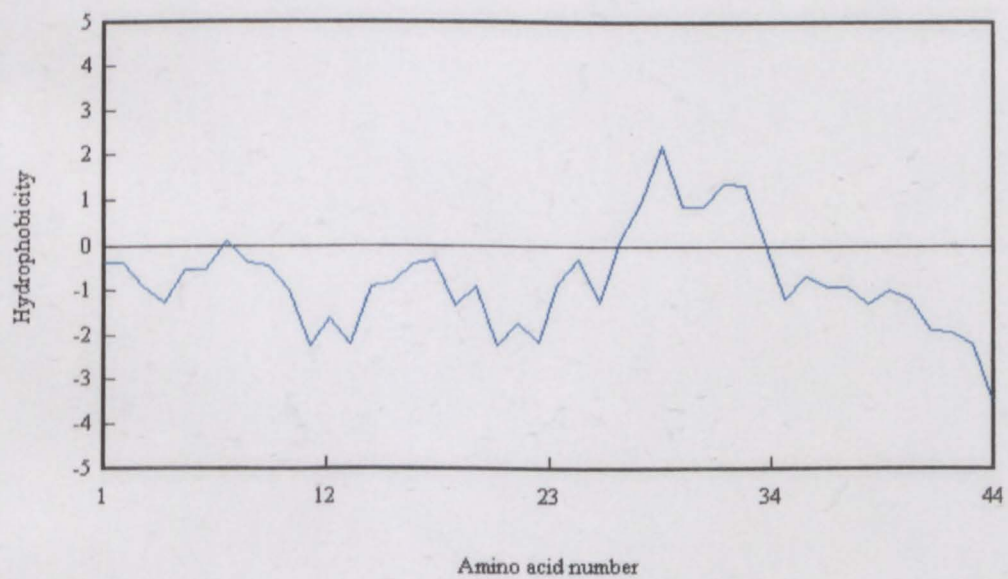
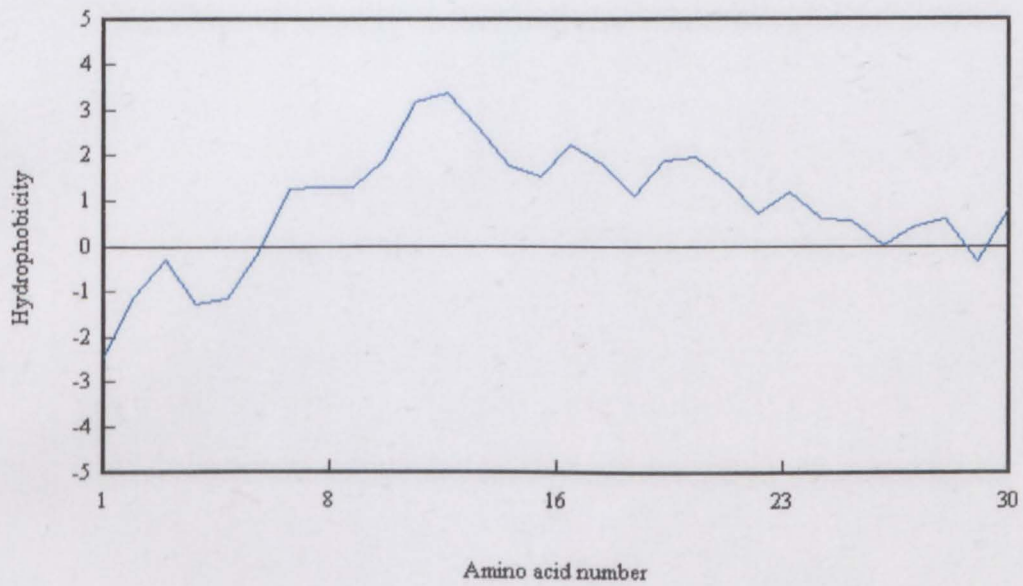


