THE SAFE, NATURAL ANTIMICROBIAL PEPTIDE SUBTILOSIN FOR CONTROL OF BACTERIAL VAGINOSIS

By

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ABSTRACT OF THE DISSERTATION

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Thesis Director: Dr. Michael Chikindas

Bacterial vaginosis (BV) is a condition characterized by an imbalance in the vaginal microflora where healthy lactobacilli are replaced by anaerobic microorganisms, especially *Gardnerella vaginalis*. It is estimated that 10-30% of North American women suffer from BV, frequently requiring medical attention. Proliferation of BV-associated organisms is known to have pathogenic effects, particularly in pregnant women. BV is associated with development of pelvic inflammatory disease, low fetal birth weight, preterm births with an elevated risk of infant death, intra-amniotic infections leading to fetal brain damage, and spontaneous abortion. Furthermore, BV (and *G. vaginalis* in particular) has been shown to increase the probability of contracting HIV and to stimulate viral replication. The antibiotics commonly prescribed to treat BV cause widespread inhibition of the healthy vaginal microflora, leading to a 20% recurrence rate of infection, often with newly developed antibiotic resistance(s).

One promising alternative BV treatment is the bacteriocin, or antimicrobial peptide, subtilosin A. This dissertation describes the isolation, characterization, and purification of subtilosin from a fermented dairy product isolate of *B. amyloliquefaciens*. This is the first report of the intra-species horizontal gene transfer of subtilosin (from *B.*

subtilis), and it is the first bacteriocin characterized from *B. amyloliquefaciens.* Via well diffusion assays, subtilosin was found to inhibit several human pathogens, including *G. vaginalis,* but has no effect on healthy vaginal lactobacilli. Motility and structure analyses of human spermatozoa revealed subtilosin possesses potent spermicidal activity. *In vitro* ectocervical tissue toxicity testing showed subtilosin is completely safe for human tissues. Elucidation of the molecular mechanism of action established that subtilosin specifically inhibits *G. vaginalis* by forming pores in the cell membrane, causing an efflux of ATP and dissipation of the ΔpH portion of the proton motive force. Finally, microplate checkerboard assays confirmed that subtilosin acts synergistically with other antimicrobials of varying mechanisms of action, suggesting that lower concentrations of subtilosin could be used to effectively inhibit *G. vaginalis*, thereby decreasing the likelihood of developing resistance. Taken together, the data presented herein demonstrate that subtilosin is a safe, natural antimicrobial peptide that can easily be formulated into an effective prophylaxis or treatment for bacterial vaginosis.

Dedication

For Eric: You are my strength. I couldn't have done this without your unending love and support.

For Mom and Dad: I am only able to be who I am today because of you.

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I cannot thank my entire family enough for their unending love and support. As always, you have helped carry me through this long and difficult journey, and I share this great reward with all of you.

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Chapter 1: Medical and Personal Care Applications of Bacteriocins Produced by Lactic Acid Bacteria.[1]

This review chapter examines the emerging importance of bacteriocins as alternative treatment options for a variety of medical conditions. Discussed within are the implications of bacteriocins on human health, with particular attention given to subtilosin, the focus of this dissertation, and its impact on urogenital and reproductive health.

[1] This chapter has been accepted for publication as a chapter in the textbook *Prokaryotic Antimicrobial Peptides: From Genes to Biotechnologies* (New York: Springer, 2010). All references and formatting within follow the specifications of the publisher.

Medical and Personal Care Applications of Bacteriocins Produced by Lactic Acid Bacteria

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ABSTRACT

The frequent use of antibiotics has led to a crisis in the antibiotic resistance of pathogens associated with humans and animals. Antibiotic resistance and the emergence of multi-resistant bacterial pathogens have led to the investigation of alternative antimicrobial agents to treat and prevent infections in both humans and animals. Research on antimicrobial peptides, with a special interest on bacteriocins of lactic acid bacteria, is entering a new era with novel applications other than food preservation. Many scientists are now focusing on the application of these peptides in medicinal and personal care products. However, it is difficult to assess the success of such ventures due to the dearth of information that has been published and the lack of clinical trials.

INTRODUCTION

Bacteriocins of lactic acid bacteria are ribosomally synthesized, generally cationic, have less than 100 amino acid residues (Marcus, 1999; Jenssen, 2006) and contain a substantial portion (30% or more) of hydrophobic residues (Dijkshoorn, 2004). The cationic charge of these peptides ensures electrostatic affinity with the negatively charged bacterial outer membrane, whereas the hydrophobic section of the peptide interacts with the cell membrane and enters the double lipid membrane. Most bacteriocins have a relatively narrow spectrum of antimicrobial activity, i.e. the growth of only certain species, usually those phylogenetically related to the producer strain, are affected. However, some bacteriocins exhibit a much broader spectrum of antimicrobial activity and may extend beyond the borders of bacteria to include protozoa, yeast, fungi and viruses (Reddy *et al*., 2004). A few bacteriocins are cytotoxic, with activity against sperm and tumour cells (Reddy *et al*., 2004).

Bacteriocins may be seen as defense peptides and may thus be grouped into the same category as killer toxins of yeast, defensins of mammals, cecropins of insects, tachyplesins of crabs, magainins of amphibia, pandanins of scorpions, and thionins of plants (Boheim, 1983; Corzo *et al*., 2001; de Vuyst and Vandamme, 1994; Reddy *et al*., 2004). Mature peptides are produced after the cleavage of inactive pre-peptides (Nes and Holo, 2000; Sahl and Bierbaum, 1998).

Colicins, produced by *Escherichia coli*, were the first described bacteriocins. The mode of action of these peptides varies from pore formation to nuclease activity (Braun *et al*., 1994; Riley and Wertz, 2002; Smarda and Smajs, 1998). Colicins E1 and K inhibit macromolecular synthesis without arrest of respiration. Colicin E2 causes DNA breakdown, and colicin E3 stops protein synthesis (Nomura, 1967). The genes encoding colicin production are usually located on plasmids (Pugsley and Oudega, 1987). Type A plasmids are small (6 to 10 kb), usually present as multiple copies and are conjugative. Type B plasmids are approximately 40 kb in size, occur as single copies and are also conjugative.

Microcins, also produced by *E. coli*, are smaller than colicins and share more properties with bacteriocins produced by Gram-positive bacteria (Baquero and Moreno, 1984; Gillor *et al*., 2004; Pons *et al.,* 2002). Fourteen microcins have been reported to date, of which only seven have been isolated and fully characterized (Duquesne *et al*., 2007a, b; Severinov *et al*., 2007).

Bacteriocins of Gram-positive bacteria are more diverse than bacteriocins described for Gram-negative bacteria (Riley and Wertz, 2002). Over the years many classification methods have been proposed for bacteriocins. Fredericq (1957) classified bacteriocins based on specificity of absorption and proposed subclasses according to immune responses. Reeves (1965) implemented a system consisting of 16 classes of bacteriocins based on the species that produce them. Ten years later Bradley (1967) classified

bacteriocins based on molecular weight. Klaenhammer (1993) classified bacteriocins into four classes based on structure, mechanism of action, genetics and biochemical properties. Finally, Cotter *et al*. (2005) proposed two major classes, each divided into subclasses (Table 1).

Bacteriocins of class I contain lanthionine or β-methyllanthionine and are classified as lantibiotics. They undergo post-translational modifications to produce peptides of less than 5 kDa in size. Type A lantibiotics such as nisin, epidermin and subtilin are screw-shaped, elongated, flexible, and amphipathic peptides with pore-forming activities (Kordel *et al*., 1989; Ruhr and Sahl, 1985), whereas type B lantibiotics, of which mersacidin is a typical example, are small and compact peptides which target specific components of the bacterial membrane (Brötz *et al*., 1998). Type B lantibiotics kill by interfering with cellular enzymatic reactions, such as cell wall synthesis (Pag and Sahl, 2002; Sahl and Bierbaum, 1998; Sahl *et al*., 1995).

Nisin A and mutacin B-Ny266 are active against a range of organisms, including species of *Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactococcus, Listeria, Micrococcus, Mycobacterium, Propionibacterium, Streptococcus,* and *Staphylococcus* (Mota-Meira *et al*., 2000, 2005). Activity against a number of Gram-negative pathogens such as *Campylobacter, Haemophilus, Helicobacter* and *Neisseria* spp. have also been reported (Morency *et al*., 2001).

Non-lanthionine bacteriocins are grouped in class II. These peptides are heat-stable and do not undergo extensive post-translational modifications (Cotter *et al*., 2005). The majority of bacteriocins in class II kill by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria. Two subgroups are differentiated. Class IIa peptides form the largest subgroup. They are active against *Listeria* and have a conserved amino-terminal sequence (YGNGVXaaC) that facilitates nonspecific binding to the target surface (Drider *et al*., 2006; Oppegård *et al*., 2007). Similar to type A lantibiotics, class IIa bacteriocins form pores in the cytoplasmic membrane. Typical examples include pediocin and pediocin-like bacteriocins, sakacin A, and leucocin A (Drider *et al*., 2006; Héchard and Sahl, 2002; Oppegård *et al*., 2007). Class IIb bacteriocins such as lacticin F and lactococcin G form pores and have two different proteins (Garneau *et al*., 2002; Hechard and Sahl, 2002). In the case of lacticin 3147, one of the peptides depolarizes the membrane, while the other forms pores (Martin *et al*., 2004). Sec-dependent bacteriocins, such as acidocin 1B, are placed in subgroup IIc (Han *et al*., 2007). Non-pediocin like bacteriocins are classified as subgroup IId (Cotter *et al*., 2005).

Genes encoding the biosynthesis of bacteriocins are organized in clusters located on either the chromosome, plasmids or on possibly both if located on a transposon (McAuliffe *et al*., 2001; Nes *et al*., 1996; van Reenen *et al*., 1998). The class I (lantibiotic) gene cluster consists of a structural gene that codes for a pre-bacteriocin, genes encoding accessory proteins involved in structure modifications such as proteolytic

processing of the leader peptide, transport genes that code for an ABC-superfamily of transport proteins, regulation genes, and immunity genes that confer resistance to the producer strain (de Vos *et al*., 1995; Jack *et al*., 1995; Kolter and Moreno, 1992; Sahl, 1995). The structural gene encodes an inactive pre-peptide with an N-terminal extension or leader peptide connected to the C-terminal of the pro-peptide (McAuliffe *et al*., 2001; Sahl, 1998). The pro-peptide is modified to become a mature active peptide (McAuliffe 2001). At the end of biosynthesis the leader peptide is cleaved by a protease, before or after the peptide is translocated by ABC (ATP-binding) transport proteins (Klaenhammer, 1993; Sahl, 1998). The bacteriocin is then released to execute its antibacterial activity (McAuliffe *et al*., 2001).

Genes involved in the production of class II bacteriocins are somewhat functionally similar to the genes for class I, i.e. they include a structural gene, an immunity gene and two genes encoding a membrane-associated ATP-dependent binding casette (ABC) transporter and an accessory protein (Eijsink *et al*., 1998; Ennahar *et al.*, 2006; Klaenhammer, 1993; Nes *et al*., 1996). The structural genes also encode a pre-peptide with a leader peptide, which become active once the leader peptide is cleaved by a protease (Eijsink *et al*., 2002; Nes and Holo, 2000; Nissen-Meyer and Nes, 1997). Class IIa bacteriocins have a leader peptide with double-glycine residues which acts as the processing site and is secreted once the leader peptide has been cleaved (Klaenhammer, 1993; Michiels *et al*., 2001).

Mode of action of bacteriocins

Class Ia lantibiotics, especially nisin, have been extensively studied for their mode of action because of various industrial applications. Nisin, a typical example of type A lantibiotics, binds to lipid II on the cell wall surface and prevents cell wall synthesis. Insertion of the peptide into the phospholipid bilayer of the cell membrane leads to drastic changes in permeability and may cause cell death (Wiedemann *et al*., 2001). Nisin kills sensitive organisms by disruption of the proton motive force (PMF) (Abee *et al*., 1995; Chung and Hancock, 2000; de Vuyst and Vandamme, 1994; Kraaij, 1999; Ruhr and Sahl, 1985). The PMF, composed of a chemical component (pH gradient) and electrical component (membrane potential), plays an important role in the synthesis of adenosine triphosphate (ATP) and the influx of molecules by PMF-driven transport systems (de Vuyst and Vandamme, 1994; McAuliffe *et al*., 2001). The amphiphilic nature of nisin allows it to interact with hydrophilic heads and hydrophobic regions of the plasma membrane and cause an efflux of ions, solutes and small molecules, forcing biosynthetic processes in the cell to a halt (Sahl, 1998). Although the formation of pores or channels with the depletion of the PMF is the primary activity of nisin, the peptide also forms a complex with lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc), thereby inhibiting the synthesis of lipids and peptidoglycan (de Vuyst and Vandamme, 1994; Sahl, 1998). Compared to pore formation, inhibition of cell wall biosynthesis is a relatively slow process (McAuliffe, 2001). Class Ib lantibiotics have a different mode of action. They hinder the activities of essential enzymes (McAuliffe, 2001).

Class II bacteriocins also disrupt the PMF. They recognize specific protein receptors and form voltage independent pores or channels in the plasma membrane of sensitive cells (Héchard and Sahl, 2002). This has been shown for pediocin PA-1, lactococcin, and sakacin A and B (Chikindas *et al*., 1993). Two-peptide bacteriocins such as lactococcin G have no effect on the pH gradient, but dissipate the membrane potential which leads to the efflux of cations (Moll *et al*., 1998, 1999).

Various models for the formation of pores or channels by bacteriocins have been proposed, such as the 'barrel-stave' model, the 'carpet' model and the 'wedge' model (Driesen *et al*., 1995; Héchard and Sahl, 2002; Moll *et al*., 1999; Ojcius, 1994; Sahl, 1991; van den Hooven *et al*., 1996). The 'wedge' model is proposed for lantibiotics, while the 'barrel-stave' and 'carpet' models are proposed for the class II bacteriocins (Moll *et al*., 1999). In the 'barrel-stave" model the bacteriocin forms a trans-membrane barrel of α-helices with a hydrophilic interior and hydrophobic exterior (Ojcius, 1994; Sahl, 1991). Electrostatic interactions form between the positively charged amino acids of the peptide and the negatively charged heads of the phospholipid bilayer. The hydrophobic part of the peptide forms interactions with the hydrophobic acyl chains of the membrane lipids. Once inserted in the plasma membrane, the bacteriocin molecules form pores which lead to disruption of the PMF (Ennahar *et al.*, 2006; Moll *et al*., 1999). In the 'carpet' model single peptide molecules interfere with plasma membrane organization and result in pore formation (Moll *et al*., 1999). In the 'wedge' model the

hydrophilic, positively charged amino acids of the peptide interact with the negatively charged heads of the phospholipid bilayer. The hydrophobic region of the peptide inserts itself into the outer leaflet of the bilayer (Driesen *et al*., 1995; Sahl, 1998; van den Hooven *et al*., 1996).

Medical applications of bacteriocins

While nowadays everyone expects to see bacteriocins related to food applications, their many possible uses for the control of nondesired microorganisms in the human environment are greatly underappreciated. With the advent of multi-drug resistant bacteria, it has become a priority to develop alternative medicinal treatments/preventive measures against these pathogens. Since the mode of action of bacteriocins is remarkably different from conventional antibiotics, they may be considered as a novel approach for the control of microbial pathogens. This will be discussed in greater detail in a later section of this review.

Staphylococcus aureus and methicillin resistant *S. aureus* (MRSA) are the most prevalent organisms in skin infections and have become a serious problem, especially in hospitals (Guggenheim *et al*., 2009; Lesseva and Hadjiiski, 1996; Taylor *et al*., 1992). MRSA was first reported in the 1960's after methicillin was introduced to treat *S. aureus* infections (Hackbarth and Chambers, 1989; Kim, 2009). Resistant strains have acquired the *mecA* gene that codes for a low-affinity penicillin binding protein, PBP2a (Fraise *et al*., 1997; Hiramatsu, 1995). Oxacillin, nafcillin, quinopristin-dalfopristin, rifampicin,

ciprofloxacillin, teichoplanin, cefazolin and cephalothin A are also used to treat *S. aureus* infection (Gould, 1995; Lowry, 1998), but with limited success. In many cases vancomycin is used as last resort, but also with limited success (Dicks *et al*, 2010). Mersacidin, a lantibiotic produced by *Bacillus* sp. strain HIL Y-85,54728 (Sass *et al*., 2008), inhibits the growth of MRSA strains *in vivo* in mice (Kruszewska *et al*., 2004) and may be considered an alternative treatment. The peptide inhibits cell wall synthesis of MRSA strains with an efficiency equal to that reported for vancomycin (Chatterjee *et al*., 1992; Limbert *et al*., 1991). Mersacidin is also active against *Propionibacterium acnes* and may thus be used in the treatment of acne (Jung, 1991; Kellner *et al*., 1988; Niu *et al*., 1991). The mode of action of mersacidin differs from vancomycin, which leaves the option of using the two substances in combination (Brötz, 1995).

In a study conducted by Kruszewska *et al*. (2004), two-month old female BALB/cA mice were immune suppressed, intranasally infected with *S. aureus* 99308 and then treated with mersacidin. Mersacidin effectively inhibited the growth of *S. aureus* in the mice.

Lacticin 3147, a two-peptide lantibiotic produced by *Lactococcus lactis* subsp. *lactis*, inhibits the growth of *S. aureus*, MRSA and vancomycin-resistant strains of *Enterococcus faecalis* (Galvin *et al*., 1999). Epidermin and gallidermin, also classified as lantibiotics but produced by *Staphylococcus gallinarum* and *Staphylococcus epidermidis*, proved effective in the treatment of skin infections (Kellner *et al*., 1988).

Epidermin and gallidermin are also active against *P. acnes* (Jung, 1991; Kellner, 1988; Niu and Neu, 1991). Bonelli *et al*. (2006) measured the antimicrobial activity of epidermin and gallidermin by determining the release of potassium (K^+) from membrane models they have constructed. Treatment with gallidermin resulted in K^+ release from *Staphylococcus simulans* and *Micrococcus flavus*. Epidermin was active against *M. flavus,* whereas nisin inactivated both pathogens. Nisin and IB-367, a protegrin-like cationic peptide produced by Intrabiotics (Mountain View, California), have recently entered phase I clinical trials for acne infections.

A number of studies claim that lantibiotics are effective in the prevention of tooth decay and gingivitis (Blackburn and Goldstein, 1995; Howell *et al*., 1993; McConville, 1995; Patel, 1995). Nisin exhibits antimicrobial activity against plaque and gingivitis-causing bacteria and has been included in mouth washes (van Kraaij *et al*., 1999). *In vivo* experiments were done on beagle dogs (Howell *et al*., 1993). Lacticin 3147 prevented the growth of *Streptococcus mutans* associated with dental decay (Galvin *et al*., 1999). BLIS K12, a commercial product that contains a strain of *Streptococcus salivarius* that produces salivaricin A2 and B, inhibits bacteria associated with bad breath (Tagg, 2004).

Mastitis, a bacterial infection of the mammary glands, causes huge economic losses in the dairy industry (Bradly, 2002; Riffon *et al*., 2001; Soltyns and Quinn, 1999; Sordelli *et al*., 2000; Twomey, 2000). Trials carried out by Taylor *et al.* (1949) indicated that a single intramammary infusion of nisin was effective in treating both streptococcal and

staphylococcal infections. However, infusions of the nisin preparation into the udder produced an adverse cellular response, although there was no correlation between the nisin concentration and local intolerance produced in the udder. Broadbent *et al.* (1989) showed that nisin inhibited growth of several Gram-positive, mastitis causing pathogens *in vivo*. Nisin A in combination with lysostaphin was also administered by intramammary infusions (Sears *et al*., 1992a, b). During these experiments, cure rates of 66% for *Staphylococcus aureus*, 95% for *Streptococcus agalactiae*, and 100% for *Streptococcus uberis* were observed. Nisin A has also been incorporated in teat wipes (Broadbent *et al*., 1989; Cotter, 2005; Ross *et al*., 1999).

Lacticin 3147 proved effective in the treatment of bovine mastitis and showed activity against mastitic staphylococci and streptococci (Ryan *et al*., 1999). A bacteriocin produced by *Bacillus subtilis* LFB112 inhibited the growth of *S. aureus* associated with mastitis (Xie *et al*., 2009). *Streptococcus gallolyticus* subsp. *macedonicus* ST91KM produces a bacteriocin (macedocin ST91KM) active against *Streptococcus agalactiae, Streptococcus dysgalactiae* subsp. *dysgalactiae, Streptococcus uberis, Staphylococcus aureus* and *Staphylococcus epidermidis*, including strains resistant to methicillin and oxacillin (Pieterse *et al*., 2008, 2010). Macedocin ST91KM may thus be used as an alternative in the treatment of mastitis in dairy cows.

Several cases of infections caused by contaminated biomedical implant devices have been reported (Campoccia, 2005). Nisin, adsorbed to silanized surfaces, prevented the

growth of *Listeria monocytogenes* (Bower *et al*., 1995). In a separate study, the authors coated Teflon® FEP intravenous catheters with nisin and inserted the devices in the jugular veins of sheep. In a similar experiment PVC tracheotomy tubes were coated and placed in the upper airways of ponies. The endotracheal tubes coated with nisin prevented colonisation of *S. aureus*, *S*. *epidermidis* and *Streptococcus faecalis* (Bower *et al*., 2002). The authors, however, concluded that the antimicrobial activity of nisin may be short lived *in vivo*, as intravascular catheters introduced in sheep controlled bacterial infection for only 5 h. Tracheotomy tubes treated with nisin controlled infection to some degree (Bower *et al*., 2002).

Bacteriocins for the control of upper respiratory tract infections

Pseudomonas aeruginosa is the main causative agent of nosocomial pneumonia in cystic fibrosis (CF) patients (Linden *et al*., 2003) and has been diagnosed in more than 50% of CF patients with lung infections (CF patient registry, 2008). *P. aeruginosa* is also one of the major pathogens responsible for chronic/acute otitis (Roland *et al*., 2002; Wright *et al*., 2009). Otitis media is one of the most common diseases diagnosed in children under the age of 2 years (Segal *et al*., 2005). Long term side effects of persistent otitis media include impaired hearing and delayed speech (Ryding *et al*., 1997; Teele *et al*., 1990). In addition to lung infection in CF patients and ear infections, *P. aeruginosa* is also associated with burn wound infections, especially amongst immunodeficient individuals (Deretic, 2000; Hachem *et al*., 2007). Treatment of *P. aeruginosa* depends on several factors, including age of patient and severity of disease (Doring *et al*., 2000).

Antibiotic treatments include Cefepime, Ceftazidime, Ciprofloxacin, Colistin, Piperacillin-tazobactam, Gatifloxacin, Amikacin and Gentamicin (Doring *et al*., 2000; Osih *et al*., 2007).

Nisin inhibited the growth of *S. pneumonia* associated with otitis media in *in vivo* trials (Goldstein *et al*., 1998). Peptide ST4SA, a class II bacteriocin, showed better activity towards Gram-positive middle ear pathogens compared to other antimicrobial agents (Knoetze, 2008). Peptide ST4SA remained active when incubated in blood and middle ear fluid and thus have the potential to be used in the treatment of otitis media.

Peptide IB-367 has recently undergone phase I safety trials on humans with the objective of using the peptide against chronic *P. aeruginosa* lung infections, specifically on patients suffering from cystic fibrosis.

Towards the end of the last century, approximately one third of the world's population had been infected with *Mycobacterium tuberculosis* and was at risk of acquiring tuberculosis (Bloom and Murray, 1992). In 2007 alone, more than 13 million people had been diagnosed with tuberculosis and more than nine million new cases were reported. The World Health Organization reported more than 1.8 million deaths caused by *M. tuberculosis*, of whom almost half a million were HIV positive (WHO 2009). Class II bacteriocins are active against *M. tuberculosis*. According to *in vivo* experiments, a bacteriocin–liposome complex increased the survival rate of animals challenged with

the pathogen (Sosunov *et al*., 2007). The authors created an acute TB model in C57BL/6JCit (B6) mice by injecting the animals with a *M. tuberculosis* strain into the tail vein. Six hours after infection bacteriocin Bcn5 was injected into the tail vein and repeated every day for the next 5 days. Treatment with this bacteriocin showed less mortality than the negative control group, but greater mortality than the positive control group which was treated with the clinically used antibiotic, rifampicin.

Nisin F, described by de Kwaadsteniet *et al*. (2009), inhibited the growth of *S. aureus* in the respiratory tract of rats when administered intranasally. The trachea and lungs of immunosuppressed rats that were infected with *S. aureus* and then treated with nisin F remained healthy. No significant differences were recorded in blood cell indices. The antimicrobial activity of low concentrations of nisin F (80 to 320 AU ml^{-1}) was slightly stimulated by lysozyme and lactoferrin. The authors concluded that Nisin F is non-toxic and may be used to control respiratory tract infections caused by *S. aureus*. This is, however, a preliminary study with an animal model and needs to be confirmed with human studies.

A strain of *Streptococcus mutans* that produces mutacin 1140 is active against tooth decay bacteria (Hillman *et al*., 1998). The strain has since been genetically modified to produce less lactic acid. The genetically modified strain, referred to as strain SMaRT, has been evaluated as a replacement therapy to eliminate decay-causing strains of *S. mutans.* Preclinical tests on laboratory animals have shown that the SMaRT strain

eliminates disease-causing *S. mutans* strains, but not other types of bacteria commonly found in the oral cavity.

Use of bacteriocins in systemic infections

S. aureus, *Listeria monocytogenes* and *P. aeruginosa* are often associated with systemic infections (Czuprynski *et al*., 2002, Drake and Montie, 1988; Harbarth *et al*., 1998; Klug *et al*., 1997). *Clostridium perfringens*, *Salmonella* spp., *S. aureus*, *Helicobacter* (*Campylobacter*) spp., *E. coli* and *L. monocytogenes* are the most prominent bacteria causing gastro-intestinal disorders and food poisoning (Työppönen *et al*., 2003).

L. monocytogenes is especially dangerous to young children, pregnant woman, immuno-compromised individuals and the elderly (Työppönen *et al*., 2003). The pathogen usually enters through the intestine and attaches to the epithelial cells where it is taken up via phagocytosis (Portnoy *et al*., 2002; Ramaswamy *et al*., 2007). Hemolysin secreted by the bacteria then induces cytolysis of the phagosome membrane, which initiates intracytoplasmic replication (Portnoy *et al*., 1988, 2002; Ramaswamy *et al*., 2007). *Listeria* survives in monocytes/macrophages and crosses the blood/brain barrier to cause meningitis (Drevets *et al*., 2010; Yildiz *et al*., 2007).

Listeria is resistant to β-lactams, monolactams, and cephalosporins, including cefotaxime and ceftazidime (Guinane *et al*., 2006). *Listeria* infection is usually treated with penicillin and ampicillin, which may be used in combination with an

aminoglycoside, e.g. amikacin (Charpentier and Courvalin, 1999; Yildiz *et al*., 2007). A trimethoprim-sulfamethoxazole combination is often used in patients allergic to penicillin (Charpentier and Courvalin, 1999; Yildiz *et al*., 2007). Class II bacteriocins are all active against *L. monocytogenes* and may be used to treat listeriosis (Nes and Holo, 2000). Pediocin PA-1, produced by *Pediococcus acidilactici*, revealed *in vivo* antimicrobial activity against various *L. monocytogenes* strains (Naghmouchi *et al.*, 2006, 2007). Additionally, pediocin PA-1 does not inhibit other intestinal bacteria when administered intragastrically, in contrast to nisin A and nisin Z (le Blay *et al.*, 2007).

Extensive *in vitro* studies have been done on antilisterial bacteriocins including pediocin PA-1, divergicin 35, and nisin, while only a few were done *in vivo*. When injected intravenously into the tail vein of BALB/c mice, piscicolin 126 relieved *Listeria* infection in various tissues (Ingham *et al.*, 2003). Abp118, a bacteriocin produced by *Lactobacillus salivarius* UCC118 also showed good antilisterial activity in infected mice (Corr *et al.*, 2007). Pediocin PA-1 offered protection against *L. monocytogenes* infection when administered onto the gastro-intestinal tract of ICR mice (Dabour *et al.,* 2009). Rihakova *et al.* (2010) have shown *in vivo* activity of divercin V41 against *L. monocytogenes* EGDe.

Mersacidin inhibited the growth of MRSA *in vivo* and lacticin 3147 acted against *S. aureus* and MRSA strains (Galvin *et al*., 1999; Kruszewska *et al*., 2004, Limbert *et al*., 1991). Nisin inhibited the growth of *P. aeruginosa* when used in combination with polymyxin E and clarithromycin (Giacometti *et al*., 1999). Intrabiotics claims that the

peptide protegrin PG-1 confers up to 100% systemic protection against intraperitoneal infections caused by *S. aureus,* MRSA, and *P. aeruginosa.* Another derivative of protegrin-1, iseganan, is undergoing phase II/III clinical testing for ventilator-associated pneumonia.

Clostridium botulinum causes a neuroparalytic disease known as botulism (Dolly *et al*., 2009; Franciosa *et al*., 2009; Kalb *et al*., 2009). The neurotoxins inhibit release of acetylcholine from the neuromuscular junction, bind to the cell surface of cholinergic nerve endings, and inhibit acetylcholine release, causing flaccid paralysis (Seyler *et al*., 2008). To eliminate the toxin from the body, an antitoxin is administered that binds to the toxin, rendering it inactive (Domingo *et al*., 2008; Shukla *et al*., 2004).

Most strains of *C. botulinum* are susceptible to antibiotics such as tetracycline, metronidazole and chloramphenicol. Antibiotics that may worsen the paralysis, such as aminoglycoside and clindamycin, should be avoided (Davis *et al*., 2008). Nisin proved effective in the inhibition of *C. botulinum* (Delves-Broughton *et al*., 1996) and *Clostridium tyrobutyricum* (Carvalho *et al*., 2007). Nisin also proved effective against *Clostridium difficile* (Bartoloni *et al*., 2004), leading the authors to conclude that nisin may be used in the treatment of diarrhea.

Salmonella typhi causes typhoid fever and is prevalent in underdeveloped countries (Hoffner *et al*., 2000; Ling *et al*., 1996; Perera *et al*., 2007). *S. typhi* attach to host

epithelial cells with the help of fimbriae (Baemler *et al*., 1996) and invade cells of the intestinal epithelium (House *et al*., 2001; Sukhan, 2000).

Salmonella infection is usually treated with fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin and ciprofloxacin) and chloramphenicol. However, the increase in antibiotic resistance is cause for major concern (Kadhiravan *et al*., 2005; Perera *et al*., 2007). Two mainstream vaccines are currently available for the prevention of typhoid fever; a live-attenuated oral vaccine (Ty2Ia) and a vaccine based on purified capsular polysaccharide of *S. typhi* Vi antigen (Vi polysaccharide vaccine; Fraser *et al*., 2006, 2007).

Bacteriocins of LAB are usually not active against Gram-negative bacteria and only a few papers referred to activity against *Salmonella* spp. Bacteriocin AS-48, produced by *Enterococcus faecalis*, inhibited the growth of *Salmonella choleraesuis* at pH 4.0 (Abriouel *et al*., 1998). A bacteriocin-like substance produced by a strain of *L. plantarum* inhibited the growth of *Salmonella* spp. isolated from mango (Ragazzo-Sanchez *et al*., 2009). Enhanced antimicrobial activity was observed when NaCl (40 mg/mL) was used in combination with the bacteriocin-like substance. *Enterococcus gallinarum* strain 012, isolated from the duodenum of ostrich, produced enterocin 012 (3.4 kDa in size) which is active against *Salmonella typhimurium* (Jennes *et al*., 2000).

Gastric colonization of *Helicobacter pylori* causes upper GIT disorders such as chronic gastritis, peptic ulcer disease, tissue lymphoma and gastric cancer (Correa, 1992; Israel

and Peek, 2001; Kusters *et al*., 2006). *H. pylori* is not only able to survive the acidic gastric conditions of the GIT but is also able to colonize these areas (Salama *et al*., 2001). Gastritis may protect the bacterium from host defences (Israel and Peek, 2001). Proteins CagA and VacA secreted by the bacterium increase its virulence by increasing the inflammatory responses, causing vacuolization in epithelium cells, inducing apoptosis, and promoting activation and proliferation of T-cells (Cover *et al*., 1992, 2005; Kuipers *et al*., 1995; Peek *et al*., 1995; Salama *et al*., 2001).

Nisin and Lacticins A164 and BH5 inhibited the growth of *H. pylori in vitro* and may thus be used in the treatment of peptic ulcers (Delves-Broughton *et al*., 1996; Kim *et al*., 2003). Nisin, produced by AMBI (Purchase, New York), and IB-367, a protegrin-like cationic peptide from Intrabiotics (Mountain View, California), have successfully undergone phase I (safety) clinical trials. Both these peptides are being considered for treatment of stomach ulcers caused by *H. pylori* and oral mucositis. The companies Astra and Merck have commercialized nisin for treatment of gastric *Helicobacter* infections and ulcers, while other nisin variants (nisin A and Z) have entered preclinical trials for treating vancomycin-resistant enterococci.

Phospholipase A2 plays a regulatory role in the arachidonic acid cascade, leading to the formation of potent mediators of inflammation and allergy, including the prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (Braquet *et al*., 1987; Irvine, 1982; Johnson *et al*., 1983; Page *et al*., 1984; Zipser and Laffi, 1985). The lantibiotics

duramycin, duramycin B and C, and cinnamycin inhibit phospholipase A2 indirectly by sequestering the substrate phosphatidylethanolamine (Fredenhagen *et al*., 1991; Marki *et al*., 1991) and may thus be used as anti-inflammatory drugs (van Kraaij *et al*., 1999).

The angiotensin-converting enzyme catalyzes the conversion of angiotensin I to angiotensin II and degrades bradykinin, thereby regulating blood pressure and fluid balance (Cushman and Cheung, 1980; Imig, 2004; Skeggs *et al*., 1956; Zhang *et al*., 2000). Cinnamycin-like lantibiotics and ancovenin, a type B lantibiotic, inhibit the activity of the angiotensin-converting enzyme (Jung, 1991; Shiba *et al*., 1991). Lantibiotics may thus have potential for treating high blood pressure (Kido *et al*., 1983).

Potential applications of bacteriocins in infections of the urogenital tract

High numbers of *Lactobacillus* spp. form part of the normal bacterial flora in the vagina and ensure a reduced risk of bacterial vaginosis and urinary tract infections (Nomoto, 2005). Although bacteriocin production by lactobacilli is one of their major protective mechanisms, these antimicrobial peptides are not typically active against yeasts. As such, this review is focused only on those microorganisms sensitive to the antimicrobials in question, with bacterial vaginosis (BV) as the primary focus. However, it should be noted that when bacteriocins are combined with other natural antimicrobials, there may be increased activity against pathogenic fungi in the vaginal ecosystem.

In the past, antimicrobially-active lactobacilli were commonly used to develop products for the prevention and treatment of genital infections (Barbes and Boris, 1999; Famularo *et al.,* 2001; Maggi *et al.,* 2000). The most consumer-accepted product is based on an application of lactobacilli that produce H_2O_2 (McLean and Rosenstein, 2002; Ocaña *et al.,* 1999). Based on the health-promoting action of healthy vaginal *Lactobacillus crispatus*, The Medicines Company (Cambridge, MA) and University of Pittsburgh (PA) initiated an NIH-sponsored study of the *L. crispatus* strain CTV-05 as an adjunct to standard antibiotic treatment of bacterial vaginosis. It was hypothesized that *L. crispatus* could replace vaginal pathogens and promote the re-establishment of normal vaginal microflora due to high level production of hydrogen peroxide, which is an effective antimicrobial agent. However, strain CTV-05 did not improve clinical cure rates at 30 days, the primary endpoint of the trial. This failure was not an accidental one. To succeed in a strain replacement therapy approach as described above, the healthy microorganism has to i) migrate to the surface of the epithelial cells; ii) adhere onto the epithelial cells; iii) successfully colonize the epithelial cells and develop a functional biofilm, and finally, having survived all these initial phases, iv) start producing antimicrobials such as hydrogen peroxide, bacteriocins, lactic acid etc. that will force the invading pathogens away from the ecological niche conquered back by the healthy microorganism. Failure in any of the major outlined phases (and other undescribed circumstances) will lead to failure in the replacement of non-desired pathogens by healthy bacteria. Recently published data showed that a daily oral and vaginal intake of *L. rhamnosus* GG (1010 cells per dose, Culturelle, USA) was unsuccessful in reaching the

goal of colonizing the vagina and correcting the disturbed vaginal ecology (Colodner *et al.,* 2003; Devillard *et al.,* 2005).

Lactobacilli inhibited the growth and attachment of uropathogenic *E. coli in vitro* (McGroarty and Reid, 1988; Reid *et al*., 1987; Velraeds *et al*., 1998). *L. rhamnosus* GR-1, *L. fermentum* B-54 and *Lactobacillus* strains with high adhesion and hydrogen peroxide production were weekly administered as a vaginal suppository (Reid and Bruce, 2001; Reid *et al*., 1987; 1995). Urinary tract infection was significantly decreased. Orally administered *L. rhamnosus* GR-1 and *L. fermentum* RC-14 decreased *E. coli* and fungi in the vagina. Future studies should include the inhibition of STBs by probiotics taken orally or as vaginal suppositories (Reid *et al*., 2003; Sewankambo *et al*., 1997). A proposed mechanism is the induction of an immune response via the urethra or vagina which is functional in the bladder (Reid and Burton, 2002). Probiotic colonization in the vagina might prevent infection by competition for nutrients and mannose and hydrophobic adhesion to receptors (Braun, 1999; Masuoka and Hazen, 1999) and possibly bacteriocin production with fungistatic activity (Okkers *et al*., 1999).

An overall low vaginal pH is essential for the prevention of vaginal infections. Intravaginal products such as Acidgel, BufferGel, etc. are based on the acid-producing ability of the lactobacilli, which help maintain a vaginal pH lower than 4.5 (Amaral *et al.,* 1999; Andersch *et al.,* 1990; Garg *et al.,* 2001). However, low vaginal pH alone is not sufficient to inhibit vaginal pathogens and to prevent infection.

Bacterial vaginosis (BV), a common condition found in up to 30% of women in North America (Schwebke, 2003), is typically associated with a multi-species infection, where *Gardnerella vaginalis* is often the major contributor to BV development. In addition to *G. vaginalis, Prevotella bivia* and *Peptostreptococcus* spp. contribute significantly to the development of BV (Dover *et al*., 2008; Nikolaithcouk *et al.*, 2008), which is associated with a relatively high pH, a decrease in antimicrobial activity of the vaginal fluid compared to healthy women, and local impairment of the multiple innate immune pathways (Pybus and Onderdonk, 1999; Valore *et al.,* 2006).

Almost one-third of childbearing age women in the world are diagnosed with BV (Schwebke, 1997). The harmful effects of BV range from complications with pregnancies to the development of pelvic inflammatory diseases (Hillier *et al*., 1995; Ness *et al*. 2005). Studies have also associated BV with a higher risk of acquisition of HIV infection (Cohen *et al.,* 1995; Martin *et al.,* 1999; Sha *et al.,* 2005; Taha *et al.,* 1998) and herpes simplex virus type 2 infections (Cherpes *et al.,* 2003). It is estimated that nearly 60% of HIV+ women have concurrent BV infections (Mascellino *et al.,* 1991), a statistic made especially disturbing by the fact that BV directly causes an increase in the rate of HIV replication and disease progression (Al-Harthi *et al*., 1999; Hashemi *et al.,* 2000). Toxins from BV-associated microorganisms (such as lipopolysaccharides) may also cross the placenta and cause brain injuries in fetuses. These toxins may cause permanent neurological brain damages such as cerebral palsy, a risk of developing Parkinson's

disease and schizophrenia (Grether and Nelson, 2000; Ling *et al*., 2004; Urakubo *et al.,* 2001).

While 60% of BV cases can be successfully treated with metronidazole and clindamycin, as recommended by the Centers for Disease Control and Prevention (Paavonen *et al.,* 2000; Sobel *et al.,* 2001), about 20% of these cases return with highly-developed antibiotic resistance (Boris *et al.,* 1997; Bannatyne and Smith, 1998; Ferris *et al.,* 1995; Liebetrau *et al*., 2003; Lubbe *et al*., 1999). The risk of developing antimicrobial drug resistance increases dramatically with overall increased use of antimicrobial (feminine hygiene and treatment) preparations (Uehara *et al.,* 2006). In addition, *in vitro* studies showed that clindamycin and metronidazole inhibit healthy vaginal *Lactobacillus* spp. at concentrations lower than doses topically applied for treatment (Aroutcheva *et al.,* 2001; Simoes *et al.,* 2001). Therefore, there is an interest in developing alternative treatments against BV, such as selective antimicrobials that will inhibit BV-associated bacteria without killing healthy *Lactobacillus* spp.

One promising alternative is the bacteriocin subtilosin A. Originally isolated from the wild-type *B. subtilis* 168 by Babasaki *et al.* (1985), subtilosin was recently found to be concurrently produced by *B. amyloliquefaciens*, a similar but divergent *Bacillus* species isolated from a fermented dairy beverage (Sutyak *et al*., 2008). It is a circular molecule of 35 amino acids, with the distinctive post-translational modification of three sulfur cross-links between cysteine and the α-carbon of two phenylalanines and one threonine

residue. This structure is unique among bacteriocins, suggesting it may belong in a new, undefined class of antimicrobial peptides (Kawulka *et al.* 2004).

In contrast to many bacteriocins, which have an overall positive charge at physiological pH, subtilosin A is anionic, having only one lysine and a total of three aspartate and glutamate residues (Kawulka *et al.,* 2004; Thennarasu *et al.,* 2005). Because bacterial membranes also have a net anionic charge, it has been hypothesized that subtilosin may not interact solely with the cell membrane, but may first bind a surface receptor prior to insertion into target membranes (Thennarasu *et al.,* 2005). It has been shown that at high concentrations (much greater than its MIC values), subtilosin A interacts with the lipid head group region of bilayer membranes of target cells, causing membrane perturbation, the extent of which is dependent on lipid composition (Thennarasu *et al.,* 2005). At these high concentrations, subtilosin aggregates into multimeric units, therefore its primary mode of action may be by interaction with a membrane component or receptor (Thennarasu *et al.,* 2005).

Subtilosin has proven antimicrobial activity against a wide variety of human pathogens, including *L. monocytogenes*, *G. vaginalis*, *S. agalactiae*, and *Micrococcus luteus* (Sutyak *et al.,* 2008). Its activity against *G. vaginalis*, combined with its lack of effect on probiotic vaginal *Lactobacillus* isolates (Sutyak *et al*., 2008), indicate that subtilosin could target the vaginal pathogen while leaving the healthy vaginal microflora intact. Cytotoxicity testing conducted *in vivo* using the EpiVaginal (VEC-100) human ectocervical tissue

model (MatTek, Ashland, MA) demonstrated that subtilosin caused only a 5% decrease in cell viability after 24 h of continuous exposure (20% after 48 h) (Sutyak *et al*., 2008b). Its safety for human tissue as well as the probiotic microflora of the specific ecological niche gives subtilosin great potential as a future inclusion in personal care products.

Spermicidal activity and potential contraceptive usage of bacteriocins

Some of the bacteriocins that are active against vaginal pathogens are also reported as having spermicidal activity. This feature makes them attractive for formulation in feminine health care and contraceptive products.

In order to evaluate nisin's spermicidal activity, Aranha *et al.* (2004) developed a contraceptive model in rats. Nisin, dissolved in saline, was administered into the vagina of the animals for 14 consecutive days during the proestrus–estrous transition phase. Animals were then immediately allowed to mate and none of the nisin-treated animals became pregnant. No histopathological lesions were observed in the vaginal epithelium and liver and kidney function remained normal. Fertility was also restored after experiments. According to the authors, 1 mg of nisin was able to completely halter sperm motility. This is an interesting finding, since many commonly used contraceptive products contain Nonoxynol-9 (N-9), a compound harmful to epithelium.

Subtilosin, the previously described bacteriocin produced by *B. amyloliquefaciens*, was also shown to have potent spermicidal activity. When tested against human

spermatozoa, it was able to decrease motility in a dose-dependent manner and has an IC_{50} value of 64.5 µg/mL (Sutyak *et al*., 2008b). Interestingly, subtilosin also has demonstrated significant spermicidal activity when tested on various animals, including boar, horse/pony, rat and bovine models (Silkin *et al.*, 2008). Nisin inhibited sperm motility and caused no abnormalities when applied intravaginally in rats (Aranha, 2004). Intravaginal application of nisin also prevented conception in rabbits and showed no inflammation or damage to the vaginal epithelium when applied for 2 weeks (Reddy *et al*., 2004). However, if the concentrations of nisin used in the animal model are extrapolated for human usage, they are well above the limits of what the healthy vaginal microflora can survive. Thus, nisin cannot be practically considered for use in human products.