FIG. 6. Hydrophobicity profiles of the leader peptide (A) and the mature bacteriocin (B).

	i	ii	iii	
Enterocin P	MR <b>KKL</b> FSLALIGI---FGLVVTNFGTK <b>VDA</b>			27
Bacteriocin 31	M <b>KKK</b> VICGIIGIG-----FTALGTN <b>VEA</b>			24
Bacteriocin T8 (A)	M <b>KKK</b> VLKHCVILGILGTCLAGIGTG <b>IKVDA</b>			30
Enterocin P	ATRSYNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH			44
Bacteriocin 31	ATYYNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR			43
Bacteriocin T8	ATYYNGLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAPRR			44
Consensus (B)	YYNG	YCN	KCWV W A I I GW	

FIG. 7. Comparison of amino acid sequences of the N-terminals of class IIa *sec*-dependent bacteriocins. Data for enterocin P and bacteriocin 31 were obtained from Cintas *et al.* (1997) and Tomita *et al.* (1996), respectively.

## GENERAL DISCUSSION AND FINAL CONCLUSIONS

Probiotic lactic acid bacteria may be used to treat urogenital infections. *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 reversed the microflora of patients suffering from bacterial vaginosis to normal cell numbers (Reid *et al.*, 2001; Reid *et al.*, 2003). *L. fermentum* CRL1058 protected mice against uropathogenic *E. coli*, a causative agent of urinary tract infections (Nader de Macias *et al.*, 1996). The ingestion of *Lactobacillus acidophilus* NFCM™ reduced the number of candidal and bacterial vaginosis infections (Collins and Hardt 1980; Hallen *et al.*, 1992; Hilton *et al.*, 1992; Shalev *et al.*, 1996). Bacteriocins produced by various lactic acid bacteria inhibit uropathogens such as *G. vaginalis*, *E. coli*, *N. gonorrhoeae* and clinical *E. faecalis* strains (Aroutcheva *et al.*, 2001; Morency *et al.*, 2001; Ocana *et al.*, 1999; Simoes *et al.*, 2001).

Bacteriocin ST15, plantaricin 423 and bacteriocin T8 produced by *Enterococcus mundtii* ST15, *Lactobacillus plantarum* 423 and *Enterococcus faecium* T8, respectively, displayed antimicrobial activity against uropathogenic strains of *E. faecalis* and *C. albicans*. Bacteriocin ST15 was the most effective, followed by plantaricin 423. Although bacteriocin T8 displayed low antimicrobial activity, the specificity of the peptide is higher than that recorded for of plantaricin 423, i.e. has a narrower activity spectrum (van Reenen *et al.*, 1998). The high specificity of bacteriocin T8 and bacteriocin ST15 towards lactobacilli may be advantageous, since they will not cause a rapid decline in the natural microflora of the urogenital tract (Altöparlak *et al.*, 2004; Hillier *et al.*, 1993; Klebanoff *et al.*, 1991).

The probiotic properties of *E. mundtii* ST15, *Lb. plantarum* 423 and *E. faecium* T8 were further investigated. All three bacteria produce lactic acid but not H<sub>2</sub>O<sub>2</sub>. This may be an advantage, since H<sub>2</sub>O<sub>2</sub>-producers are susceptible to the spermicide, nonoxynol-9 (McGregor *et al.* 1990). Furthermore, *E. mundtii* ST15 and *E. faecium* T8 co-aggregated with a clinical strain of *C. albicans* and can thereby inhibit the growth of *C. albicans*.

A novel class IIa *sec*-dependent bacteriocin, T8, was identified and characterized. The producer strain, T8, revealed 94% 16S rDNA homology with *Enterococcus faecium* and *Enterococcus durans*. However, strain T8 ferments glycerol and melibiose and is thus more closely related to *E. faecium* (Holt, 1994). Bacteriocin T8 is a heat stable protein and is active against *Lactobacillus sakei*, *E. faecalis* and a *Propionibacterium* sp. The mode of activity is bactericidal.