Recombinant antimicrobial peptides

Because of their low resistance to plasma and serum proteolytic activity (Bracci *et al*., 2003; Pini *et al*., 2005), cationic peptides show high *in vitro* activity and limited *in vivo* activity. It is thus necessary to resort to different strategies to increase peptide stability for therapeutic application. Multimeric peptides have a remarkably increased half-life *in vivo* and enhanced antimicrobial activity with respect to linear homologues (Pini *et al*., 2005; Tam *et al*., 2005). This topic is covered by another chapter in the book.

Bacteriocins, immunity, and resistance: Issues and concerns

Genes involved in the biosynthesis of bacteriocins are organized in a cluster on the genome, plasmid or on a transposon (McAuliffe *et al*., 2001; Nes *et al*., 1996; Van Reenen *et al*., 1998). In some instances the immunity gene is located in the same operon as the structural gene that codes for the bacteriocin (Gasson, 1984). However, in some cases immunity genes are located on plasmids or transposons and thus not linked to bacteriocin production (Froseth *et al*., 1988; Klaenhammer and Sanozky, 1985; McKay and Balwin, 1948). Mobile genetic elements can, however, be exchanged with other organisms of the same or different species, rendering sensitive strains insensitive to some bacteriocins.

Resistance in spontaneous mutants can be ascribed to changes in the membrane charge and fluidity, cell wall thickness, cell wall charge as well as combinations of the aforementioned changes (Abachin *et al*., 2002, Bierbaum, 1987; Crandall and Montville, 1998; Li *et al*., 2002; Maisnier-Patin and Richard, 1996; Mantovani and Rusel, 2001; Vadyvaloo *et al*., 2002, 2004; Verheul *et al*., 1997). Spontaneous nisin resistance frequency in *L. monocytogenes* varied from 10^{-2} to 10^{-7} in a strain dependent manner (Gravesen, 2002, Davies and Adams, 1994) and *L. monocytogenes* mutants were detected at a frequency of 10^{6}-10^{8} that were resistant to 50 g/ml nisin (Harris *et al*., 1991). Nisin-resistant *S. pneumoniae* has also been found when this organism is exposed to nisin over long periods of time, with the minimum inhibitory concentration (MIC) increasing from 0.4 mg/ml to 6.4 mg/ml (Severina *et al*., 1998). Spontaneous mutants of *P. acidilactici* resistant towards pediocin AcH are lost when grown in the

absence of the bacteriocin. Pediocin-resistant mutants had a reduction of up to 44% of the maximum specific growth rate as compared to the wild-type strain (Gravesen, 2002). Nisin resistance has also been reported in *C. botulinum* spores and vegetative cells (Mazzotta, 1997).

Most bacteriocin resistance studies have been carried out by using resistant *L. monocytogenes* strains. Resistance of *L. monocytogenes* towards nisin can be ascribed to changes in fatty acid and phospholipid composition, a lower ratio of C-15 to C-17 fatty acids, more zwitterionic phosphatidylethanolamine, less anionic phosphatidylglycerol and cardiolipin and a requirement for divalent cations (Crandall and Montville, 1998; Mazzotta, 1997; Ming and Daeschel, 1993, 1995; Verheul *et al*. 1997). An enzyme, nisinase, can also confer resistance to nisin. Nisinase activity, which inactivates nisin, has been reported in several *Bacillus* spp. (Jarvis 1967).

L. monocytogenes resistance towards class II bacteriocins correlates with a reduction in the expression of a mannose permease of the phosphotransferase system (man-PTS) (Cotter *et al*., 2005). Because class II bacteriocin-resistant mutants display this reduction in the expression of man-PTS, it was speculated that the man-PTS serves as a target site for some class II bacteriocins (Ramnath *et al*., 2000). This was confirmed by Diep *et al.* (2007), who reported that class II bacteriocins use the IIC and IID components of the man-PTS as receptors. They also reported that the immunity proteins form a complex with the receptor proteins, thus rendering the producer strain resistant to its

own bacteriocin. Grasesen *et al.* (2002) reported that *L. monocytogenes* resistance towards class II bacteriocins is characterized by the upregulation of EII^{Bgl} and phospho-β-glucosidase, as well as the prevention of EII_t^{Man} synthesis. Inhibited *mpt* expression confers the resisitance, while upregulated EII^{Bgl} and phospho-β-glucosidase expression are merely a result of inhibited *mpt* expression.

Cross-resistance has been reported between different bacteriocins. Nisin resistance conferred cross-resistance to pediocin PA-1 and leuconocin S in *L. monocytogenes* (Crandall and Montville, 1998). Leucocin A-resistant strains of *L. monocytogenes* showed no significant cross-resistance towards other bacteriocins, including nisaplin and ESF1-7GR, although they were shown to be resistant to pediocin PA-2 (51, 200 AU/ml) (Ramnath *et al*., 2000). Studies have demonstrated that resistance to bacteriocins is still relatively weak, because low levels of resistant strains are being isolated and in some cases bacteriocin resistance is unstable. Resistance can easily be lost if the strains are cultured without the bacteriocins (Breuer and Radler 1996; Dykes and Hastings, 1998; Ming and Daeschel, 1993; Rasch and Knøchel 1998; Rekhif *et al*. 1994; Song and Richard 1997). Resistant mutants have also been shown to have a lower growth rate than wild-type strains and were unable to outgrow them (Gravesen, 2002; Dykes and Hastings, 1998; Maisnier-Patin *et al*., 1995). This indicates that a resistance mechanism towards bacteriocins has a negative influence on the strain and when grown without bacteriocin this organism is outcompeted by other organisms (Dykes, 1995; Noerlis and Ray, 1994). No resistance has been reported to lacticin 3147 in sensitive gram-positive organisms

(Ross *et al*., 1999), which is especially desirable with the increase in antibiotic resistance seen in numerous human pathogens.

Although as yet there are no reports in the literature on the acquisition of bacteriocin immunity gene(s) by pathogenic bacteria, there are examples where these genes have been cloned and expressed by Venema *et al.* (1995) and other groups (Fimland *et al.,* 2002), in a bacteriocin-sensitive host resulting in resistance to the antimicrobial protein by the bacteria. Also, a number of examples of the production of identical bacteriocins (and their cognate immunity proteins) by bacteria of different species (Ennahar *et al.,* 1999; Sutyak *et al.,* 2008) raises additional concern regarding the emergence of bacteriocin-resistance via interspecies gene transfer. In addition, it was reported for *Streptococcus mutans* that the microorganism's increased sensitivity to antimicrobials such as tetracycline, penicillin, and triclosan is triggered by the repression of the bacteriocin immunity gene (Matsumoto-Nakano and Kuramitsu, 2006). All of these render the elucidation of the mechanism of immunity even more important, since it will contribute to our general understanding of how antimicrobial-resistance may be prevented. Furthermore, strains of *Enterococcus faecium* and *Bacillus licheniformis* were found to carry gene homologues which provide protection against a bacteriocin. This phenomenon was named "resistance through immune mimicry" (Draper *et al.,* 2009).

Almost no or very low levels of cross-resistance between bacteriocins and antibiotics have been found (Bower, 2001; Mantovani and Russel, 2001). Bacteriocins and antibiotics have different modes of action. Antibiotics can inhibit cell wall synthesis, protein synthesis, DNA synthesis, RNA synthesis and can cause the competitive inhibition of folic acid synthesis, while bacteriocins forms pores in the membrane of sensitive cells, leading to leakage of intracellular material and cell death (Ennahar *et al*., 1999; Kraaij, 1999; Levy and Marshall, 2004; Nes and Holo, 2000; Neu, 1992; Sahl and Bierbaum, 1998). Bacteriocins and antibiotics can potentially be used together to prevent or hamper the emergence of resistant pathogens, because they have different modes of action and acquiring resistance towards two different antimicrobials is very unlikely (Diep *et al*., 2007)

Intelligent delivery systems for antimicrobial peptides

Bacteriocins show the potential to have various applications in the biomedical industry. However, the stability of bacteriocins can become a problem in a complex *in vivo* environment. Polymeric delivery systems can help overcome the stability problems of peptides in an *in vivo* system and can release the bacteriocins in a bioactive form to a specific site of interest. A variety of biodegradable biomedical polymers are available that can be used as delivery systems. Poly-lactic acid is one such polymer that has received FDA approval for use in humans (Nair and Laurencin, 2007). Most studies aimed at the delivery of bacteriocins have dealt with antimicrobial packaging films or materials aimed at food preservation, and very few studies have been conducted to

generate delivery systems for biomedical applications of bacteriocins (Cutter *et al*., 2001, Malheiros 2010; Marcos *et al*., 2007; Scannell *et al*., 2000). For the most recent comprehensive review see Balasubramanian et al. (2009).

Nisin has been encapsulated into poly(L-lactide) (PLA) nanoparticles by semicontinuous compressed CO_2 anti-solvent precipitation (Salmaso *et al*., 2004). Nisin was released in the active form for up to 1000 h and release was dependent on the salt concentration and the pH of the release medium. Nisin released from the PLA nanoparticles was able to inhibit the growth of *Lactobacillus delbrueckeii* subsp. *bulgaricus* when nisin-loaded PLA nanoparticles were incubacted in MRS containing the sensitive strain. These nanoparticles could potentially be used in antimicrobial pharmaceutical products.

Encapsulation of bacteriocins in electrospun nanofibers was recently reported (Heunis *et al*., 2010). Electrospinning is the process where a high voltage is applied to a polymer solution, which forms a Taylor cone when charged (Taylor, 1969; Yarin *et al*., 2001). When the electric forces overcome the surface tension of the solution, a charged polymer jet is ejected from the Taylor cone and will start to accelerate towards the collector. The solvent will evaporate during this process and very thin fibers are formed (Agarwal *et al*., 2008, Liang, 2007). Plantaricin 423, produced by *Lactobacillus plantarum* 423, was electrospun into polyethylene oxide (PEO) nanofibers. A slight decrease in bacteriocin activity was seen in the fibers, however enough activity was retained to inhibit the growth of *Lactobacillus sakei* DSM 20017 and *Enterococcus faecium* HKLHS.

The activity decreased from 51 200 AU/ml to 25 600 AU/ml and from 204 800 AU/ml to 51 200 AU/ml after electrospinning, as determined against *L. sakei* DSM 20017 and *E. faecium* HKLHS, respectively. Thus, nanofibers could also be used in various biomedical applications.

Drug delivery systems will play an integral role for the use of bacteriocins in the biomedical industry. These systems will help to protect and keep the peptides active and will release them in a controlled manner to exert activity. Drug delivery systems will have added potential with regards to the localized release of bacteriocins, which would be desired if the bacteriocins are to be used as a topical treatment for skin infections or as coatings for biomedical devices to combat device-related infections. More studies need to be conducted on the controlled release of bacteriocins from drug delivery systems. These studies will help to not only increase the already huge potential biomedical applications of bacteriocins, but will bring bacteriocins into a new era of biomedicine.

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FIGURES AND TABLES

Table 1. Classification of bacteriocins (Cotter 2005)

Classes	Characteristics
Class I	**Lantibiotics**
Class Ia	Small (19-38 amino acids), elongated, positively charged peptides that form pores
Class Ib	Globular peptides that interfere with essential enzymes
Class II	**Non-lanthionine containing bacteriocins**
Class IIa	Pediocin-like peptides that contain the YGNGVXCXXXXVXV consensus sequence in their N-terminal
Class IIb	Two-peptide bacteriocins, require both peptides for activity
Class IIc	Cyclic peptides, N- and C-terminal are covalently linked
Class IId	Single non-pediocin like peptides

Chapter 2: Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens.*[2]

This chapter details the initial discovery, purification, and characterization of subtilosin. Our laboratory centers on the belief that many bacteria, especially those found within yogurts and other fermented foods, have beneficial effects on human health. One such product, the fermented beverage Yogu Farm, was examined for the presence of *Lactobacilli* based on the product advertising. No such organisms were found, and the only culture able to be isolated was an unknown later identified as *Bacillus amyloliquefaciens*. This organism was eventually found to produce an antimicrobial compound with remarkable activity against a variety of pathogens. Through the processes detailed within this chapter, the substance was later identified as subtilosin, the first bacteriocin identified from *B. amyloliquefaciens*.

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Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*

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ABSTRACT

Aims: To purify and characterize the *Bacillus subtilis*-produced antimicrobial protein (bacteriocin) subtilosin from the dairy product-derived *Bacillus amyloliquefaciens* in a potential case of horizontal gene transfer (HGT).

Methods and Results: An unknown bacterial species cultured from the Yogu Farm™ probiotic dairy beverage was identified through 16S rRNA analysis as *Bacillus amyloliquefaciens*, a phylogenetically close relative of *B. subtilis*. The cell-free supernatant (CFS) of overnight cultures was active against *Listeria monocytogenes* and also against clinical isolates of *Gardnerella vaginalis* and *Streptococcus agalactiae*. At the same time, several isolates of vaginal probiotic *Lactobacilli* were resistant to the CFS. The nature of the compound causing inhibitory activity was confirmed as proteinaceous by enzymatic digestion. The protein was isolated using ammonium sulfate precipitation, and further purified via column chromatography. PCR analysis was conducted to determine relatedness to other bacteriocins produced by *Bacillus* spp.

Conclusion: The antimicrobial protein isolated from *B. amyloliquefaciens* was shown to be subtilosin, a bacteriocin previously reported as produced only by *B. subtilis*.

Significance and Impact: This is the first report of intra-species horizontal gene transfer for subtilosin and the first fully-characterized bacteriocin isolated from *B. amyloliquefaciens*. Finally, this is the first report on subtilosin's activity against bacterial vaginosis-associated pathogens.

Keywords: *Bacillus amyloliquefaciens*, *Bacillus subtilis*, subtilosin, bacteriocin, antimicrobial

INTRODUCTION

Bacteriocins are ribosomally-synthesized proteins produced by a diverse group of microorganisms that elicit bactericidal activity, usually against closely-related species (Drider *et al*. 2006, Guinane *et al*. 2005, Klaenhammer 1993, Nagao *et al.* 2005). *Bacillus subtilis* is a known producer of many antibiotic and antimicrobial compounds, including the bacteriocin subtilosin A (subtilosin) (Stein 2005). Subtilosin A was originally isolated by Babasaki *et al*. (1985) from the wild strain *B. subtilis* 168, and an early, incomplete amino acid sequence was reported. A complete amino acid sequence was later published by Zheng *et al*. (1999), and further elucidation was provided by Marx *et al*. (2001) using ^{1}H-NMR to produce a 3-D image of the molecule's structure. It was determined to be a circular molecule of 35 amino acids with a very unique posttranslational structure, namely three sulfur cross-links between cysteine and the α-carbon of two phenylalanines and a threonine residue. Since this configuration is

unparalleled in other antimicrobial peptides, it is speculated that subtilosin may belong to a unique class of bacteriocins (Kawulka *et al*. 2004).

Horizontal gene transfer (HGT) is a mechanism employed by bacteria as a means of acquiring new genetic properties. Though it was once difficult to establish instances of HGT, genetic analysis now provides unmistakable supportive evidence. The evolutionary modification of traits is typically a slow and lengthy process defined by point mutations that inactivate or activate new regions of genes. In comparison, HGT can rapidly change whole features of a species for generations to come. In order to occur, bacteria must possess a means of acquiring the new information from a neighboring species, *e.g.* competency (Ochman *et al*. 2000). In both *B. subtilis* and *Streptococcus mutans* competency has been linked to bacteriocin production, suggesting that this mechanism may be prevalent among organisms in multispecies environments (Hamoen *et al.* 2005, D'Souza *et al.* 2005, Kreth *et al.* 2005).

There have been several documented cases of HGT involving bacteriocins, specifically those involving the well-characterized and utilized class I bacteriocin nisin. Primarily produced by *Lactococcus lactis* subsp. *Lactis* (Klaenhammer 1993), genes encoding for the production of and resistance to this small protein have also been isolated from several other *L. lactis* subspecies (Gireesh *et al*. 1992), as well as *Leuconostoc dextranicum* (Tsai and Sandine 1987). Muriana and Klaenhammer (1992) also reported on the conjugal transfer of bacteriocin production determinants in *Lactobacillus acidophilus* 88. While many strains of *B. subtilis* have been identified as subtilosin producers (Stein *et al*. 2004), there are no documented cases of the presence

of its structural and functional genes, or reported production of it, in another species. Here, we describe the production, purification, antimicrobial activity and genetic identification of subtilosin from a *Bacillus amyloliquefaciens* culture.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and culture media

B. amyloliquefaciens was isolated from the yogurt-flavored cultured beverage Yogu Farm™ (JSL Foods, Los Angeles, CA) purchased from Hong Kong Market, New Brunswick, NJ, by aliquoting 1 ml of the product into 20 ml of MRS broth (Difco^{TM}, Detroit, MI). The culture was incubated for 48 hours at 37°C in 5% CO_2 atmosphere without agitation. Inoculated plates were also incubated in the same conditions. Samples of the liquid culture were examined with phase microscopy to visualize basic cell characteristics. Culture samples were sent to the Laboratory for Molecular Genetics (Cornell University, Ithaca, NY) for ribotyping and to Accugenix (Newark, DE) for 16S ribosomal RNA (rRNA) analysis to confirm the identity of the unknown organism. *Micrococcus luteus* ATCC 10420, *Listeria monocytogenes* Scott A and *Salmonella* Typhimurium ATCC 14028-1s were grown in Tryptic Soy Broth supplemented with 0.6% Yeast Extract (Difco^{TM}) at 30°C under aerobic conditions. *Pediococcus pentosaceus* ATCC 43200 was cultivated in MRS broth at 37°C for 24 hours under aerobic conditions. *Gardnerella vaginalis* ATCC 14018 was grown on HBT agar (BD, Franklin Lakes, NJ), while *Streptococcus agalactiae* (Group B Streptococcus) was grown on Columbia agar with 5% Sheep Blood (BD). Both organisms were incubated at 36°C in 5% CO_2 atmosphere without agitation. The

indicator strains used in well diffusion assays were obtained from either ATCC collections or as clinical isolates from the Rush Presbyterian Medical Center in Chicago, IL (Table 1).

Sample preparation

Cell-free supernatant (CFS) harvested from MRS broths was incubated for 48 hours at 37°C in 5% CO_2 atmosphere (until approximately 10^6 CFU ml^{-1}). Cells were removed from the culture by centrifugation (Hermle Z400K, LabNet, Woodbridge, NJ) for 25 min at 4500 x *g* and 4°C. Supernatants were filter-sterilized using 0.45 µm microfilters (Fisher, Pittsburgh, PA).

Assay of antimicrobial activity

Well diffusion inhibition assays were conducted as described by Cintas *et al*. (1995) with the following modifications. The efficacy of the *B. amyloliquefaciens* product at inhibiting the growth of various microorganisms was tested using CFS against MRS broth as a negative control and nisin (10 mg ml^{-1}) (Sigma, St. Louis, MO, 2.5% bacteriocin preparation [10^6 IU g^{-1}] dissolved in ddH_2O) as a positive control. The indicator organism was grown overnight according to its specific growth requirements, with *M. luteus* used as a standard based on its known sensitivity to bacteriocins (Pongtharangkul and Demirci, 2004) . Soft agar was made by adding 0.7% agar to either TGY or MRS; solid base plates were dried in a sterile hood for approximately 90 min prior to use in order to remove any extraneous moisture. To create an overlay, the indicator organism was

added to the soft agar in a ratio of 100 µl bacterial culture per 10 ml soft agar (*ca.* 10^6 CFU ml^{-1}). From this mixture, 4 ml was overlaid onto each base plate and allowed to completely solidify. Pasteur pipettes were used to create 5 mm wells in the overlaid base plates. These wells were then allowed to dry for approximately 30 min. Then, 50 µl of each sample was added to the wells and allowed to freely diffuse for 45-60 min. All plates were then incubated overnight at the optimal growing conditions for the indicator organism (Table 1). The procedure for testing activity against the clinical isolates varied slightly from the previously described method. The indicator organism was inoculated as a lawn using a sterile swab, and after air-drying for 5 min, 17-mm wells were punched into the agar using a sterile glass test tube, and 400 µl of CFS was added. The plates were kept at room temperature for 2 h to allow for absorption of the supernatant, and then incubated overnight at 36°C with 5% CO_2 atmosphere.

Determination of lactic acid concentrations

Lactic acid concentrations in the CFS were determined using a D-Lactic acid/L-Lactic acid test kit and according to the manufacturer's protocol (Roche Boehringer, Mannheim, Germany). After completing the steps of the protocol, the gathered data was applied to the provided equations in order to accurately calculate the quantities of each acid form in the sample.

Enzymatic digestion to confirm proteinaceous nature of antimicrobial compound

The CFS was exposed to seven different enzymes (Sigma; Table 2) overnight to determine the type of compound causing bacterial growth inhibition. Aliquots (250 µl)

of the CFS were combined with equal volumes of the enzymes and the pH of the mixture and the incubation temperature were adjusted to those optimal for enzymatic activity. Two controls were used: (i) the enzyme mixed with sterile MRS media, and (ii) the CFS and enzyme diluent (Table 2). After 24 h the pH of all samples was readjusted to ~6 to attain maximum antimicrobial activity. Well diffusion assays were conducted in triplicate against the indicator *Micrococcus luteus.*

Protein visualization

SDS-PAGE was conducted using a Tris-Tricine gel made in a Bio-Rad casting apparatus (Bio-Rad, Hercules, CA) according to the protocol given by Schägger and Von Jagow (1987). The gels were loaded with either 20 µl of marker or 200 µl of sample [1:1 sample + loading buffer (Bio-Rad)]. Nisin (10^6 IU g^{-1}, 2 mg ml^{-1}) was used as the positive control. The procedure was conducted in 0.2 mol l^{-1} Tris-base anode running buffer (pH=8.9) and 0.1 mol l^{-1} Tris/0.1 mol l^{-1} Tricine/0.1% SDS cathode running buffer (pH=8.25) in a Mini-Protean 3 (Bio-Rad) chamber with Power-Pac 300 power source (Bio-Rad).

Upon completion of electrophoresis, the gel was cut into identical halves; one half was treated for the overlay process while the other was used in the staining procedure. The overlay gel was fixed for 2 h in 100 ml of 10% acetic acid/20% isopropanol buffer, rinsed 3 times over 2 h in 100 ml ddH_2O, and stored overnight in ddH_2O at 4°C (all steps occurred under rotation). The following day, it was laid onto a dried enriched TSA plate and overlaid with *M. luteus*. The staining gel was processed according to the manufacturer's Silver Stain protocol (Bio-Rad).

Protein purification

Ammonium sulfate precipitation. Using a stock overnight culture, *B. amyloliquefaciens* was inoculated (*ca.* 10^6 CFU ml^{-1}) and grown in 500 ml MRS under normal conditions. Cells were removed by centrifugation for 25 min at 12120 x *g*. The CFS was filter-sterilized as previously described. A nomogram (Dixon 1953) was used to calculate the amount of solid ammonium sulfate needed to achieve 30% saturation, which was added to the solution incubated at 4°C overnight while stirring. The following day, the precipitate was gathered by centrifugation as described above and re-dissolved in 20 ml ddH_2O. Activity of both the precipitate and the supernatant were tested in a well diffusion assay against *M. luteus*. The precipitate was used to conduct all further experiments and is designated as the "sample".

Column chromatography. Further purification of the 30% ammonium sulfate precipitate was achieved with Sep-Pak® Light C18 Cartridges (Waters, Milford, MA) to separate the protein of interest based on an assumed hydrophobic nature. In each instance, 0.5 ml of liquid was passed through the column at a flow rate of 0.2 ml min^{-1}. The cartridge was initially rinsed with 0.5 ml 100% methanol and equilibrated by four 0.5 ml washes with ddH_2O to remove any traces of the methanol. Following the water washes, the sample was loaded onto the cartridge and the flow-through was collected. This was followed by another four 0.5 ml washes with ddH_2O, with each fraction collected individually. Immediately after the water washes, the column was washed sequentially with 1 ml of

50%, 70%, 90% and 100% methanol, and individual 0.5 ml fractions were collected. Antimicrobial activity was confirmed by the well diffusion assay.

Effect of temperature and pH on antimicrobial activity

Heat shock. The ability of the compound to retain activity under elevated temperatures was tested by incubating the sample at a given temperature for 0-60 min. After each time point 200 µl was aliquoted and used to create 2-fold serial dilutions in ddH_2O. Each dilution was used in a well diffusion assay; the reciprocal value of the lowest dilution that maintained activity is considered the protein concentration in arbitrary units (AU) ml^{-1}.

pH stress. The level of antimicrobial activity of the sample was tested at varying pH levels. The pH of the solution was adjusted to fall within the range of 2-10 using either 3 mol l^{-1} HCl or NaOH. The samples were incubated at room temperature for 1 min before conducting a well diffusion assay against *M. luteus*.

Genetic analysis

DNA extraction. DNA was extracted from overnight cultures of *B. amyloliquefaciens* and *B. subtilis* ATCC 6633 using the Promega Wizard SV Genomic DNA Kit (Promega Corp, Madison, WI) with the following modifications. Cells were harvested from the culture (2 x 1.5 ml) in a microfuge tube at 13,000 *×g* for 3 min and resuspended in 382 µl 0.5 mol l^{-1} EDTA (pH 8.0). To this, 100 µl of lysozyme (20 mg ml^{-1}), 10 µl proteinase K (20 mg ml^{-1})

and 8 µl mutanolysin (2.5 U μl^{-1}) was added. The mixture was incubated for 60 min at 37°C, following which 200 µl of nuclei lysis solution and 5 µl RNase A were added, and incubated for 20 min at 65°C. Two hundred-fifty µl of lysis buffer was immediately added, and DNA was subsequently purified using the provided spin columns according to the manufacturer's specifications and eluted in 100 µl nuclease-free water.

PCR testing. Polymerase chain reactions (PCRs) were performed to assess the relatedness between the bacteriocin produced by *B. amyloliquefaciens* and the *B. subtilis* products subtilin and subtilosin. Primers (listed in Table 3) were designed using the *B. subtilis* genome (GenBank Accession #AJ430547) to specifically recognize the functional genes of subtilin (*spaS*) and subtilosin (*sboA*). Genomic DNA from *B. amyloliquefaciens* and *B. subtilis* ATCC 6633 was added to a master mix consisting of each primer, nucleotides, buffer and HotMaster Taq (Eppendorf, Hamburg, Germany). PCR was conducted using an Applied Biosystems GeneAmp PCR System 2400 apparatus (Applied Biosystems, Foster City, CA) under the following parameters: denaturation for 30 s at 94°C, annealing for 30 s at 55°C (*spaS*) or 50°C (*sboA*), and elongation for 1 min at 65°C for a total of 30 cycles. PCR products were sequenced using ABI Prism 3730*xl* DNA analyzers (GeneWiz, Inc., South Plainfield, NJ), and the resulting sequences were analyzed using the Vector NTI software suite of programs (Invitrogen, Carlsbad, CA). The sequence obtained for *B. amyloliquefaciens* has been submitted to GenBank under the accession no. EU105395.

RESULTS

Characterization of unknown isolate

While the Yogu Farm™ beverage was purported to contain Lactobacillus cultures, *B. amyloliquefaciens* was the only organism recovered from four individual lots of the product. Phase microscopy of each sample of bacterial growth revealed a single organism that was a very motile endospore-producing bacillus. On solid agar, the colonies tended to spread quickly into lawn formation, with an extremely wrinkled texture. The organism appeared to secrete a thick, opaque slime from the colonies, which was later revealed to be a byproduct of the starch-hydrolyzing enzyme amylase. Ribotyping and 16S rRNA analyses determined the bacterium to be *Bacillus amyloliquefaciens*, a closely related species to *Bacillus subtilis*.

Range of antimicrobial activity

The CFS of a *B. amyloliquefaciens* culture was determined to have antimicrobial activity against a wide range of bacterial species, including the pathogens *L. monocytogenes, G. vaginalis* and *S. agalactiae*. There was no activity against several strains of vaginal probiotic *Lactobacilli* also gathered from the clinical setting (Table 1).

Determination of lactic acid concentrations

Using equations provided by the manufacturer's protocol, it was determined that *B. amyloliquefaciens* produced very low levels of both D- and L-lactic acid in three separately conducted assays. Calculations revealed that there was an average of 0.17 g l^{-}

[1] D-lactic acid per sample, a value equal to that of the tested blank. The average concentration of L-lactic acid rose to 2.22 g l^{-1}, which was slightly higher than the blank's concentration of 0.15 g l^{-1} (Table 4). The very low basal concentrations of both forms of lactic acid suggest they do not play a significant role in microbial inhibition, and that all detected activity may be attributed to the bacteriocin.

Effect of enzyme digestion, temperature and pH on antimicrobial activity

Inhibition assays revealed that activity was completely lost in the presence of pepsin and proteinase K, and significantly decreased by trypsin and chymotrypsin, confirming the proteinaceous nature of the compound (Table 2). Exposure to increasingly high temperatures had no apparent effect on the protein, with activity still present (64 AU) after the sample had been heated for 60 min at 100°C (Table 5). There was also no reduction in activity at any of the pH values ranging from 2-10, despite the fact that the pH of the CFS was typically neutral (~6.5) (data not shown).

Protein purification

Ammonium sulfate precipitation. The protein was fully precipitated out of solution at 30% ammonium sulfate concentration, and the presence of the bacteriocin was confirmed on SDS-PAGE gels with a large zone of inhibition in the overlay portion corresponding to the known size of subtilosin (data not shown).

Column chromatography. Inhibition assays indicated that the protein was solely and completely eluted from the columns by 90% methanol. They also confirmed activity was wholly due to the antimicrobial peptide and not background activity from the methanol.

Genetic analysis

PCR analysis showed *B. amyloliquefaciens* to be negative for the functional gene encoding subtilin (*spaS),* but positive for the functional gene encoding subtilosin (*sboA)* (Stein *et al*. 2004). The DNA sequence of the PCR product amplified from *B. amyloliquefaciens* was compared to that from *B. subtilis* ATCC6633 , and was shown to be 91.7% identical. There were only three base pair changes in *sboA*, none of which affected the amino acid sequence of the protein. A homolog of *sboX* (95% identical), a gene which putatively encodes a bacteriocin-like substance and overlaps *sboA*, was also identified. The gene encoding YwiA (*albA*) is downstream of the gene encoding SboA, and is believed to have a role in the posttranslational modifications of subtilosin (Stein *et al*. 2004). Due to the overwhelming similarity of the two gene products, the sequence preceding the gene and the intergenic sequence were compared, and found to be 95.6% and 85% similar, respectively.

DISCUSSION

Bacillus amyloliquefaciens was isolated from a fermented dairy beverage purported to contain *Lactobacillus* cultures. The bacteria produced a compound, later determined to be a protein, which possessed potent antimicrobial activity against such pathogens as *L.*

monocytogenes and *S. agalactiae.* Of particular importance is the fact that while activity was strong against the vaginal pathogen *G. vaginalis*, there was no activity against the clinically-isolated probiotic organisms tested.

The bacteriocin was shown to be remarkably stable under extreme temperature and pH stresses, with full activity retained after an hour at 100°C and across the pH range of 2-10. These observations increase the likelihood of this compound being considered for food preservation and personal care applications, as it can be adapted to, and function in, a variety of harsh environments.

Through genetic analysis, the unknown protein was later discovered to be identical to the bacteriocin subtilosin, which is produced by *B. subtilis*. Though there are well-documented cases of subtilosin production by several *B.subtilis* subspecies and the closely related species *B. atrophaeus* (Stein *et al*. 2004), this is the first report of production originating in *B. amyloliquefaciens*. Recent advents in genetic analysis have allowed for a comparison of the 16S rRNA regions and 16S-23S internal transcribed spacer (ITS) regions of the two species, demonstrating their extreme similarity (Xu and Côté 2003). However, *B. amyloliquefaciens* is generally accepted as a separate and individual species based on its higher GC content (~44% vs. ~42%), ability to grow in 10% NaCl, and increased production of α-amylase (Welker and Campbell 1967). The possibility that *B. amyloliquefaciens* is a closely related, yet diverged, species is supported by the research of Hoa *et al* (2002). They utilized transcriptional profiling to reveal that the *B. subtilis* Rok protein (encoded by *rok*) had an ortholog in *B. amyloliquefaciens*, but not in other bacilli or Gram-positive, spore-forming bacteria, and

suggested that *rok* could have been introduced into the *B. amyloliquefaciens* genome by HGT (Albano et al 2005).

With the recent surge in bacteria developing antibiotic resistance, there has been a marked increase in the level of attention given to bacteriocins. These proteins are considered ideal candidates for food preservation and personal care applications because the range of their activity is limited to only closely-related species. Therefore, while they may target a specific pathogen, they would theoretically have no harmful effects on humans and their normal microbiota. One of the most studied bacteriocins, nisin, is the only bacteriocin given GRAS (Generally Recognized as Safe) status by the U.S. Food and Drug Administration, leading to its use as a mainstream preservative. Despite the potential of nisin, organisms such as *L. monocytogenes* have developed complex mechanisms rendering them resistant to such treatments (Crandall and Montville, 1994; Gravesen *et al*. 2001; Bonnet *et al*. 2006). Subtilosin has a proven track record of efficacy against *L monocytogenes*, as indicated by this study and others (Zheng *et al*. 1999; Stein *et al*. 2004). This posits it as an attractive option that should be investigated by the food industry.

Very recently, Shelburne *et al*. (2007) conducted the most comprehensive study to date on the spectrum of antimicrobial activity of subtilosin. Their findings suggest that while the bacteriocin has the ability to act on a wide range of organisms, its inefficacy against capsulated organisms limits its practical value. The authors greatly diminish the importance of subtilosin as an antimicrobial agent; this, however, is contradicted by our results indicating its usefulness against vaginal pathogens such as *G.*

vaginalis or *S. agalactiae*. The ability of subtilosin to inhibit these species without damaging the healthy microbiota of that niche leads us to propose it may be an effective and safe way to treat infections that, in the case of *G. vaginalis*, have proven adept at developing antibiotic resistance to the drugs of choice for treating bacterial vaginosis (McLean and McGroarty 1996). Our future research with subtilosin will investigate its safety and toxicity to vaginal tissues as a means of determining its potential in practical care applications.

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FIGURES AND TABLES

Table 1. Growth conditions and subtilosin sensitivity of indicator organisms.

Organism	Growth Media	Growth Temperature (°C)	Range of antimicrobial activity (mm)
Micrococcus luteus ATCC 10420	TGY	30	25±1.5
Listeria monocytogenes ScottA	TGY	30	18±0.5
Pediococcus pentosaceus ATCC 43200	MRS	37	0
Salmonella Typhimurium ATCC 14028-1s	TGY	30	0
Lactobacillus L711 (clinical isolate)	MRS	30	0
Lactobacillus L735 (clinical isolate)	MRS	30	0
Lactobacillus L807 (clinical isolate)	MRS	30	0
Gardnerella vaginalis ATCC 14018	HBT	36	22
Gardnerella vaginalis (clinical isolate)	HBT	36	28
Streptococcus agalactiae (clinical isolate)	Columbia Agar with 5% Sheep Blood	36	20

Table 2. Effect of enzymatic digestion on antimicrobial activity.

Enzyme	Enzyme Diluent	Enzyme Concentration (mg ml^{-1})	Zone of Inhibition (mm)*
Catalase	50 mmol l^{-1} KPi (pH=7)	10	10
Pepsin	10 mmol l^{-1} HCl	10	0
Proteinase K	ddH_2O	10	0
Trypsin	1 mmol l^{-1} HCl	20	5
Chymotrypsin	1 mmol l^{-1} HCl	10	6
Lipase	ddH_2O	20	9
Protease	ddH_2O	10	7

∗Average zone of inhibition for undigested CFS was 10 mm.

Table 3. Specific primers for the functional genes of subtilin and subtilosin.

Bacteriocin	Primer	Primer sequence (5' to 3')	Reference
Subtilin	spaSFwd	CAAAGTTCGATGATTTCGATTTGGATGT	(Klein *et al*,
	spaSRev	GCAGTTACAAGTTAGTGTTTGAAGGAA	1992)
Subtilosin	sboAFwd	CGATCACAGACTTCACATGGAGTGT	(Stein *et al*,
	sboARev	CGCGCAAGTAGTCGATTTCTAACA	2004)

Table 4. Concentrations of D- and L-lactic acid in CFS.

Sample	ΔA_D	[D-lactic acid] (g l^{-1})	ΔA_L	[L-lactic acid] (g l^{-1})
Blank	0.053	0.17	0.047	0.15
Sample 1	0.046	0.15	0.692	2.24
Sample 2	0.054	0.17	0.679	2.19
Sample 3	0.059	0.19	0.686	2.22

Table 5. Effect of temperature stress on antimicrobial activity.

Temperature (°C)	Exposure Time (min)	Highest Active Dilution (AU)
60	5	64
	60	64
80	5	64
	60	64
100	5	64
	60	64

Chapter 3: Spermicidal activity of the safe natural antimicrobial peptide subtilosin.[3]

As described in Chapter 2, subtilosin was initially purified and characterized from a *B. amyloliquefaciens* isolate from a fermented dairy product. Subtilosin's spectrum of antimicrobial activity, particularly against the BV-associated pathogen *Gardnerella vaginalis*, raised the possibility of the peptide's potential as an ingredient in medical and/or personal care products. However, in order for formulation to occur, it was imperative to assess subtilosin's safety for human tissues, especially those in the targeted vaginal ecosystem. The work described within Chapter 3 describes the tissue toxicity testing conducted on subtilosin, as well as an investigation into its spermicidal capabilities.

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Spermicidal activity of the safe natural antimicrobial peptide subtilosin

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Keywords: subtilosin, spermicidal activity, tissue toxicity, bacteriocin, bacterial vaginosis, contraception, spermatozoa

ABSTRACT

Bacterial vaginosis (BV), a condition affecting millions of women each year, is primarily caused by the Gram-variable organism *Gardnerella vaginalis*. A number of organisms associated with BV cases have been reported to develop multi-drug resistance, leading

to the need for alternative therapies. Previously, we reported the antimicrobial peptide subtilosin has proven antimicrobial activity against *G. vaginalis*, but not against the tested healthy vaginal microbiota of lactobacilli. After conducting tissue sensitivity assays using an ectocervical tissue model, we determined that human cells remained viable after prolonged exposures to partially-purified subtilosin, indicating the compound is safe for human use. Subtilosin was shown to eliminate the motility and forward progression of human spermatozoa in a dose-dependent manner, and can therefore be considered a general spermicidal agent. These results suggest subtilosin would be a valuable component in topical personal care products aimed at contraception and BV prophylaxis and treatment.

INTRODUCTION

Subtilosin is a ribosomally-synthesized cyclopeptide produced by *Bacillus subtilis* ATCC 6633 and a recently identified natural isolate of *Bacillus amyloliquefaciens* (Babasaki et al., 1985; Sutyak et al., 2008). This protein has a unique structure among bacteriocins (Kawulka et al., 2004), and possesses antimicrobial activity against a variety of pathogenic organisms, including *Gardnerella vaginalis*, *Listeria monocytogenes*, and *Streptococcus agalactiae* (Group B *Streptococcus*) (Sutyak et al., 2008). The ability to inhibit the growth of *G. vaginalis* is of particular importance as it is one of the primary causative agents for bacterial vaginosis (BV), a common condition found in up to 30% of women in North America (Allsworth and Peipert, 2007). BV is characterized by the replacement of normal vaginal lactobacilli with anaerobic bacteria (e.g. *Prevotella*,

Bacteroides, and *Mobiluncus*) and mycoplasmas, as well as a several log increase in overall bacterial growth (Falagas et al., 2007; Lin et al., 1999; Gibbs, 2007). BV is asymptomatic in approximately one-half of all cases, but is associated with a wide variety of symptoms and problems ranging from complications with pregnancies (i.e. preterm births, low fetal birth weight) to the development of pelvic inflammatory disorder (Hillier et al., 1995; Ness et al., 2005; Pararas et al., 2006; Svare et al., 2006; Gibbs, 2007). Recent studies have estimated that nearly one in three women in the United States (29.2%) suffer from BV, with varying prevalence according to ethnicity and education levels (Allsworth and Peipert, 2007). One of the most troubling aspects of BV infection is the association with an increased risk of several sexually transmitted diseases (STDs), including chlamydia, herpes, gonorrhea, trichomoniasis, and HIV/AIDS (Sewankambo et al., 1997; Taha et al., 1998; Moodley et al., 2002; Cherpes et al., 2003; Kaul et al., 2007). The treatments recommended by the Centers for Disease Control are clindamycin and metronidazole administered either orally or intravaginally (Workowski et al., 2002). Following these guidelines successfully treats 60% of BV cases, but 20% of these cases return with highly developed antibiotic resistances (Bannatyne and Smith 1998; Lubbe et al., 1999; Liebetrau et al., 2003). In such cases, it would be extremely desirable to have an alternative form of treatment that could fully eradicate the infection.

Bacteriocins are typically divided into two major, yet diverse, classes: class I, or lantibiotics, contain unusual amino acids and are subject to extensive post-translational modifications, and class II, the heat stable non-lantibiotics (Maqueda et al., 2008).

Subtilosin has a unique post-translational structure that is unmatched among bacteriocins, which has led to speculation that it may belong in a distinct and separate class (Kawulka et al., 2004; Sutyak et al., 2008). Bacteriocins have been widely considered for use in medicinal and pharmaceutical applications, particularly for their bactericidal activity against multi-drug resistant organisms (van Kraaij et al., 1999). They are especially appealing alternatives since their cost of production is so comparatively low to conventional pharmaceutical treatments. For example, commercial grade nisin costs approximately $100/lb through chemical distributors. Much attention has recently been focused on the spermicidal activity of bacteriocins, due to their targeted antibacterial activity and lack of effect on human tissues. Nisin A, a well-studied class I bacteriocin produced by *Lactococcus lactis* subsp. *lactis* (Klaenhammer 1993), has human spermicidal activity (Aranha et al., 2004; Reddy et al., 2004), and subtilosin has spermicidal activity against boar, bovine, horse, and rat spermatozoa (Silkin et al., 2007). The results of these previous studies suggest that subtilosin may also have spermicidal activity against human spermatozoa, prompting our investigation.

Since subtilosin has proven antimicrobial activity against the pathogen largely responsible for BV, its toxicity to human tissues was assayed to determine its potential as a safe alternative remedy. For the human *in vitro* study, the EpiVaginal ectocervical tissue model (MatTek Corporation, Ashland, MA, USA) was employed to examine the relationship between prolonged exposure to subtilosin and cell viability. Spermicidal evaluations were conducted to investigate the ability of the peptide to restrict or eliminate sperm mobility, leading to its classification as a spermicidal agent.

MATERIALS AND METHODS

Production of subtilosin

Subtilosin was prepared as previously described (Sutyak et al., 2008). To prepare a cell free supernatant (CFS), cells were removed by centrifugation (Hermle Z400K; LabNet, Woodbridge, NJ, USA) for 25 min at 4500 *g* and 4°C. The supernatant was filter sterilized using 0.45 µm filters (Fisher, Pittsburgh, PA, USA). The protein of interest was precipitated from the supernatant by adding 30% ammonium sulfate (w/v) while stirring overnight at 4°C and was resuspended in 20 mL of double distilled H_2O. The column chromatography method described by Sutyak et al. (2008) was used to purify subtilosin from the CFS, producing a near-pure isolate in the 90% methanol eluate. The antimicrobial activity of all samples was confirmed by the well diffusion assay according to the protocol of Cintas et al (1995) with additional modifications (Sutyak et al., 2008). The active fraction was concentrated to dryness using a Savant SC110 Speed Vac and UVS400 Universal Vacuum System (Savant Instruments, Farmingdale, NY, USA), then resuspended in 1.5 mL ddH_2O.

Determination of protein concentration

Column-purified sample concentration. The concentration of subtilosin in the column-purified fraction was determined using the Micro BCA Protein Assay Kit according to the manufacturer's protocol (Pierce, Rockford, IL, USA). In brief, the assay measures the reduction of Cu^{2+} to Cu^{1+} by colorimetric detection of Cu^{1+} by bicinchoninic acid. Bovine

serum albumin (BSA) was used to develop a standard curve with concentrations ranging from 0.5 to 20 µg/mL; the concentration of subtilosin was calculated using the R value from the trendline of the standard curve graph.