The bacteriocin determinant is located on a plasmid and consists of two ORFs. The first ORF encodes a 74 amino acid bacteriocin precursor that consists of a leader signal peptide and a mature peptide. The leader peptide fulfills Von Heijne's rules for a signal peptide, namely three positively charged amino acids, a span of hydrophobic amino acids and a cleavage region (VDA) (von Heijne and Abrahmsen, 1989). The conserved YGNGX<sub>2</sub>CX<sub>4</sub>CXV-sequence, typical of all class IIa bacteriocins, is present in the mature peptide. The mature peptide has a theoretical molecular weight of 5.07 kDa. This corresponds to the tricine-SDS-PAGE where the partially purified peptide is between 3.5 and 6.5 kDa in size. The second ORF encodes a potential immunity protein and is located immediately downstream of the structural gene. Bacteriocin T8 has 69% amino acid similarity with bacteriocin 31 and 47% with enterocin P, respectively. Bacteriocin 31, enterocin P and enterocin P-like bacteriocin, produced by *E. faecium* P (Cintas *et al.*, 1997), *E. faecalis* Y1717 (Tomita *et al.*, 1996) and *E. faecium* GM-1 (Kang and Lee, 2005), are, to our knowledge, the only three *sec*-dependent class IIa bacteriocins thus far described.

A mobilization protein and relaxase mobilization nuclease domain is located on the same plasmid as the bacteriocin operon. Production of bacteriocins by enterococci is associated with pheromone-responsive conjugative plasmids (Clewell, 1993; Clewell *et al.*, 2002). Bacteriocin 31 is encoded on a pheromone-responsive conjugative plasmid (Tomita *et al.*, 1996).

Future research is needed to optimize the production of bacteriocin T8. The problem can be approached in two different ways. Optimizing growth and bacteriocin production conditions of *E. faecium* T8 can increase the production of bacteriocin T8, as observed in the case of the *sec*-dependent class III bacteriocin, enterolysin A (Nilsen *et al.*, 2003). The alternative is to clone the bacteriocin structural gene into a bacterium or yeast that is already used in the pharmaceutical industry for large-scale production of proteins (Gutierrez *et al.*, 2005). It is interesting to note that active enterocin P was detected after the structural gene of the *sec*-dependent bacteriocin was cloned into *Pichia pastoris*. All other attempts with bacteriocins secreted with ABC-transporters delivered poor results (Beaulieu *et al.*, 2005; Schoeman *et al.*, 1999; van Reenen *et al.*, 2002). The bacteriocins were detected in the supernatants, but at very low levels.

The adhesion of *Lb. plantarum* 423, *E. mundtii* ST15, and *E. faecium* T8 to uro- and vaginal epithelial cells will have to be studied with cell lines and in clinical trials. Similar studies have been conducted by Lee (2005), Reid *et al.* (2001) and Fichorova *et al.* (2001).



*In vivo* studies are needed to conclude if the selected lactic acid bacteria are safe to be consumed and whether they can serve as an effective probiotic for women. The safety of *E. mundtii* ST15 and *E. faecium* T8 must be thoroughly investigated because of the pathogenic behaviour of certain enterococci (Franz *et al.*, 1999; Moreno *et al.*, 2005). Developing a microbicidal gel containing only the active bacteriocin is an alternative to the use of the bacteria as urogenital probiotics for treating and preventing urogenital tract infections.