Assay of subtilosin concentration in CFS. The concentration of subtilosin in the CFS was not measurable with the Micro BCA Protein Assay due to the high level of background proteins in the solvent (MRS medium). As an alternative, the protein concentration was calculated by comparing the antimicrobial activity of known concentrations of column-purified protein to equal volumes of CFS. Five two-fold dilutions were made from the stock samples of both the CFS and the column-purified fraction. Well diffusion assays were performed using 50 µl of each dilution against *Micrococcus luteus* ATCC 10420, which is commonly used as a reference microorganism for the determination of a bacteriocin's biological activity (Pongtharangkul and Demirci, 2004).

Determination of the presence of weak organic acids

As reported previously, the concentration of lactic acid in the CFS was measured to assess its potential effects on antimicrobial activity and cell viability (Sutyak et al., 2008). The quantity of each form of the acid in the sample was measured using a commercially available D-lactic acid/L-lactic acid kit (Roche Boehringer, Mannheim, Germany) according to the manufacturer's instructions.

EpiVaginal ectocervical tissue model

The EpiVaginal (VEC-100) ectocervical tissue model (MatTek Corporation, Ashland, MA, USA) was used and maintained as fully described by Dover et al. (2007). The tissues were exposed to 83 µL of subtilosin CFS (~136 µg/mL) for 4, 24, and 48 hours. For exposure times over 24 hours, the tissues were aerated by placing them on two metal washers (MatTek Corporation) and fed with 5 mL of the assay medium. Double distilled water (ddH_2O) was used as a negative control, and was applied to cells after 6, 24, and 48 hours. A spermicidal product containing 4% Nonoxynol-9 (Ortho Options CONCEPTROL Vaginal Contraception Gel, Advanced Care Products, Skillman, NJ, USA) was used as a positive control based on its documented cytotoxic properties (Ayehunie et al., 2006; Ozyurt et al., 2001; Sawyer et al., 1975; Davis et al., 1974). A cream (Monistat-3, Ortho McNeil Pharmaceutical, Inc., Raritan, NJ, USA) containing 4% of the nontoxic, BV-active compound miconazole nitrate (Ayehunie et al., 2006; Davis et al., 1974; Ozyurt et al., 2001; Sawyer et al. 1975) was used as a negative control.

Following the designated exposure times, the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine overall cell viability. The data were used to approximate an effective time (ET) that would reduce cell viability to 50% (ET-50).

MTT viability assay

The MTT assay was carried out according to the protocol outlined by Dover et al. (2007). Briefly, the viability of ectocervical cells after exposure to subtilosin was measured as a direct proportion of the breakdown of the yellow compound tetrazolium to the purple

compound formazan, since only living cells can cause this reaction to occur (Mosmann, 1983). Tissues were exposed to subtilosin and the two controls for several designated time points; at the conclusion of each, the liquid in the plate wells was combined with the liquid from the tissue inserts. This mixture was then assayed spectrophotometrically using a 96 well plate reader (MRX revelation, Dynex Technologies, VA, USA) to determine the level of tetrazolium degradation.

The viability (%) of the treated tissue inserts was calculated according to an equation provided by the manufacturer: % viability = OD_{570} (treated tissue)/OD_{570} (negative control tissue). The exposure time that reduced tissue viability by 50% (ET-50) was calculated according to the equation [$V=a+b*log(t)$] described by Ayehunie et al. (2006), where V=% viability, t=time in minutes, and "a" and "b" are constants representing the viability data from the time-points preceding and following 50% viability. On the whole, there is a direct relationship between the length of the ET-50 and the toxicity of the tested application (i.e. a shorter ET-50 corresponds to a more harmful compound).

Semen sample collection and analysis

The CFS gathered from a *B. amyloliquefaciens* culture was used to test the effect of subtilosin exposure on the motility of human spermatozoa. Initially, the CFS was diluted with normal saline (0.9%) so that 200 µL of the final material was equivalent to 50 µL, 100 µL, or 200 µL of undiluted CFS.

Two semen samples were collected on the day of experimentation. Each sample was collected by self-masturbation in a polypropylene specimen container (Fisher) prior to transport to the laboratory. Within 1 hour of collection, the samples were pooled. Total sperm count was calculated using bright field light microscopy (Olympus BX50; 400x) after dilution (1:50) of the semen in normal saline. The initial percentage of motile sperm was calculated prior to testing with a Neubauer hemacytometer (Anderson et al., 1994). The determination of motile sperm % was made using randomly selected field views (400x) from a count of between 104-201 cells. Any visibly moving spermatozoa (directional or stationary) was counted as a motile cell.

The percentage of forward progressing spermatozoa was subjectively determined based on the assumption that 70% of the sperm in a normal sample would behave in such a manner. The samples used in this experimentation fell into such a "normal" category.

Treatment of spermatozoa with subtilosin

A modified Sander-Cramer test was used to determine the effect of column-purified subtilosin on human spermatozoa motility (Anderson et al., 2000). This measured the effect of subtilosin after 30-second exposure times of 5 volumes (200 µL) of the solution at each dilution (25% and 50% in normal saline, and 100%) with one volume (40 µL) of whole semen. The motilities of cells from random high magnification fields (400x) of the sample were determined in duplicate as described above.

Data analysis

The % motility data were arcsine transformed (Sokal and Rohlf, 1981) prior to further examination. StatMost32 (version 4.1) statistical software (DataMost Corporation, Sandy, UT, USA) was used to calculate all statistical parameters. The % values of motility were presented as averages and 90% confidence limits. Any differences between treatment groups were assessed by the Newman-Keuls multiple range test. Differences were deemed significant at the 0.05 level of confidence.

RESULTS

Determination of protein concentration

The concentration of subtilosin in the column-purified sample was estimated at 135.7 µg/mL. The CFS and column-purified sample produced identical zones of inhibition at each dilution (data not shown); therefore, the concentrations of protein in both solutions were assumed to be equivalent. While it is improbable that a 100% yield would be attained from column chromatography, previous work has shown that protein concentrations can be precisely calculated based on the comparisons we conducted (Chi-Zhang, 2004). Due to the difficulty in measuring the CFS protein concentration via other assays, the chosen method was deemed the most accurate and reproducible.

Cell viability % and ET-50 values

After 48 hrs of exposure to subtilosin, the EpiVaginal ectocervical tissues retained a high level of viability compared to the positive control, Nonoxynol-9, and the negative

control, miconazole nitrate (Table 1). Due to the lack of toxicity of the antimicrobial, the ET-50 value for subtilosin could not be established since the total cell viability did not drop below 50% at any of the given time-points. However, a projection of the ET-50 value is possible by an extrapolation of the data. Data presented in Table 1 can be fit to a curve described by the equation $Ln(V)=a + bt^2$, where a = 4.605995356 and b = -0.00014151 (coefficient of determination, or r^2, = 0.9998), from which the ET-50 is estimated at 70 hours.

Quantitative observations of motile spermatozoa

Subtilosin reduces human sperm motility in a dose-dependent manner (Figure 1). The motility of the treated spermatozoa ranged from 0 to 88% of control motility levels over the four-fold range of subtilosin concentrations. All of the subtilosin concentrations tested reduced motility compared to the control samples. The differences in the proportion of motile spermatozoa in all samples (28.3, 56.7 and 113.3 μg/mL protein equivalents) were found to be significant ($p<0.05$) according to the Newman-Keuls multiple range test. TableCurve 2D (ver 5.0) curve-fitting software (SPSS Scientific Software, Chicago, IL) was used to fit the data to a dose-response curve described by the equation $Ln (\% Motility) = a + b[Subtilosin A]_3$, where a = 4.20781; b = -2.5814e-06; and [subtilosin A] is expressed as μg/mL protein equivalents. The curve had a coefficient of determination (r_2) = 0.9959. The IC_{50} value, or the amount of subtilosin required to reduce the motility of spermatozoa in whole semen by 50%, was calculated to be 64.5 μg/mL.

Semi-quantitative observations of spermatozoa: forward progression

Similar to motility, forward progression of spermatozoa is reduced in a dose-dependent fashion by subtilosin. In control samples, 70% of sperm exhibited forward progression; in the presence of 50 µl subtilosin this decreased to 50-70%, while 100 µl caused a decline to only 10% forward progression. All forward progression was eliminated after treatment with 200 µl subtilosin, with most sperm tails becoming coiled.

DISCUSSION

The *B. amyloliquefaciens*-produced bacteriocin subtilosin has proven antimicrobial activity against the vaginal pathogen *G. vaginalis*, but was not harmful to the normal and healthy *Lactobacillus* vaginal microbiota. Data from human vaginal cell viability assays convincingly demonstrated the safety of subtilosin for human applications in comparison to other accepted and available products, indicating it could be safely incorporated into personal care applications aimed at the treatment of bacterial vaginosis. Prior research in our laboratory that involved similar studies with the EpiVaginal model was also carried out in conjunction with *in vivo* testing of the rabbit vaginal irritation (RVI) system, which doubly confirmed the safety of another antimicrobial peptide, Lactocin 160 (Dover et al., 2007). Therefore, we are confident that using the EpiVaginal model instead of animal testing to demonstrate the safety of subtilosin has provided reliable and valid results.

Subtilosin was also found to significantly reduce the motility of human spermatozoa in a concentration-dependent manner for all concentrations tested. The effect of subtilosin on the forward progression of spermatozoa was also observed to be a dose-dependent interaction. Serial dilutions showed a steady decline in forward progression, with all progression halted at the highest concentration tested. It was also noted that at the highest concentration, the tails of the sperm cells were curved or coiled, indicating the cells were damaged beyond a simple restriction of movement. Coiling of the cells is considered to be a sperm abnormality, and may indicate damage to the plasma membrane (Bakst and Sexton, 1979). Tail coiling has been observed after *in vitro* exposure of monkey spermatozoa to methyl mercury (Mohamed et al., 1986). These results suggest subtilosin can be established as a general spermicidal agent. It is worth noting that nisin, a bacteriocin given GRAS (Generally Recognized As Safe) status by the Food and Drug Administration (FDA), was also shown to have impressive spermicidal activity (Reddy et al., 2004). However, the allure of these results is diminished due to the fact that it also has potent antimicrobial activity against healthy vaginal microbiota (Chikindas et al., unpublished data), a strong detraction from its commercial applicability. Therefore, subtilosin's spermicidal activity, combined with its overall safety to both human tissues and healthy human microbiota, make it a highly recommendable compound for inclusion in topical BV treatments and human contraceptive products. To facilitate the process of its incorporation into contraceptive treatments, additional analysis will be done to determine the reversibility of damage

done to the spermatozoa, as well as a time-course assay to further elucidate the exact changes effected by subtilosin.

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FIGURES AND LEGENDS

Figure 1. *Subtilosin A immobilizes human spermatozoa in a dose-dependent manner*.

The percentage of motile spermatozoa in pooled whole semen was determined 30 seconds after mixing with subtilosin A, at different final concentrations, as indicated. All data were adjusted to a normal control motility of 70% and subjected to arcsine transformation before further analysis. Values are expressed as average % motility. Error bars are 90% confidence limits.

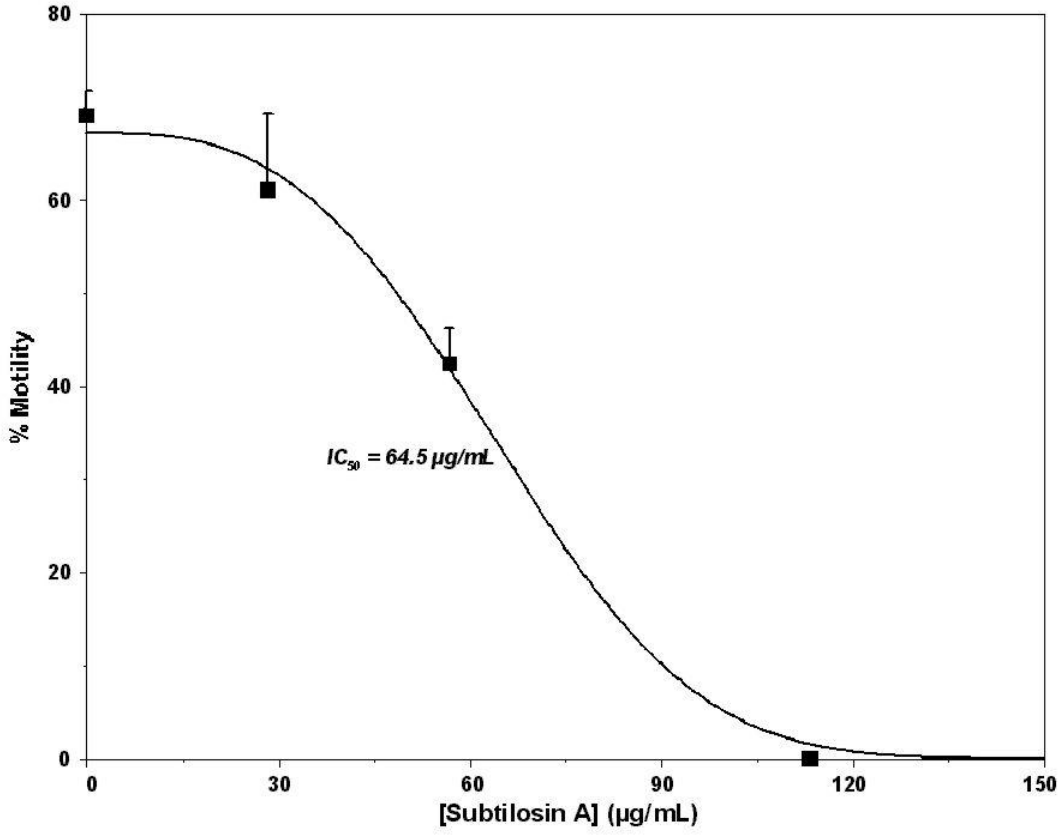

Table 1. Ectocervical cell viability after prolonged exposure to subtilosin.

Post-Exposure Cell Viability (%)					
Exposure Time (hrs)	**Assay #**	**Subtilosin**	**Nonoxynol-9 (4%)**	**Miconazole Nitrate (4%)**	**ddH_2O**
4	**1**	99.1	14.1	ND	100
	2	100	16.5	ND	100
24	**1**	94.8	0	58.4	100
	2	89.8	0	58.3	100
48	**1**	73.4	0	79.3	100
	2	70.9	0	83.5	100

Chapter 4: Elucidation of the molecular mechanisms of action of the natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen *Gardnerella vaginalis*.[4]

The promising results described within Chapter 3 established that subtilosin is safe for human use, and should be considered as a general spermicidal agent. Taken together with the data related in Chapter 2, it became very clear that subtilosin would be a safe and valuable addition to products aimed at BV prophylaxis/treatment. However, in order for such a product to be intelligently designed, it was necessary to determine the precise method by which subtilosin inhibits the primary causative agent of BV, *G. vaginalis*. As such, the research detailed within the following chapter elucidated subtilosin's molecular mechanism of action against the pathogen by examining its direct effect on the various components of the proton motive force (PMF) and intracellular ATP levels. The knowledge gained from these assays would thus allow us to understand the most effective time and way to target the pathogen, and what other compounds maybe be successfully combined with subtilosin for more efficient control.

[4] This chapter has been submitted for publication as an article in the journal *Antimicrobial Agents and Chemotherapy* in 2010. All references and formatting within follow the specifications of the journal.

Elucidation of the molecular mechanisms of action of the natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen *Gardnerella vaginalis*

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ABSTRACT

Subtilosin A is a 35-amino acid long cyclical peptide produced by *Bacillus amyloliquefaciens* that has potent antimicrobial activity against a variety of human pathogens, including the bacterial vaginosis-related *Gardnerella vaginalis*. The specific mode of action of subtilosin against *G. vaginalis* was elucidated by studying its effects on the proton motive force's (PMF) components: transmembrane electric potential ($\Delta\Psi$), transmembrane pH gradient (ΔpH), and intracellular ATP levels. The addition of subtilosin to *G. vaginalis* cells caused an immediate and total depletion of the ΔpH, but had no effect on the $\Delta\Psi$. Subtilosin also triggered an instant but partial efflux of intracellular ATP that was two-fold higher than that of the positive control bacteriocin, nisin. Taken together, these data suggest that subtilosin inhibits *G. vaginalis* growth by

creating transient pores in the cells' cytoplasmic membrane, leading to an efflux of intracellular ions and ATP, and eventually cell death.

INTRODUCTION

Bacterial vaginosis (BV) is a common condition characterized by an imbalance in the vaginal microflora, where healthy lactobacilli are replaced by a proliferation of facultative and anaerobic microorganisms, most notably *Gardnerella vaginalis* and *Prevotella*, *Peptostreptococcus*, *Porphyromonas*, and *Mobiluncus* spp. (8, 9, 22, 33). It has been estimated that between 10-30% of women in North America are afflicted by this ailment, frequently prompting them to seek medical attention (33). Although BV often remains asymptomatic (1), the unrestricted growth of these organisms has been demonstrated to have pathogenic effects, particularly in pregnant women. BV is associated with the development of pelvic inflammatory disease (13), as well as a variety of pregnancy-related complications, including low fetal birth weight (17), preterm births with an elevated risk of infant death (28), intra-amniotic infections leading to fetal brain damage (10, 27), and spontaneous abortion (7, 26). Also of great concern is the well-established connection between BV infection and sexually transmitted diseases (STDs). Bacterial vaginosis, and *G. vaginalis* in particular, has been shown to increase the probability of contracting HIV and to stimulate its proliferation in multiple cell lines (14, 15, 29, 37).

BV is typically treated by administering the antibiotics metronidazole and clindamycin orally or intravaginally. Although effective, these drugs do not specifically

target the pathogens involved in BV, causing widespread inhibition of the healthy vaginal microbiota. In turn, this leads to a high (~20%) recurrence rate of BV shortly after cessation of treatment (40), often with newly arisen developed antibiotic resistances (3, 21, 23). As such, it is critical that new treatments target the pathogens without affecting the host's healthy vaginal microflora.

Bacteriocins are ribosomally-synthesized peptides produced by bacteria that have antimicrobial activity against organisms closely related to the producer species (20). Bacteriocins have garnered much attention for their use as safe, natural food preservatives, as well as their potential in medical applications. One bacteriocin, subtilosin A, has strong potential for inclusion in alternative BV therapies. Produced by both *Bacillus subtilis* (2, 34) and *Bacillus amyloliquefaciens* (35), subtilosin A (commonly referred to as subtilosin) has a cyclical, cross-linked structure unique among characterized bacteriocins. It has demonstrated antimicrobial activity against a wide variety of human pathogens (30), including *G. vaginalis* (35), and was recently shown to possess potent spermicidal activity while remaining completely nontoxic to human vaginal epithelial cells and healthy vaginal lactobacilli (30, 35, 36). However, the inclusion of subtilosin in products aimed at BV prophylaxis or treatment requires a more detailed understanding of its specific mechanism of action against *G. vaginalis*, one of the primary pathogens involved in BV.

The primary mechanism of action for many bacteriocins is permeabilization of their target cell's cytoplasmic membrane. Electrostatic interactions between the membrane and bacteriocin provide a temporary linkage that allows the hydrophobic

peptide to insert itself into the membrane. In many cases, these transient pores produce leakage of intracellular ions, amino acids, and other low molecular weight molecules, and eventually cause a total depletion of the cell's transmembrane ion potential (ΔpH) (5). The disruption of this component of the Proton Motive Force (PMF) may then lead to either a subsequent degradation of or efflux of intracellular ATP and/or disruption of the transmembrane electric potential ($\Delta\Psi$) (5, 6). *In vitro* studies conducted by Thennarasu *et al*. (38) demonstrated that at high concentrations subtilosin is able to bind to lipid bilayers, although this binding is modulated by lipid composition. Due to its cyclical, cross-linked structure and uncommon net anionic charge (24), subtilosin can partially penetrate the hydrophobic core of lipid bilayers, disrupting their structure and thereby causing membrane permeabilization. Based on this knowledge, we hypothesized that subtilosin would selectively inhibit the growth of *G. vaginalis* by depleting cells' ATP levels and by dissipating one or more components of the Proton Motive Force (PMF).

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Stock cultures of *G. vaginalis* ATCC 14018 were kept at -80°C in BHI broth (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, Lenexa, KS) and 15% glycerol. Cultures of *G. vaginalis* were grown anaerobically in BHI broth + 3% horse serum at 37°C without shaking. *B. amyloliquefaciens* cultures were grown overnight in MRS broth

(Difco) at 37°C without shaking. The initial cultures were subcultured multiple times before use in experimental testing.

Preparation of antimicrobial solutions.

The partially purified preparation of subtilosin was prepared as previously described (35). Nisin (Sigma-Aldrich, St. Louis, MO; 100 AU/mL) was prepared according to the protocol given by Turovskiy *et al.* (39).

ATP efflux assay.

The effect of subtilosin on ATP depletion in *G. vaginalis* cells was assessed by the previously established bioluminescence method (12) and modifications of Turovskiy *et al*. (39) using an ATP Bioluminescent Assay Kit (Sigma-Aldrich) and a Luminoskan™ single-tube luminometer (Labsystems, Helsinki, Finland). This kit correlates ATP release with relative fluorescence as a result of oxidation of the D-luciferine molecule by the firefly luciferase enzyme in the presence of ATP and Mg^{2+}.

G. vaginalis cells were grown overnight in 15 mL BHI broth supplemented with 3% horse serum to an $OD_{660}\approx0.6$. Once they reached the appropriate growth stage, cells were centrifuged for 15 min at 4500 *g* (Hermle Z400K; LabNet, Woodbridge, NJ) at room temperature, and then washed once with 50 mmol/L MES buffer (pH 6.5). The cells were then maintained at room temperature for 5 min prior to an energization period, in which the cells were resuspended in half their original volume of 50 mmol/L MES buffer (pH 6.5) with 0.2% glucose and held at room temperature for 20 min. Following

energization, the cells were collected by centrifugation under the aforementioned conditions and resuspended in half their original volume in 50 mmol/L MES buffer (pH 6.5). This suspension was aliquoted in 100 µL volumes into sterile 1.5 mL microcentrifuge tubes, to which 20 µL of the appropriate treatment was added. Subtilosin was used at a final concentration of 2 µg/mL, while the positive control (bacteriocin nisin) reached a final concentration of 1.5 µg/mL, as per Winkowski *et al*. (41). Subtilosin diluent (ddH_2O) and nisin diluent (0.02M hydrochloric acid, pH 1.7) were used as negative controls. Each sample then remained at room temperature for 5 min prior to recording bioluminescent measurements.

The total ATP concentration in *G. vaginalis* cells was measured by combining 20 µL of the final cell suspension with 4.9 mL ice-cold ddH_2O and 80 µL DMSO. DMSO was chosen for its known ability to completely lyse bacterial cells, thus releasing all intracellular ATP. The data obtained for the negative controls were extremely uniform, allowing all other results to be normalized to their average and expressed as a percentage value.

Effect of subtilosin on Proton Motive Force (PMF) in *G. vaginalis*.

<u>$\Delta\Psi$ dissipation assay.</u>

The ability of subtilosin to affect the transmembrane electric potential ($\Delta\Psi$) of *G. vaginalis* cells was assessed according to the protocol given by Sims *et al.,* (1974) and the modifications outlined by Turovskiy *et al.* (39).

Briefly, *G. vaginalis* cells were grown as previously described to an OD_{600} of 0.6, harvested, then washed once and resuspended in $^{1}/_{100}$ of their original volume of fresh medium. The $\Delta\Psi$ of the cells was monitored as a function of the fluorescent intensity of the probe 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3$ (5)] (Molecular Probes, Eugene, OR) at 22°C using a PerkinElmer LS-50B spectrofluorometer (PerkinElmer Life and Analytical Science, Inc., Boston, MA) with a slit width of 10 nm and excitation and emission wavelengths of 643 and 666 nm, respectively. Initially, 5 µL of probe was added to 2 mL of fresh BHI broth supplemented with 3% horse serum in quartz cuvettes (10 mm light path) at a final concentration of 5 µmol/L. This was followed by addition of 20 µL of cell suspension, which caused an immediate decrease in fluorescence. Once the signal had equilibrated, the cells were exposed to 2µL of 5 mM nigericin (Sigma) in order to convert the ΔpH into $\Delta\Psi$. After stabilization of the signal, subtilosin, the positive control nisin, or the negative control nisin diluent was added. Finally, any remaining $\Delta\Psi$ was dissipated by the addition of 2 µL of 2 mmol/L valinomycin (Sigma).

<u>ΔpH dissipation assay</u>.

The ability of subtilosin to affect the transmembrane pH gradient (ΔpH) of *G. vaginalis* cells was analyzed according to the protocol given by Molenaar *et al.,* (1991) and the modifications described by Turovskiy *et al.* (39).

Initially, *G. vaginalis* cells were grown overnight to an OD_{600} of 0.6, harvested, then washed twice and resuspended in a hundredth of their original volume of 50 mmol/L potassium phosphate buffer (PPB, pH 6.0). The cells were then exposed for 5

min to the pH sensitive probe BCECF-AM (MP Biomedicals, Inc., Solon, OH) at ambient temperature to allow the probe to diffuse into the cytoplasm. Following exposure, the cells were washed twice with 1 mL of 50 mmol/L PBS (pH 6.0) and resuspended in 200 μL of the same. To measure dissipation of the transmembrane pH gradient, quartz cuvettes containing 2 mL of PPB (pH 7.0) were treated with 10 μL of the cell suspension. Fluorescence was read using a PerkinElmer LS-50B spectrofluorometer with slit widths of 5 nm for excitation and 15 nm for emission, and wavelengths of 502 and 525 nm, respectively. After signal stabilization, the cells were energized with 4 μL of 2.2 mmol/L glucose; the fluorescence subsequently rises as a result of an increase in intracellular pH. After again allowing for the signal to even out, 2 μL of 5 μmol/L valinomycin was added to convert the $\Delta\Psi$ component of the PMF into ΔpH. The cells were then treated with either subtilosin, the positive control (nisin), or the negative control (nisin diluent). Two μL of 2 μmol/L nigericin was added to dissipate any remaining ΔpH.

RESULTS

Subtilosin causes an efflux of ATP from *G. vaginalis* cells.

The effect of subtilosin on intracellular ATP levels in *G. vaginalis* cells was assessed as a function of bioluminescence, via the oxidation of the luminescent D-luciferine molecule by luciferase in the presence of extracellular ATP and Mg^{2+}. Subtilosin caused an efflux of intracellular ATP, denoted by the increased intensity of the luminescence in Figure 1A. On the other hand, the positive control (nisin) did not cause an efflux of ATP but instead triggered internal hydrolysis of the molecule, evidenced by a decrease in the

luminescence in the total ATP sample (Figure 1B). It was not possible to determine the effect of exposure to subtilosin and the controls past the single 5 min time point as the fastidiously anaerobic *G. vaginalis* cells poorly tolerated prolonged aerobic conditions (data not shown).

Subtilosin has no effect on *G. vaginalis* transmembrane electrical potential ($\Delta\Psi$).

The ability of subtilosin to dissipate the transmembrane electrical potential ($\Delta\Psi$) in *G. vaginalis* cells was observed using the fluorescent probe 3,3'-dipropylthiadicarbocyanine iodide [$DiSC_3$ (5)]. The ionophore nigericin (a K^+/H^+ exchanger) was added to the *G. vaginalis* cells in growth medium in order to convert the ΔpH to $\Delta\Psi$. The addition of nisin caused an instantaneous increase in the fluorescent signal of the probe as a result of the cellular membrane being depolarized by the bacteriocin (Figure 2 B). Subsequent introduction of the ionophore valinomycin had little effect, indicating nisin caused a complete collapse of this PMF component. However, the addition of subtilosin or the negative controls (nisin diluent and ddH_2O) did not cause an elevation in the probe's fluorescence, signifying they had no effect on the $\Delta\Psi$ (Figure 2 A,B). For both nisin diluent and subtilosin, subsequent addition of valinomycin fully depleted the $\Delta\Psi$, resulting in a fluorescence increase comparable to that seen after the addition of nisin (Figure 2 A,B). Unlike the positive control nisin, which caused a complete dissipation of the $\Delta\Psi$, subtilosin does not cause *G. vaginalis* cell damage by depleting this component of the PMF.

Subtilosin causes an immediate depletion of the transmembrane pH gradient (ΔpH). Subtilosin had an immediate impact on the transmembrane pH gradient (ΔpH). Prior to exposure to subtilosin and the controls, the ionophore valinomycin was introduced to convert all $\Delta\Psi$ to ΔpH. Addition of subtilosin caused an instant drop in the signal intensity of the pH dependent, fluorescent probe BCECF-AM, indicating an immediate intracellular decrease in pH in the *G. vaginalis* cells (Figure 3 A). Nisin also caused a decrease in the fluorescent signal, although at a slower, more gradual rate (Figure 3 B). Since the assay buffer was designed to have a pH lower than the intracellular pH of *G. vaginalis* cells (39), the decrease in intracellular pH is due to a depletion of the ΔpH. Adding nigericin to deplete any remaining ΔpH did not cause a further drop in fluorescence for either sample, indicating both nisin and subtilosin caused a total depletion of the ΔpH (Figure 3 A, B) through formation of transmembrane pores.

DISCUSSION

In this study, we clarified the molecular mechanism of action of the bacteriocin subtilosin against the vaginal pathogen *G. vaginalis*. To the best of our knowledge, this is the first report detailing subtilosin's mode of action against a specific target microorganism.

Our results clearly show that subtilosin acts by fully depleting the transmembrane pH gradient (ΔpH) and causing an immediate efflux of intracellular ATP, but has no effect on the transmembrane electric potential ($\Delta\Psi$). The fact that subtilosin does not affect both portions of the PMF is not surprising, as several other bacteriocins

are known to selectively dissipate only one PMF component. For example, enterocin P is able to dramatically decrease intracellular ATP concentrations and membrane potential ($\Delta\Psi$) in *Enterococcus faecium* T136 without affecting the ΔpH (16). In contrast, pediocin PD-1 creates pores in target cell membranes that allow for leakage of K^+ ions but not ATP and other essential metabolites (4). Taken together, the current results strongly suggest that the changes in the PMF brought about by subtilosin are due to the formation of transient pores in the cytoplasmic membrane of *G. vaginalis*. To our knowledge, this is the first report of the mode of action of subtilosin conducted on live cells. The *in vivo* results from this study support those of Thennarasu *et al*. (38), who demonstrated via an *in vitro*, cell-free system that subtilosin binds and inserts itself into the lipid bilayer of their target cell membrane. Their work showed that only the hydrophobic region of the cyclic peptide is submerged in the bilayer, while the anionic portion of the molecule remains free above the membrane. Further, they found that membrane permeabilization occurred at subtilosin concentrations well above the MIC level for the tested strain of *E. coli*, a phenomenon that has also been reported for the bacteriocins nisin and mersacidin (18, 19). At these high concentrations, aggregation occurs that creates multimeric units of subtilosin, thereby greatly increasing destabilization of the cell membrane. Thus, this destabilization event likely leads to subsequent formation of transient pores, which then cause the ΔpH dissipation and ATP efflux seen in the current investigation.

Our previous research (35, 36) established the antimicrobial activity of subtilosin against a variety of pathogens involved in human health, as well as its safety for human

tissues. However, the possibility of its inclusion in personal care products aimed at the treatment and/or prophylaxis of BV required that its specific mode of action against the target pathogen, *G. vaginalis*, be fully characterized. The data gathered in this study will allow for an intelligent design of subtilosin-based formulations for the effective control of BV-associated microorganisms. The growing problem of bacterial resistance to common antimicrobials is often due to prolonged exposure that allows the microorganisms to develop resistance. This can be avoided or delayed through the use of multiple compounds with differing modes of action that synergize to more effectively control the growth of the pathogen. The target cells will have less time to adapt to the various stresses, and it is far less likely that resistant organisms will appear. Now that subtilosin's mode of action has been clarified, future research will focus on evaluating potential synergies with other natural antimicrobials that possess differing mechanisms of action in a multiple-hurdle approach (11) against *G. vaginalis*. These assays will provide a final key piece of information in the intelligent design and formulation of a novel, safe product for the treatment of BV.

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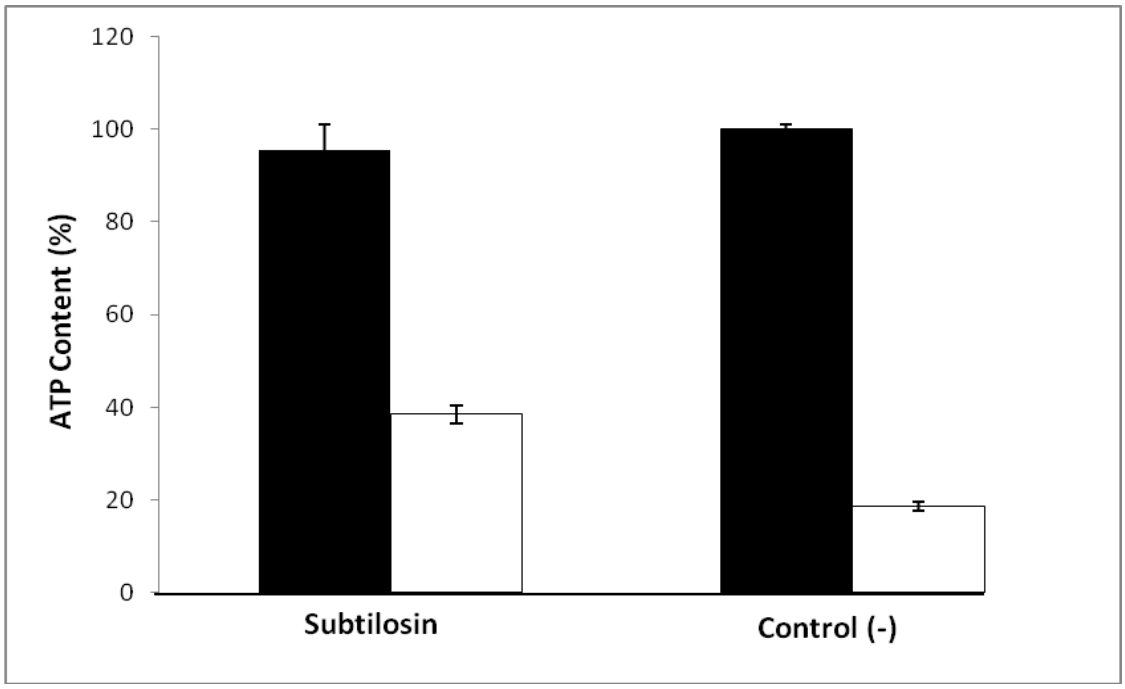
FIGURES AND LEGENDS

A.



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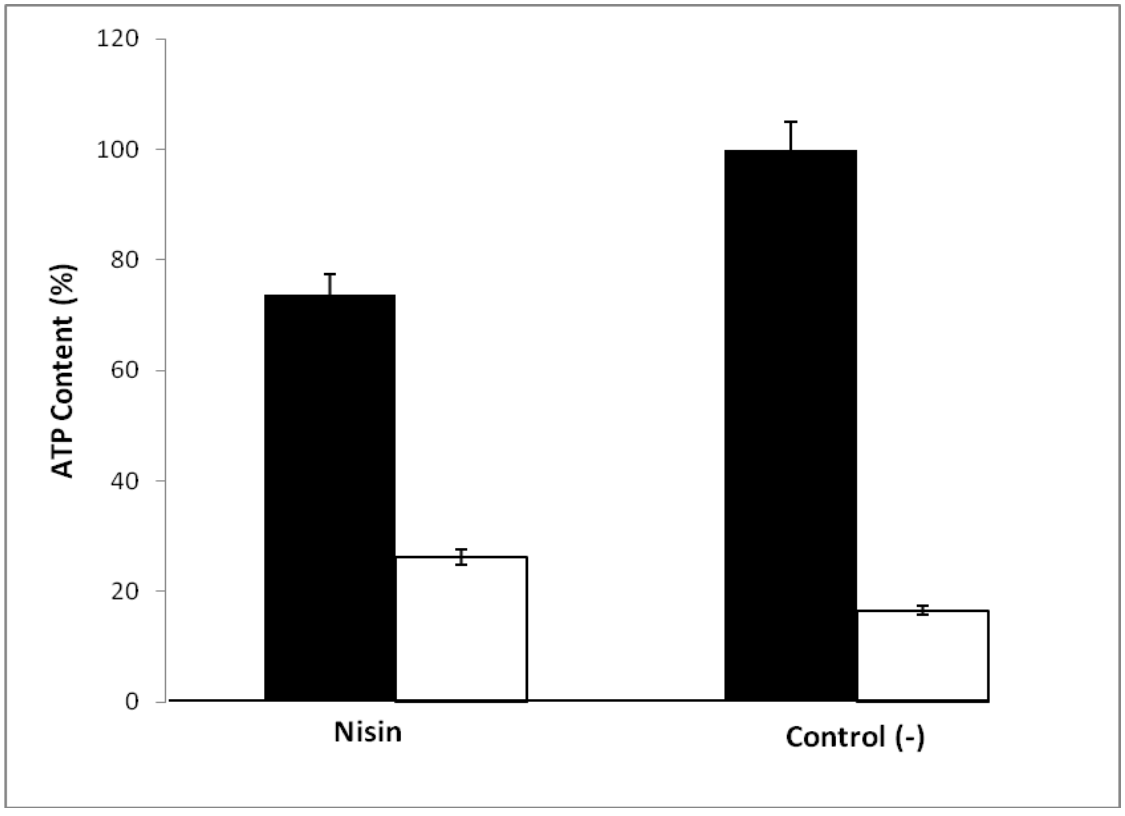
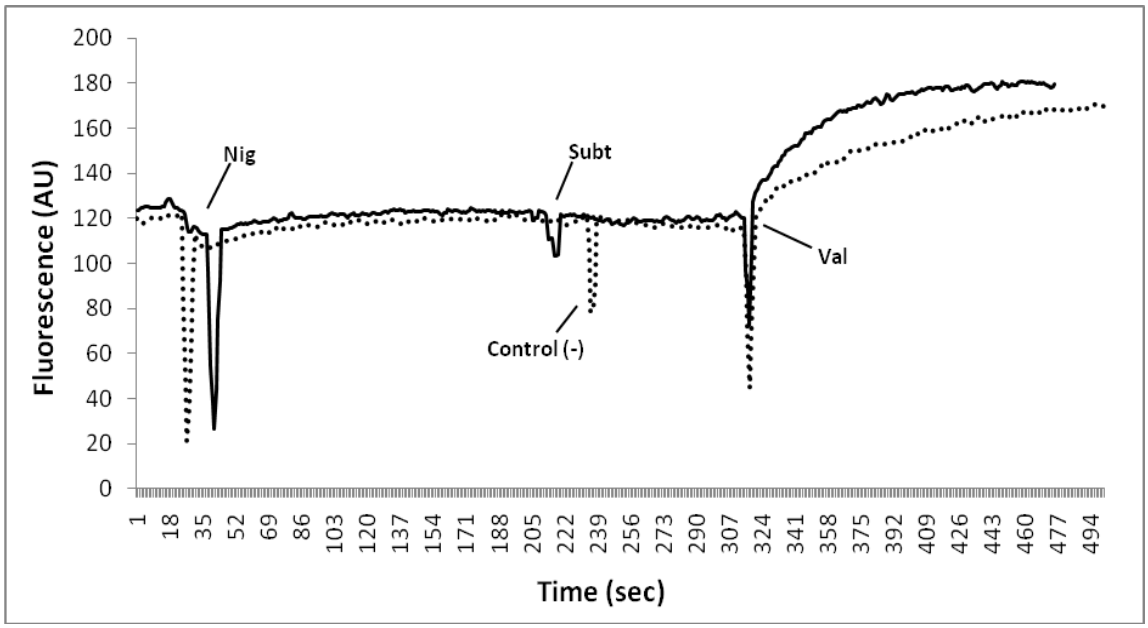


Figure 1. Subtilosin causes an efflux of intracellular ATP from *G. vaginalis* cells. Closed bars represent the total ATP content (intracellular + extracellular), while open bars represent extracellular ATP. Subtilosin (**A**) caused an efflux of ATP approximately 1.5-fold higher than that of nisin (**B**) and 2-fold higher than the negative control. Total ATP levels for nisin (**B**) were 20% lower than that of subtilosin (**A**) and both negative controls (**A,B**), indicating intracellular hydrolysis of ATP.

A.


Fluorescence (AU)
Time (sec)
Nig
Subt
Val
Control (-)

B.

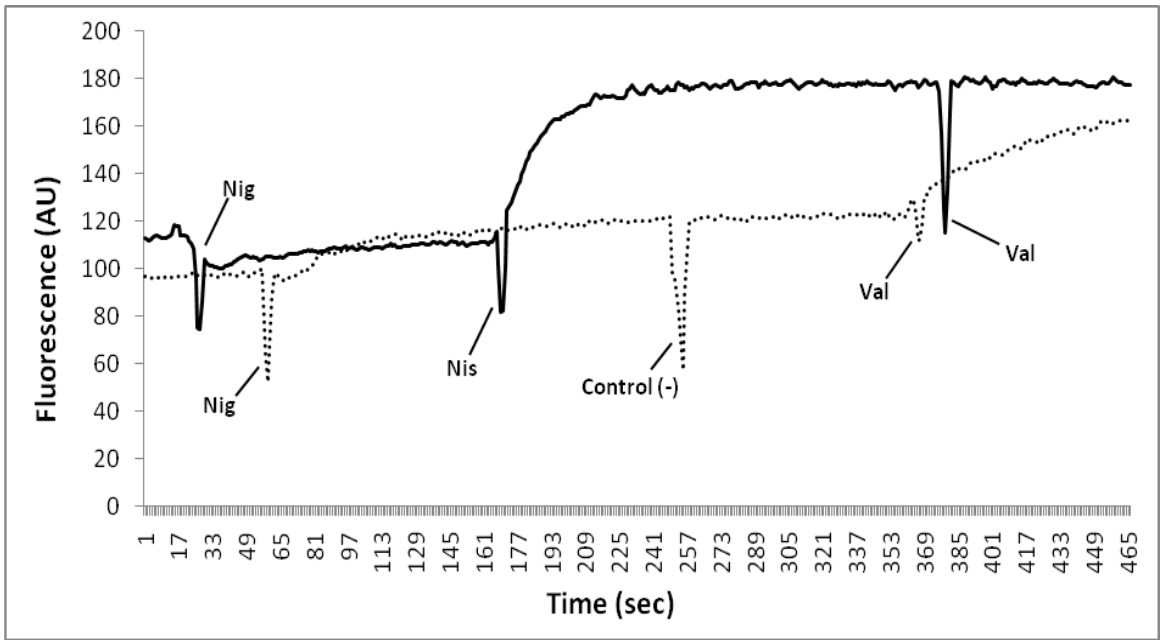
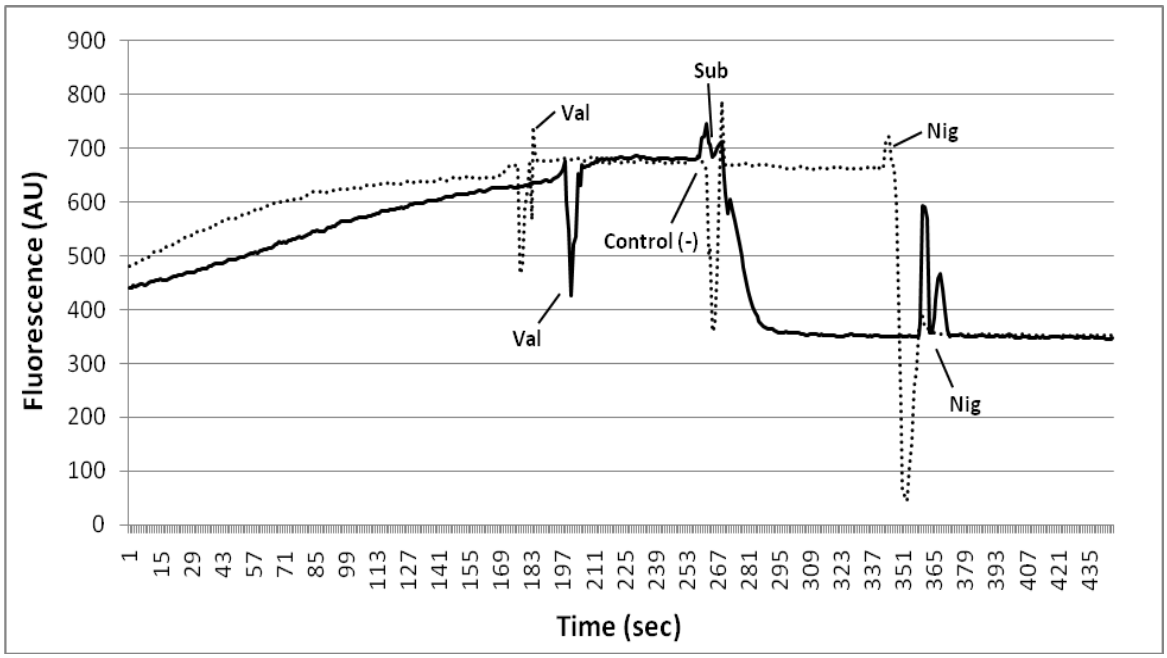

Fluorescence (AU)
Time (sec)
Nig
Nig
Nis
Control (-)
Val
Val

Figure 2. Subtilosin has no effect on transmembrane electric potential ($\Delta\Psi$) in *G. vaginalis* cells. Two µmol/L nigericin (Nig) was used to convert the ΔpH portion of the PMF into $\Delta\Psi$ (**A**, **B**). Subtilosin (Sub, **A**) caused no fluctuation in the fluorescent signal, indicating it has no effect on the $\Delta\Psi$. Addition of nisin (Nis, **B**) caused a corresponding spike in fluorescence, due to release of the $DiSC_3$ (5) probe and dissipation of the $\Delta\Psi$. In both cases, the negative controls (Control (-)) subtilosin diluent (**A**) and nisin diluent (**B**) had no effect on the $\Delta\Psi$. Two µmol/L valinomycin (Val) was used to dissipate any remaining $\Delta\Psi$ (**A**, **B**). There was no increase of fluorescence in the nisin sample (**B**), demonstrating that nisin caused a total depletion of the $\Delta\Psi$ portion of the PMF.