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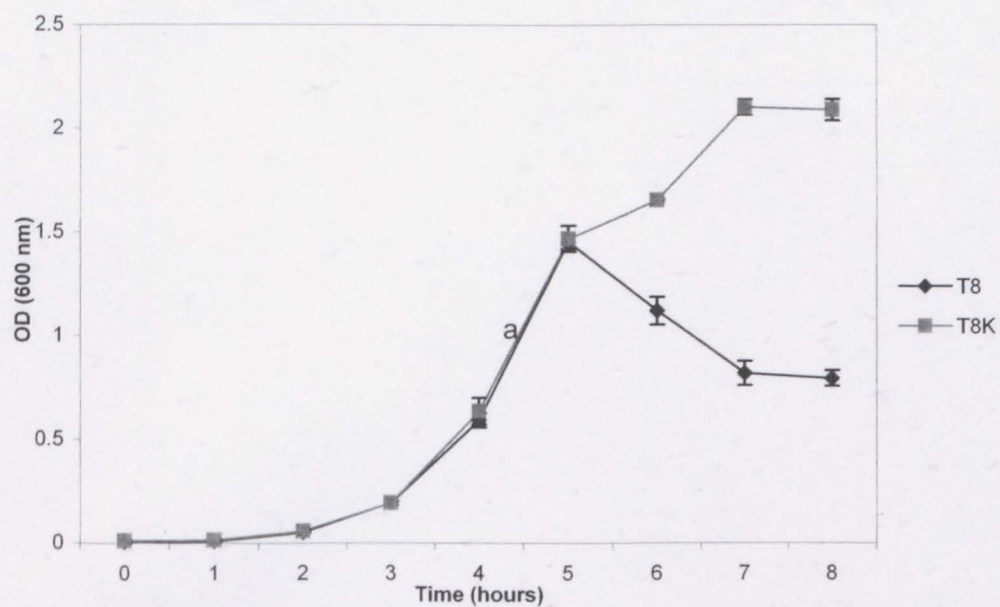


FIG. 1. Cell lysis of *E. faecalis* MDK2 following addition of bacteriocin T8 at mid-exponential phase (5 hours).

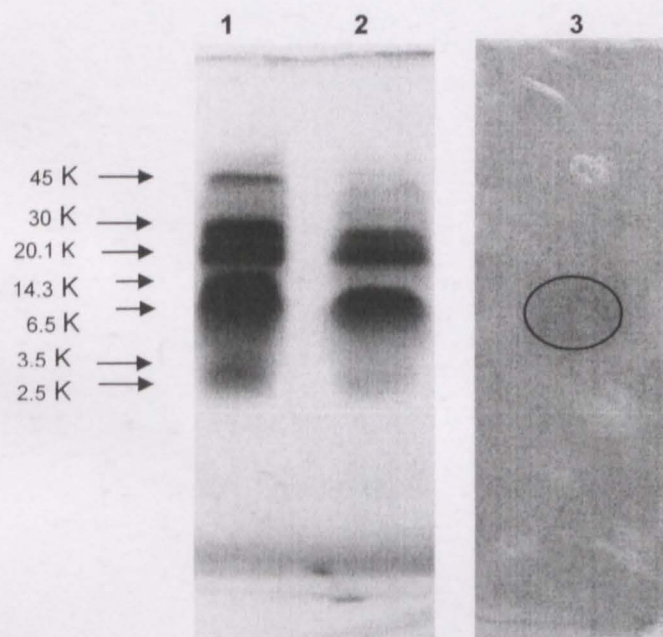


FIG. 2. Separation of partially purified bacteriocin T8 by SDS-PAGE. Lane 1, Rainbow protein size marker (Amersham); lane 2, bacteriocin T8 stained with Coomassie blue R-250; lane 3, bacteriocin T8 overlaid with cells of *E. faecalis* MDK2 embedded in BHI soft agar (1%). The circle indicates the inhibition zone.