A.


Fluorescence (AU)
Time (sec)
Val
Sub
Nig
Control (-)
Val
Nig

B.

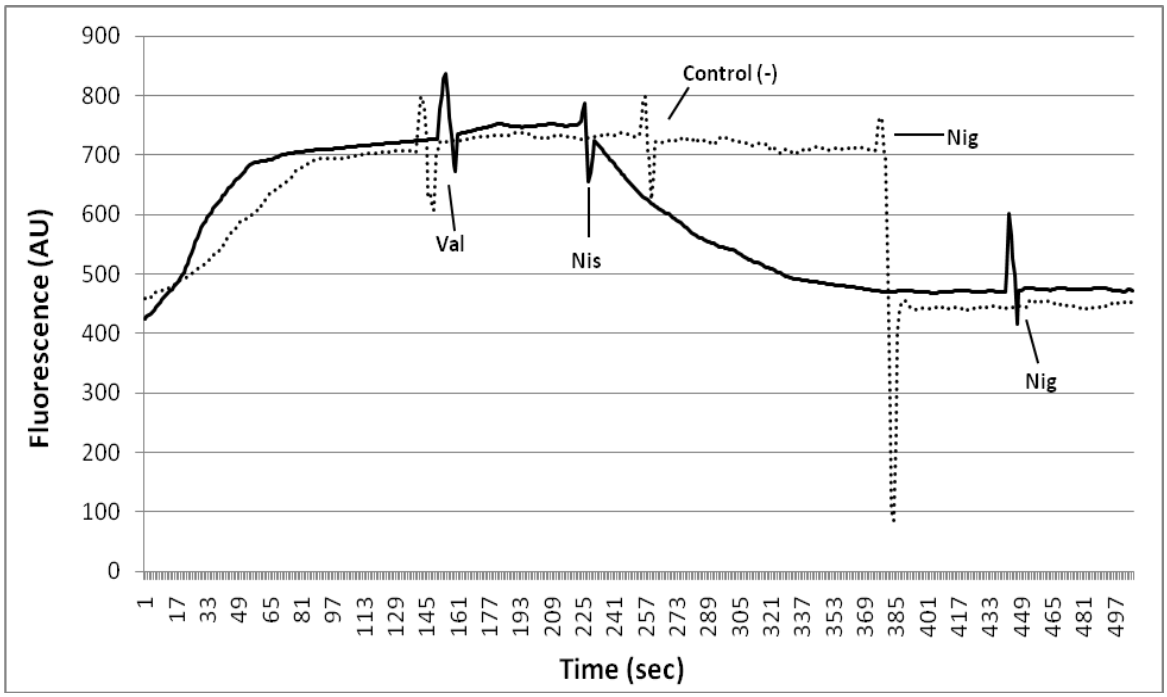

Fluorescence (AU)
Time (sec)
Control (-)
Nig
Val
Nis
Nig

Figure 3. Subtilosin immediately depletes the transmembrane pH gradient (∆pH) in *G. vaginalis* cells. Cells were energized with 2.2 mM glucose at start of fluorescence readings. Two µmol/L valinomycin (Val) was used to transform the $\Delta\Psi$ of the PMF into ∆pH (**A**, **B**). Subtilosin (Sub, **A**) caused an immediate decrease in the fluorescent signal, indicating a depletion of the ∆pH. Nisin (Nis, **B**) triggered a slower, gradual decrease in fluorescence. Both negative controls, (Control (-)) subtilosin diluent (**A**) and nisin diluent (**B**), had no effect on the ∆pH. Two µmol/L nigericin (Nig) was used to dissipate any remaining ∆pH (**A**, **B**). For both subtilosin and nisin, there was no subsequent drop in fluorescence, signifying that both bacteriocins totally depleted the ∆pH portion of the PMF.

Chapter 5: The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, ε-poly-L-lysine, and zinc lactate against the human pathogen *Gardnerella vaginalis*.[5]

Through the research described in Chapter 4, it was determined that subtilosin inhibits the growth of *G. vaginalis* by causing a total depletion of the cell's transmembrane pH potential (ΔpH) and an efflux of intracellular ATP. With this knowledge, subtilosin's effects on *G. vaginalis* were fully characterized, allowing for an informed and intelligently designed product aimed at BV prevention and treatment. However, the known threat of BV recurrence and resistance to conventional therapies led us to consider a product containing multiple "hurdles" for the pathogen to overcome. These hurdles would be comprised of multiple antimicrobial compounds, particularly those with differing mechanisms of action. In theory, *G. vaginalis* would not be able to survive exposure to subtilosin alone, but any resistant cells would then be faced with another effective opponent. Ideally, these two (or more) compounds would have synergy, meaning they could be used together in concentrations lower than on their own. Chapter 5 describes the culmination of this dissertation's research, and provides the final information necessary for subtilosin to be included in a personal care product designed to treat BV.

[5] This chapter has been submitted for publication as an article in the journal *Probiotics and Antimicrobial Proteins* in 2010. All references and formatting within follow the specifications of the journal.

The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, ε-poly-L-lysine, and zinc lactate against the human pathogen *Gardnerella vaginalis*

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ABSTRACT

Subtilosin is a cyclical antimicrobial peptide produced by *Bacillus amyloliquefaciens* that has antimicrobial activity against the bacterial vaginosis-associated human pathogen *Gardnerella vaginalis*. The ability of subtilosin to inhibit *G. vaginalis* alone and in combination with the natural antimicrobials glycerol monolaurate (Lauricidin®), lauric arginate, ε-poly-L-lysine, and zinc lactate was tested using a checkerboard approach. Subtilosin was found to synergize with all of the chosen antimicrobials. These promising results indicate that lower concentrations of subtilosin in combination with other compounds could effectively be used to inhibit growth of the pathogen, thereby decreasing the risk of developed antimicrobial resistance. This is the first report on the effects of subtilosin combined with other natural antimicrobials against *G. vaginalis*.

INTRODUCTION

In recent years, the advent of multi-drug resistant pathogens has been a subject of great concern among both the scientific community and the general public. The urgent need for new therapy options has led to a growing interest in bacteriocins. Bacteriocins are bacterially produced, ribosomally-synthesized peptides that have antimicrobial activity against organisms closely related to the producer species [18]. Although they have traditionally been used for food preservation purposes, bacteriocins have shown promise as safe, natural alternatives to conventional antibiotics. One such bacteriocin, subtilosin, has great potential for treating the condition known as bacterial vaginosis (BV), and is the subject of our current study.

Subtilosin A (referred to hereafter as subtilosin) is a cyclical peptide of 35 amino acids that has a complex, cross-linked structure unique among bacteriocins [17]. First isolated from a *Bacillus subtilis* culture by Babasaki *et al*. [3], it was recently shown to be produced by the dairy-product derived *Bacillus amyloliquefaciens* [32]. Subtilosin can inhibit the growth of a several human pathogens, including the human pathogen *G. vaginalis*, the primary causative agent of BV [32]. Most importantly, it is completely safe for human vaginal epithelial cells and healthy vaginal lactobacilli [32, 33], indicating its inclusion in personal care products would not adversely affect human health.

Bacterial vaginosis (BV) is a serious yet common health condition characterized by the replacement of healthy vaginal lactobacilli with facultative and anaerobic microorganisms, especially *Gardnerella vaginalis* and *Prevotella*, *Peptostreptococcus*,

Porphyromonas, and *Mobiluncus* spp. [10, 11, 20, 30]. Estimates predict that 10-30% of North American women are affected by BV, although many of these cases remain asymptomatic [2]. This poses a significant risk for women of a reproductive age, as the uncontrolled proliferation of organisms linked to BV has been associated with the development of pelvic inflammatory disease [13] and a variety of pregnancy-related complications, including intra-amniotic infections leading to fetal brain damage [12, 23], preterm births with an elevated risk of infant death [25], low fetal birth weight [16], and spontaneous abortion [9, 22]. BV, and particularly its causative agent *G. vaginalis*, is also associated with an elevated probability of contracting HIV and increased proliferation of the virus in multiple cell lines [15, 16, 28, 34].

The common antibiotics metronidazole and clindamycin are typically prescribed for oral and/or intravaginal BV treatment. While effective, these broad-spectrum medicines do not specifically inhibit BV-associated pathogens, often resulting in eradication of the healthy vaginal microbiota. Subsequently, there is a high BV recurrence rate of ~20% [37], which is often characterized by newly developed antibiotic resistances [6, 19, 21]. It has become critically important to develop new treatments specifically targeted at BV-associated pathogens that carry a low risk of developed resistance and are safe for human use.

One way to circumvent the development of bacterial drug resistance is through the use of combinations of antimicrobials. In this approach, low concentrations of antimicrobials with varying molecular mechanisms of action are combined to create "multiple hurdles" against microbial growth. The mixing of these compounds may

reveal compositions with synergistic or additive effects that allow for the use of each compound in amounts lower than that of their individual effective concentrations. There have been several recent reports on the synergy of bacteriocins with other natural antimicrobials. Badaoui Najjar *et al*. [4] showed that nisin, a bacteriocin Generally Recognized as Safe (GRAS) for food preservation purposes, synergizes with ε-poly-L-lysine (hereafter referred to as poly-lysine) against the foodborne pathogens *Listeria monocytogenes* and *Bacillus cereus*. On a related note, Amrouche *et al*. [1] demonstrated that subtilosin has synergy with nano-encapsulated curcumin, a plant phytochemical with antimicrobial activity, zinc lactate, and poly-lysine against *L. monocytogenes*.

In light of these results, we investigated the combinatorial relationship of subtilosin with four natural antimicrobials: glycerol monolaurate, lauric arginate, poly-lysine, and zinc lactate. Glycerol monolaurate (GML), a common ingredient in food and cosmetic industry preparations, is a monoglyceride that the FDA has given GRAS status for oral use (Title 21, Code of Federal Regulations [CFR], Part 184) [31]. Low concentrations of GML have been shown to inhibit the growth of *G. vaginalis* [31], likely through inhibition of signal transduction at microbial plasma membranes [26, 27, 36]. Lauric arginate (LAE), a derivative of lauric acid, L-arginine, and ethanol, is a GRAS compound with antimicrobial activity against a broad spectrum of microorganisms. LAE is known to cause disruptions and instability in the plasma membrane lipid bilayer without causing cell lysis, leading to inhibition of bacterial growth [5]. Poly-lysine is a short polypeptide comprised of repeating lysine subunits that adsorbs to cell surfaces

and interferes with cellular membranes [29]. Zinc lactate and other lactic acid salts, which are typically used in food preservation, are thought to inhibit pathogens such as *L. monocytogenes* by crossing the cell membrane as undissociated species and acidifying the intracellular components [24]. The varying mechanisms of action of these antimicrobials make them strong candidates for synergistic activity with subtilosin, the discovery of which could lead to more effective formulations of personal care products targeted at BV prophylaxis and/or treatment. This is the first report investigating the synergy of subtilosin combined with various natural antimicrobials against *G. vaginalis*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Gardnerella vaginalis ATCC 14018 cultures were grown anaerobically in BHI broth (Difco, Sparks, MD) + 3% horse serum (JRH Biosciences, Lenexa, KS) at 37°C without shaking. *B. amyloliquefaciens* cultures were grown overnight in MRS broth (Difco) at 37°C without shaking. Initial cultures of both organisms were subcultured multiple times before use. For all experiments, *G. vaginalis* was grown overnight to an approximate cell concentration of 10^8 CFU/mL, then diluted 100-fold in growth medium for a working concentration of 10^6 CFU/mL. Stock cultures of both organisms were kept at -80°C in their appropriate growth medium supplemented with 15% v/v glycerol.

Preparation of Antimicrobial Solutions

The partially purified preparation of subtilosin was prepared as previously described [32]. Sterile Lauricidin® (glycerol monolaurate) was a gift from Dr. Alla Aroutcheva of Rush Medical Center, Chicago, IL. A 2 mg/mL stock solution of glycerol monolaurate was prepared in BHI + 3% horse serum broth pre-warmed to 37°C. MIRENAT-CF was a gift from Vedeqsa Corp. (Barcelona, Spain), and contained 1 mg/mL lauric arginate (N^{α}-lauroyl-L-arginine ethyl ester monohydrochloride, LAE). A stock solution containing 25% ε-poly-L-lysine (250 mg/mL) was a gift from Chisso America, Inc. (Lot #2090501; Rye, NY). A solid stock supply of zinc lactate (Puramex Zn) was a gift from Purac America, Inc. (Lot #0807000376; Lincolnshire, IL). A 5.45 mg/mL stock solution of zinc lactate was made using ddH_2O. All antimicrobial solutions were filter-sterilized using a 0.45 µm filter (Nalgene, Rochester, NY) prior to use.

Determination of Minimal Inhibitory Concentrations (MICs)

The ability of each antimicrobial to individually inhibit *G. vaginalis* growth was determined using the broth microdilution method as per Amrouche *et al*. [1] with slight modifications. From the stock solutions, 10-fold serial dilutions of each antimicrobial (subtilosin: 230-0.023 ug/mL; glycerol monolaurate: 200-0.02 ug/mL; lauric arginate: 10,000-10 ug/mL; poly-lysine: 25,000-25 ug/mL; zinc lactate: 5450-0.545 ug/mL) in the proper diluent. *G. vaginalis* cells were grown overnight and prepared as previously described. A sterile, 96-well microplate (Corning, Inc., Corning, NY) was prepared by adding the serial dilutions of antimicrobials in horizontal rows, descending from highest concentration to lowest concentration tested. The antimicrobials were tested in 20 µL

increments (0-100 µL), with each volume tested in duplicate. The volume of each well was raised to 100 µL total with the addition of sterile ddH_2O, and the contents of each well were mixed by gentle pipetting. One hundred µL of *G. vaginalis* cells were added to each well; wells containing cells alone, antimicrobial alone, water alone, and growth medium alone were used as controls. Fifty µL of sterile mineral oil was pipetted onto the top of each well to form an airtight seal that would allow for anaerobic growth of the *G. vaginalis* cells. Each plate was then transferred into a Coy Type C Anaerobic Chamber (Coy Laboratory Products, Inc., Grass Lake, MI) and placed in a Bio-Rad Model 550 Microplate Reader (Bio-Rad Life Sciences, Hercules, CA). The turbidity of each well was recorded at 595 nm every 30 min for 48 hrs at 37°C. In order to prevent mixing of the mineral oil seal with the contents of each well, the plate was not shaken prior to each measurement. Data was gathered and analyzed using Microplate Manager (version 5.1.2) software (Bio-Rad). The lowest concentration of each antimicrobial that showed no increase in optical density (no bacterial growth) was designated as the MIC. Each assay was performed at least twice in duplicate.

Checkerboard Assay

The interaction between subtilosin and the chosen antimicrobials was tested via a "checkerboard" assay that allowed for testing of multiple antimicrobials at various concentrations at the same time. The assays were performed according to Badaoui Najjar *et al*. [4] with the following modifications. In each experiment, a sterile 96-well microplate (Corning) was prepared so that subtilosin (horizontal rows) would be

combined with one of the chosen antimicrobials (vertical columns). Using a stock solution of a 10-fold higher concentration than its respective MIC, each compound was aliquoted into the appropriate row or column. Each plate was designed to test concentrations directly above, equal to, and, particularly, below that of the individual MIC of each antimicrobial (Table 1). The volume of each well was raised to 100 µL using sterile ddH_2O. *G. vaginalis* were grown overnight and prepared as previously described; 100 µL of this preparation was added to each well. The first row and column of the microplate served as controls (no antimicrobials), as did a row of water alone and growth medium alone. Fifty µL of sterile mineral oil was pipette onto the top of each well to ensure anaerobic conditions. Each plate was run using the same equipment and under the same conditions as described in the previous section. Each assay was performed at least twice in duplicate.

Graphical Presentation of the Data

The kinetic growth curve data from all assays was analyzed using Microsoft Excel 2007 (Microsoft, Redmond, WA). Isobolograms were created for each synergy assay as a way to visualize the presence of synergy, additive effect, or antagonism. In an isobologram, the *x*- and *y*- axes represent the concentrations of each antimicrobial; the MIC of each substance is then plotted on the graph, and the two points are joined by a line. The mixed concentrations of antimicrobials that caused complete inhibition of microbial growth are then plotted on the graph. Points that fall below the line indicate synergy,

points on the line show an additive effect, and points above the line demonstrate antagonism [7].

RESULTS

Determination of MICs

The MIC of subtilosin, GML, LAE, poly-lysine, and zinc lactate against *G. vaginalis* was tested by the broth microdilution method in BHI broth supplemented with 3% horse serum. As seen in Table 1, all of the tested substances were able to completely inhibit the growth of the selected vaginal pathogen Subtilosin proved to be quite effective with an MIC of only 9.2 µg/mL , while GML and poly-lysine had MICs of 20 µg/mL and 25 µg/mL, respectively. The MIC of GML is supported by the findings of Strandberg *et al*. , who demonstrated that GML had an MIC of 10 µg/mL against a clinical isolate of *G. vaginalis* [31]. At 1.0901 mg/mL, the MIC of zinc lactate was 40-fold higher than that of the other tested compounds. As previously stated, all MIC assays were run at least two times in duplicate. The results for each compound did not deviate between assays, despite the extensive range of tested concentrations; thus, there was no standard deviation recorded for these results (Table 1).

Determination of synergy between antimicrobial substances

Once the individual MICs of all the chosen compounds were calculated, a checkerboard assay was performed using subtilosin in combination with one other substance. Each assay was designed to test a wide range of concentrations, beginning with one slightly

above that of each compound's individual MIC and decreasing in a serial manner to a zero concentration (negative control). Combinations of concentrations below each of the MIC levels that caused complete inhibition of microbial growth were analyzed with isobolograms to determine the presence of synergy, additive effect, or antagonism.

Interaction between subtilosin and glycerol monolaurate (GML)

Since GML has demonstrated antimicrobial activity against the BV-associated pathogen *G. vaginalis*, it was the first substance tested for synergy with our target peptide, subtilosin. To visualize synergy between combinations of the two compounds, an isobologram was constructed by plotting the individual MICs of subtilosin on the *x*-axis and GML on the *y*-axis and connecting the two points (Figure 1). From the checkerboard assay, the lowest combined concentrations of subtilosin and GML that caused total growth inhibition of *G. vaginalis* were 4.6 and 2 µg/mL, respectively (Table 2). When used in combination, there was a two-fold reduction in subtilosin's MIC and a four-fold reduction in GML's MIC. The point representing these two concentrations was added to the isobologram and falls well below the trendline, indicating synergy. While the concentration combinations of 2.3 µg/mL subtilosin and 10 µg/mL GML also caused complete inhibition of *G. vaginalis* growth, the corresponding graph point fell closer to the trendline, indicating weaker synergy (Figure 1).

Interaction between subtilosin and lauric arginate (LAE)

The second natural antimicrobial, lauric arginate, has previously been shown to synergize with the *Lactobacillus rhamnosus*-produced bacteriocin lactocin 160 against *G. vaginalis* (Y. Turovskiy, personal communication). As described for GML, its potential synergy with subtilosin was assessed and an isobologram was constructed using the individual MICs of subtilosin and LAE (Table 1). The checkerboard assay showed the lowest concentration combination of subtilosin and LAE that caused complete inhibition of *G. vaginalis* growth to be 4.6 µg/mL and 25 µg/mL, respectively (Table 2). This combination caused a two-fold decrease in subtilosin's individual MIC and a four-fold reduction in LAE's MIC. When plotted on the isobologram, the point representing these two concentrations also falls below the trendline, indicating synergy between the two compounds (Figure 2).

Interaction between subtilosin and ε-poly-L-lysine

The third antimicrobial compound, poly-lysine, was previously demonstrated to synergize with both nisin and subtilosin against the foodborne pathogen *L. monocytogenes* [1, 4], supporting the possibility of synergy with subtilosin against *G. vaginalis*. As previously described, an isobologram was constructed using the individual MICs of subtilosin and poly-lysine (Table 1). The checkerboard assay exhibited the lowest concentration combination of subtilosin and poly-lysine to completely inhibit *G. vaginalis* growth as 4.6 µg/mL and 2.5 µg/mL, respectively (Table 2). This combination caused a two-fold decrease in subtilosin's individual MIC and a significant ten-fold reduction in poly-lysine's MIC. When plotted on the isobologram, the point representing

these two concentrations also falls below the trendline, indicating synergy between the two compounds (Figure 3).

Interaction between subtilosin and zinc lactate

Amrouche *et al*. [1] also demonstrated that subtilosin acts synergistically with zinc lactate against *L. monocytogenes*, prompting our investigation of their combined activity against *G. vaginalis*. As previously described, an isobologram was constructed using the individual MICs of subtilosin and zinc lactate (Table 1). The checkerboard assay demonstrated that the combination of the lowest concentrations of subtilosin and zinc lactate that fully prevented *G. vaginalis* growth were 2.3 µg/mL and 272.5 µg/mL, respectively (Table 2). This combination caused a four-fold decrease in subtilosin's individual MIC and a five-fold decrease in zinc lactate's MIC. When plotted on the isobologram, the point representing these two concentrations falls below the trendline, indicating synergy between the two compounds (Figure 4). While two other concentration combinations (2.3 µg/mL subtilosin and 545 µg/mL poly-lysine; 4.6 µg/mL subtilosin and 272.5 µg/mL zinc lactate) also caused complete inhibition of *G. vaginalis* growth, the corresponding graph points were closer to the trendline, indicating weaker synergy (Figure 4).

DISCUSSION

The antimicrobial activity of subtilosin and four natural antimicrobials were investigated alone and in combination against the BV-associated pathogen *G. vaginalis*. A

checkerboard assay was utilized to study multiple concentrations of subtilosin and another antimicrobial compound for the presence of synergy, additive effect, or antagonism against the target microorganism. Individually, subtilosin had the lowest MIC against subtilosin at 9.2 µg/mL, although GML, LAE, and poly-lysine also had MICs in the µg/mL range. On its own, zinc lactate was shown to be less effective against *G. vaginalis* with an MIC of slightly over 1 mg/mL (Table 1). However, when each of the four compounds were tested in combination with subtilosin, there was a dramatic reduction in their MIC. Both GML and LAE's MICs were reduced four-fold, while subtilosin's MIC decreased by half. The ten-fold drop in poly-lysine's MIC was the most significant change, while zinc lactate's relatively high individual MIC was decreased five-fold (Table 2). While the ability to use considerably smaller amounts of each compound to inhibit *G. vaginalis* growth was a promising result, our main interest lay in whether these interactions were the result of synergy between the two compounds. As seen in each of the isobolograms (Figures 1-4), the points representing the combinatorial MICs of subtilosin and the secondary antimicrobial all fall well below the trendlines connecting the points depicting each compound's individual MIC. As such, it is apparent that subtilosin synergizes with all of the tested antimicrobials.

The presence of synergy between subtilosin and these substances is a promising result that creates a wide range of possibilities for future formulations of personal care products targeted at the prophylaxis and treatment of BV. There are many documented instances of drug-resistant cases of BV developing after treatment with the regularly prescribed antibiotics [6, 19, 21], indicating the need for new treatment options.

Considering the multitude of health risks associated with BV, there would ideally be a low possibility of BV-associated organisms developing resistance to these new alternatives. The multiple hurdle approach is therefore ideal, since it uses combinations of synergistic substances in concentrations lower than their individual effective doses [1]. The use of more than one antimicrobial, especially those with differing mechanisms of action, makes it very difficult for the pathogen to overcome each 'hurdle', and lowers the chances of significant cell numbers surviving [4]. Due to its cyclic structure, subtilosin has a unique mechanism of action. Kawulka *et al*. [17] first posited that subtilosin may bind to a surface receptor rather than solely interacting with the cell membrane. Later, Thennarasu *et al*. [35] suggested that subtilosin may in fact attach to the target cell's lipid bilayer, causing the leakage of unilamellar vesicles. However, our own research has shown that, at least for *G. vaginalis*, subtilosin forms transient pores in the cell membrane that disrupt components of the cell's proton motive force and allow for efflux of ATP [manuscript in preparation]. As detailed in the Introduction, the varying mechanisms of action of GML, LAE, poly-lysine, and zinc lactate would all be suitable counterparts to that of subtilosin, and would indeed provide the multiple 'hurdles' required to effectively control the growth of BV-associated pathogens like *G. vaginalis*.

While a BV treatment containing two antimicrobials of differing mechanisms of action would be an improvement over the currently prescribed, resistance-prone antibiotics metronidazole and clindamycin, it would be far more preferable there were three or more 'hurdles' to stymie pathogenic growth. Our future research will focus on

evaluating combinations of subtilosin with two or more of the antimicrobials investigated in this report, and similarly determining the presence of synergy or antagonism. The presence of an additional antimicrobial would make it difficult to test the wide array of concentrations needed to accurately assess synergistic behavior in a single microplate. Thus, we will employ the CompuSyn for Drug Combinations and General Dose-Effect Analysis program (ComboSyn, Inc., Paramus, NJ) to determine each substance's MIC when in combination, and to generate isobolograms from the data (see Chou [7, 8] for further information). While the possibility of discovering synergy between subtilosin and two other antimicrobials would open exciting new avenues of research and drug formulation, the data from our current investigation is the first step in that process. This is the first report on the synergy of subtilosin with natural antimicrobials against *G. vaginalis*, the primary causative agent of BV.

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FIGURES AND LEGENDS

Antimicrobial Compound	**Starting Concentration**	**MIC for *G. vaginalis***
Subtilosin	229.5 µg/mL	9.2 µg/mL
Glycerol monolaurate (GML)	2 mg/mL	20 µg/mL
Lauric arginate (LAE)	1 g/mL	100 µg/mL
Poly-lysine	250 mg/mL	25 µg/mL
Zinc Lactate	5.45 mg/mL	1090.1 µg/mL

Table 1. Minimal inhibitory concentrations (MICs) of subtilosin, glycerol monolaurate, lauric arginate, poly-lysine, and zinc lactate against the BV-associated pathogen *G. vaginalis*. Each MIC assay tested a wide range of concentrations for each compound, and was conducted at least twice in duplicate. All assays conducted resulted in identical results for all substances (no standard deviation).

Antimicrobial Compound	Combinatorial Synergy MIC (µg/mL)	Subtilosin Synergy MIC (µg/mL)
Glycerol monolaurate (GML)	2	4.6
Lauric arginate (LAE)	25	4.6
Poly-lysine	2.5	4.6
Zinc Lactate	272.5	2.3

Table 2. Minimal inhibitory concentrations (MICs) of antimicrobial compounds tested in a checkerboard assay against *G. vaginalis*. Subtilosin was combined with one other antimicrobial per assay; the data in this table represents the minimum concentration of each compound required to inhibit *G. vaginalis* growth in a combinatorial manner. When combined with subtilosin, GML, LAE, and zinc lactate all had a 4-fold reduction in their MIC, while poly-lysine had a dramatic 10-fold reduction. The MIC of subtilosin was reduced two-fold when combined with GML, LAE, and poly-lysine, and 4-fold when combined with zinc lactate. Each checkerboard assay tested a wide range of concentrations for each compound, and was conducted at least twice in duplicate. All assays conducted resulted in identical results for all substances (no standard deviation).

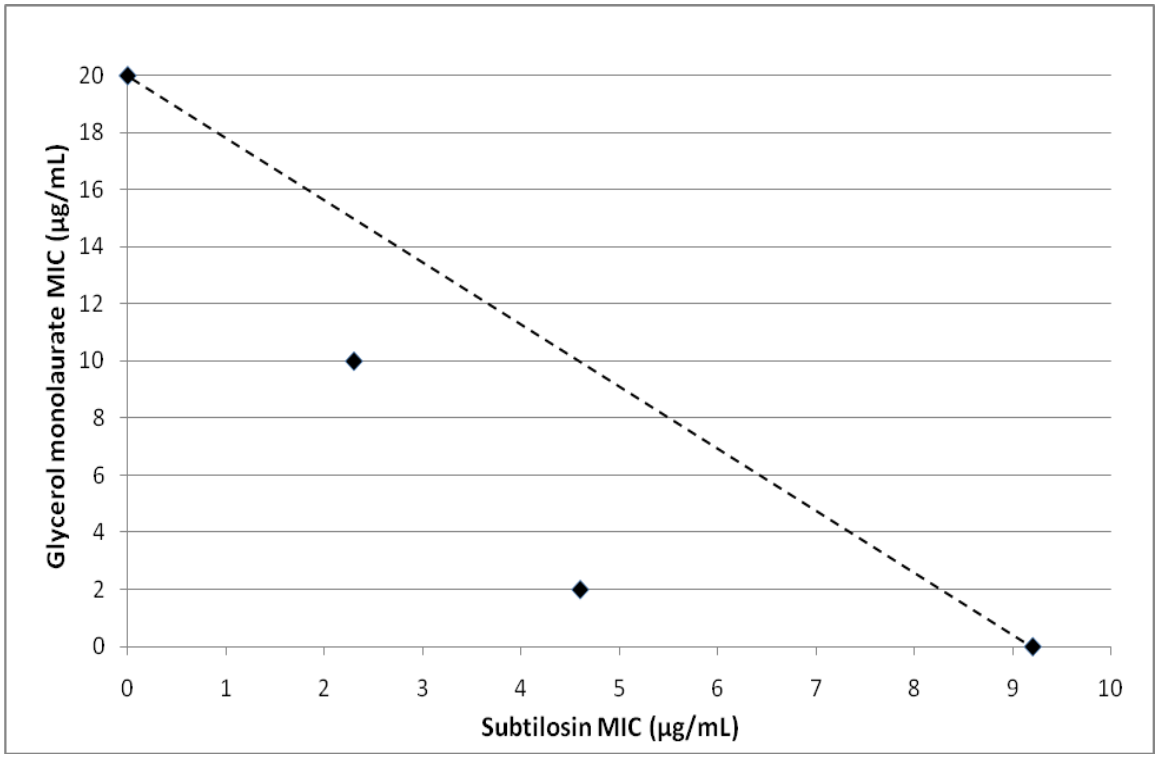

Figure 1. Subtilosin and glycerol monolaurate (GML) act synergistically against *G. vaginalis*. This isobologram shows the individual MICs for GML (20 µg/mL) and subtilosin (9.2 µg/mL) connected by a trendline. The points below the trendline represent the combinatorial concentrations of subtilosin and GML that completely inhibited *G. vaginalis* growth. While both combinations fall below the line, indicating synergy, the lower point (4.6 µg/mL subtilosin, 2 µg/mL GML) demonstrates the stronger synergy between the two substances.

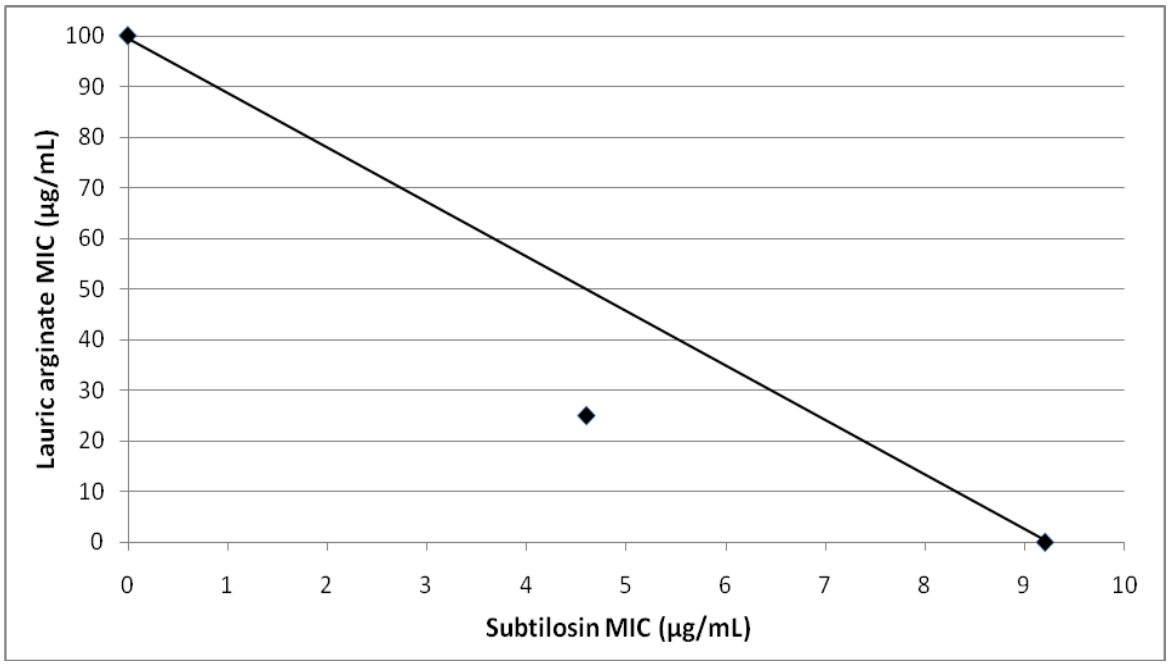

Figure 2. Subtilosin and lauric arginate (LAE) act synergistically against *G. vaginalis*. This isobologram shows the individual MICs for LAE (100 µg/mL) and subtilosin (9.2 µg/mL) connected by a trendline. The point below the trendline represents the combinatorial concentration of subtilosin and LAE that completely inhibited *G. vaginalis* growth (4.6 µg/mL subtilosin, 25 µg/mL LAE). The location of the point below the trendline indicates that synergy does occur.

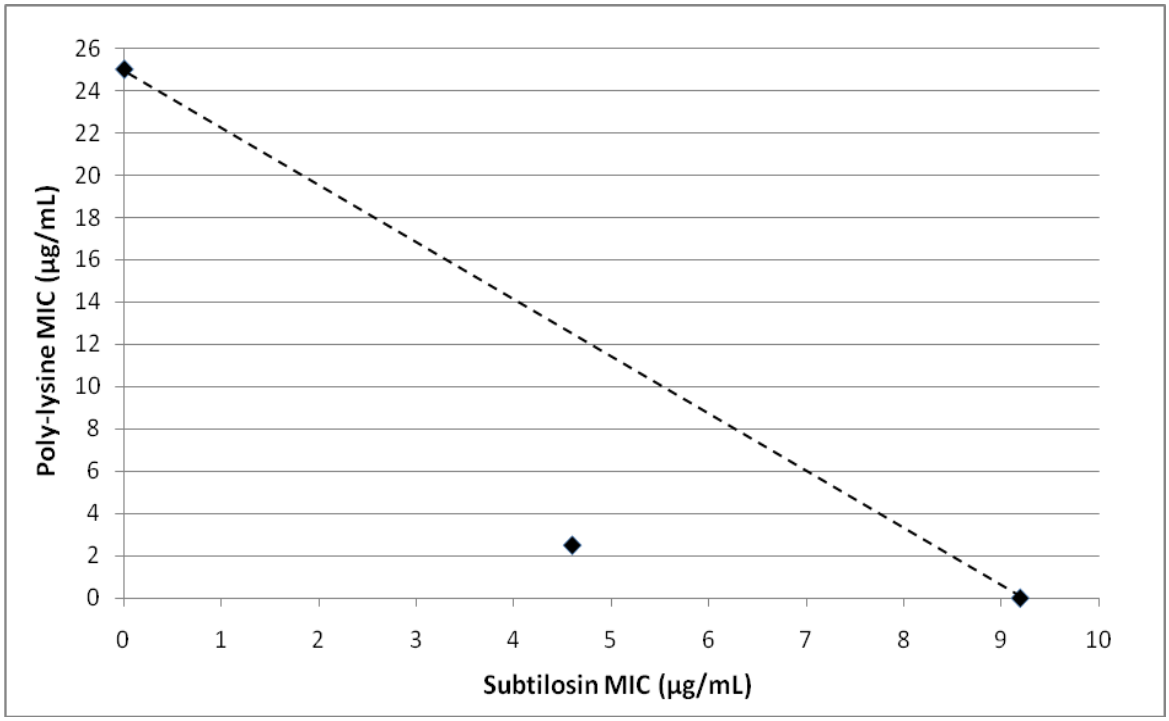

Figure 3. Subtilosin and poly-lysine act synergistically against *G. vaginalis*. The isobologram demonstrates the individual MICs for subtilosin (9.2 µg/mL) and poly-lysine (25 µg/mL) connected by a trendline. The point below the trendline represents the combinatorial concentration of subtilosin and poly-lysine that completely inhibited *G. vaginalis* growth (4.6 µg/mL subtilosin, 2.5 µg/mL poly-lysine). Since the point falls below the trendline, there is synergy between subtilosin and poly-lysine.

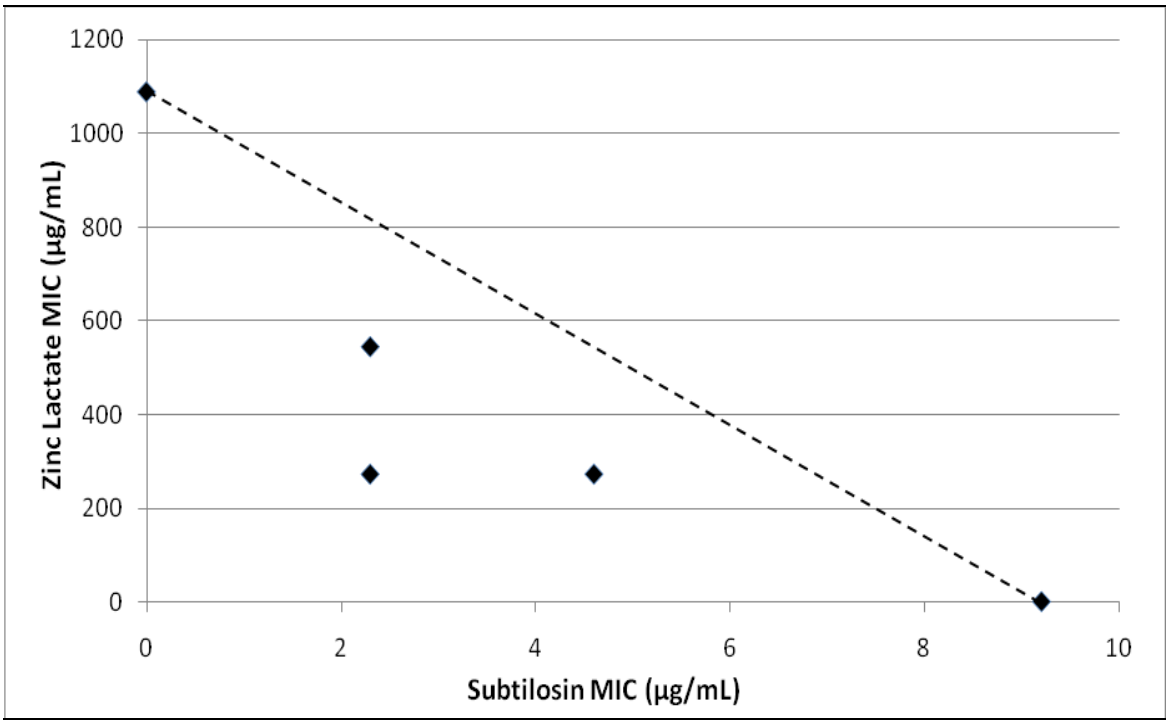

Figure 4. Subtilosin and zinc lactate act synergistically against *G. vaginalis*. The isobologram presents the individual MICs for zinc lactate (1090.1 µg/mL) and subtilosin (9.2 µg/mL) connected by a trendline. The points below the trendline represent the combinatorial concentrations of subtilosin and zinc lactate that completely inhibited *G. vaginalis* growth. While the combinations representing the upper point (2.3 µg/mL subtilosin, 545 µg/mL zinc lactate) and the far right point (4.6 µg/mL subtilosin, 272.5 µg/mL zinc lactate) both fall below the line, indicating synergy, the lowest point (2.3 µg/mL subtilosin, 272.5 µg/mL zinc lactate) demonstrates the strongest synergy between the two substances.

SUGGESTIONS FOR FUTURE STUDIES

The research described in Chapters 2-5 of this dissertation focused on the complete characterization of the *B. amyloliquefaciens*-produced bacteriocin subtilosin. Subtilosin is a prime candidate for inclusion in personal care products and alternative therapies designed to prevent and treat BV, a serious gynecological condition. The data from Chapter 5 showed that subtilosin has synergy with the four selected natural antimicrobials, and future research will continue to study subtilosin's synergy with other antimicrobial compounds, such as saponins. The effects of combining subtilosin with two or more synergistic compounds are unknown; therefore, future checkerboard assays are necessary to determine the relationship between subtilosin and multiple synergistic antimicrobials.

Once the combinatorial studies are completed, the preliminary stages of product formulation will commence. Along with collaborators from the Rutgers University Pharmacy Department, thin hydrogel buffers containing purified subtilosin and other antiviral/antimicrobial compounds will be constructed. These hydrogels are designed with the intention of preventing HIV acquisition, and those containing subtilosin will hopefully prevent BV development. The gels will be assayed using the same vaginal tissue model described in Chapter 3 for their safety for human tissues, as well as their ability to impede *G. vaginalis* biofilm formation. Eventually, it is hoped that these hydrogels will be affordably manufactured and delivered to third world countries, where social and cultural stigmas prevent the use of condoms and other contraceptives, and can help slow the progress of two incredibly serious and widespread diseases.

Finally, recent funding from the Rutgers University Technology Commercialization Fund will allow for scale-up fermentation and production of subtilosin. In order for any BV-targeted products to be generated, sufficient quantities of purified subtilosin will be required. Thus, it will be necessary to define the growth medium that gives optimal subtilosin production from *B. amyloliquefaciens* cultures; for safety purposes, it will also be important to guarantee that all ingredients in the newly-designed medium are of non-animal origin. The fermentation facilities at Rutgers University should make this process easily achievable, ensuring that the process of formulating a subtilosin-based personal care product remains unimpeded.

Curriculum Vitae

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Education

September 2005 -- September 2010	Rutgers University, Microbiology and Molecular Genetics
September 2001 – May 2005	University of Connecticut (Storrs), Molecular and Cell Biology, B.Sc.

Positions Held

June-August 2009, 2010	Koft-Umbreit Graduate Fellow, Rutgers University
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Publications

1. **Sutyak Noll, K**., Sinko, P., and M. L. Chikindas. (2010) The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, ε-poly-L-lysine, and zinc lactate against the human pathogen *Gardnerella vaginalis*. Probiotics Antimicrob Prot (prepared for submission).
2. **Sutyak Noll, K.**, Sinko, P., and M. L. Chikindas. (2010) Elucidation of the molecular mechanisms of action of the natural antimicrobial peptide subtilosin against the bacterial vaginosis-associated pathogen *Gardnerella vaginalis*. Antimicrob Agents Chemother (submitted).
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