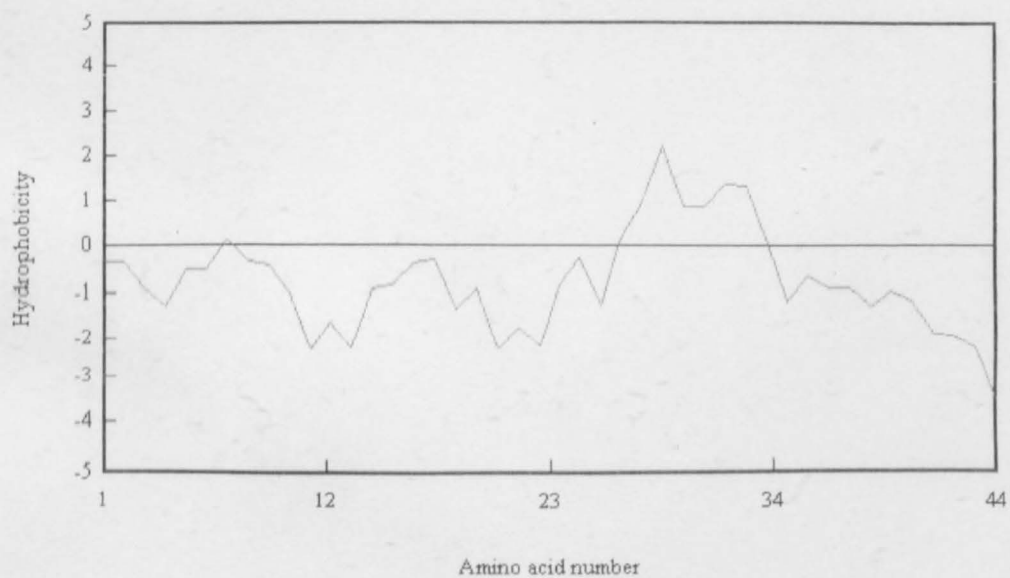
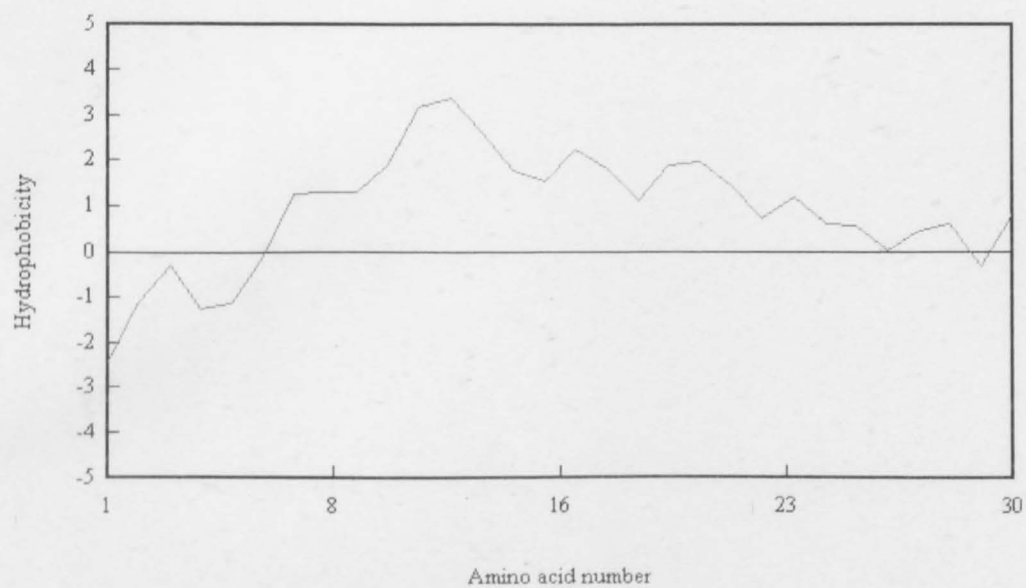


FIG. 6. Hydrophobicity profiles of the leader peptide (A) and the mature bacteriocin (B).

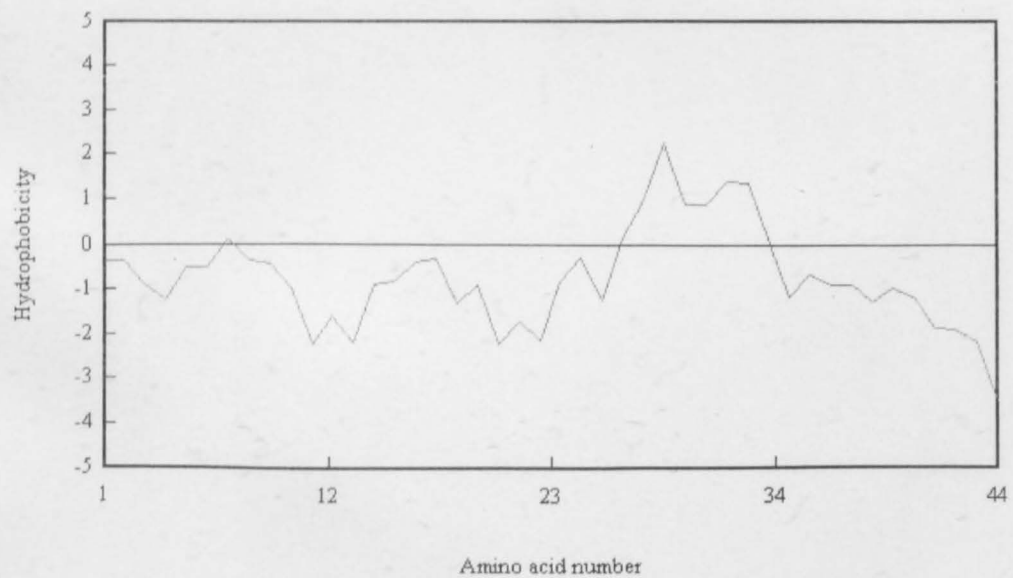
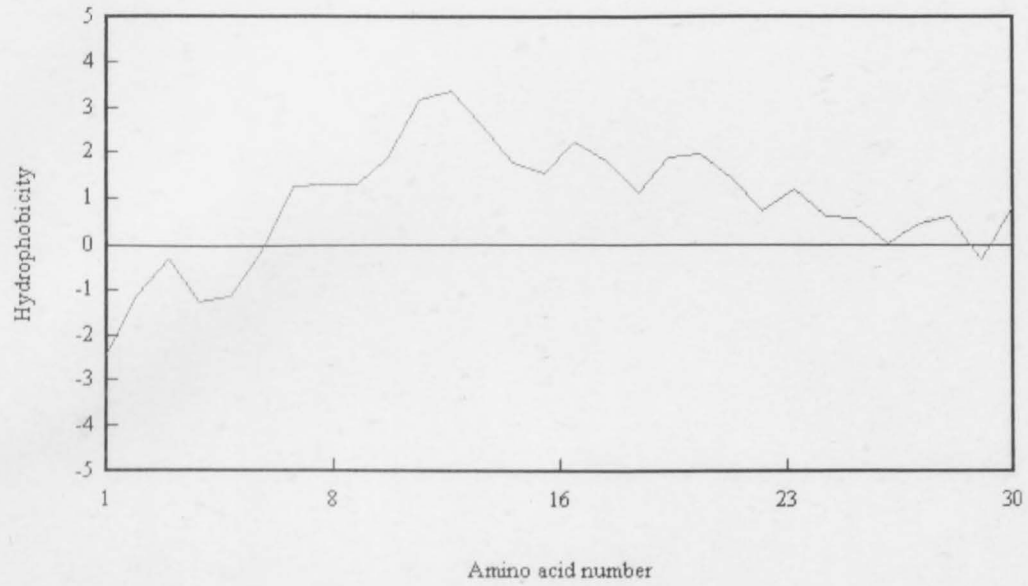


FIG. 6. Hydrophobicity profiles of the leader peptide (A) and the mature bacteriocin (B).

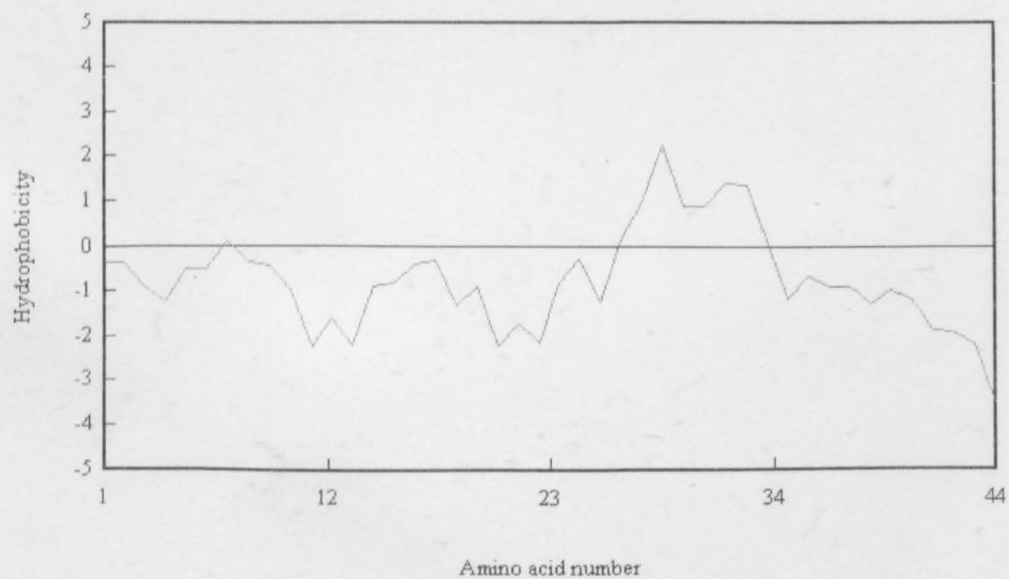
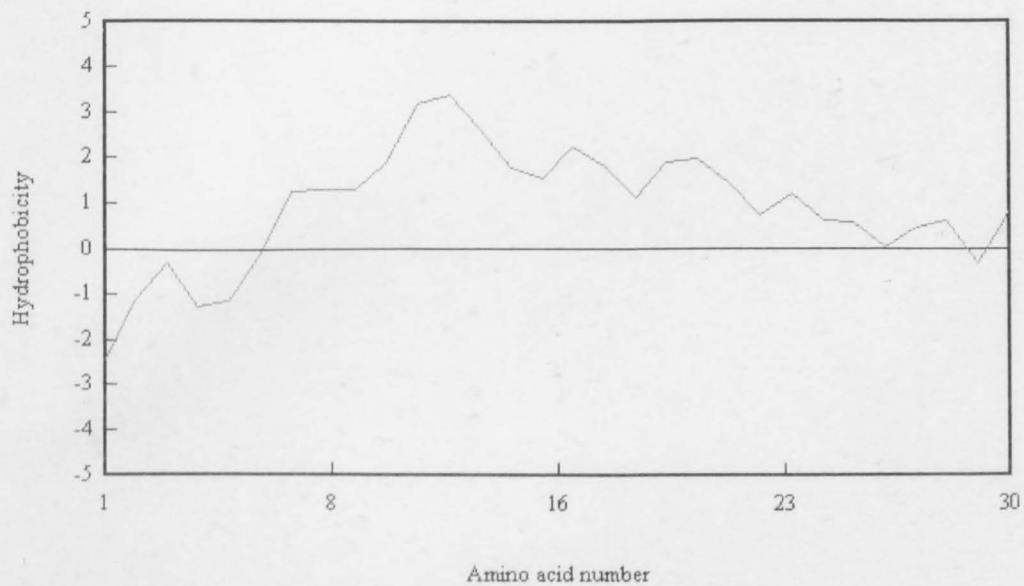


FIG. 6. Hydrophobicity profiles of the leader peptide (A) and the mature bacteriocin (B).