LACTOSPORIN, A NATURAL ANTIMICROBIAL PROTEIN FOR CONTROL OF BACTERIAL VAGINOSIS

by

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ABSTRACT OF THE DISSERTATION

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Bacterial vaginosis (BV) is one the most common infections in women of reproductive age which results from the replacement of healthy vaginal microflora mostly by anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella bivia* and *Peptostreptococcus* spp. Conventional treatment of BV with the antibiotics metronidazole and clindamycin, as recommended by the Centers for Disease Control and Prevention, does not eradicate all of the microorganisms associated with BV and many women remain colonized with *G. vaginalis* and other anaerobes and inhibit the healthy vaginal microflora such as *Lactobacillus spp*. Lactosporin, a natural antimicrobial protein produced by *Bacillus coagulans* ATCC 7050 have shown to be an effective and safe alternative for BV treatment. This dissertation describes the isolation, characterization, mode of action and safety of lactosporin. Lactosporin was found to be an anionic (pI=3.5-4), heat stable protein that its activity is highly pH dependent. Lactosporin inhibited *G. vaginalis* in well diffusion assay while it had no effect on healthy vaginal lactobacilli. Mode of action studies established that lactosporin inhibits *G. vaginalis* by forming pores in the cytoplasmic membrane, causing efflux of intracellular material and dissipation of the ΔpH component of the proton motive force (PMF). The saferty of lactosporin was

evaluated in-vitro by using EpiVaginal™ ectocervical tissue model where it was shown to be a safe, non-toxic preparation for feminine care application.

To Mom and Dad
for their love,
endless support
and encouragement

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CHAPTER I.

LITERATURE REVIEW

Probiotic bacteria: how the enemy is killed?

1. Introduction

Numerous research studies have highlighted the health benefits associated with consumption of probiotic bacteria. In the past decade, there has been an increasing consumer demand for so-called functional foods such as food products supplemented with probiotic organisms (Shendereov *et al*. 1997). FAO and WHO have defined probiotic bacteria as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". There is a large volume of literature supporting the use of probiotics in the management and treatment of a diverse range of gastrointestinal disorders such as lactose intolerance, diarrhea, food allergies, gastroenteritis, intestinal infections, as well as high cholesterol and inhibiting bacterial enzymes in the intestine that are involved in the synthesis of carcinogens in the colon (Fuller, 1992; Kailasapathy and Chin 2000; Salminen 1996; Daly *et al*. 1998). Most probiotic bacteria commonly used in dietary supplements or food products are lactic acid bacteria that belong to the genera *Lactobacillus* and *Bifidobacterium, however,* recently some spore-forming bacteria (mostly *Bacillus* spp.) have been exploited as possible probiotics (Hyronimus *et al*. 2000; Cutting, 2011).

There are a number of mechanisms by which probiotics achieve their associated health benefits however; the mode of action of probiotic bacteria is not fully understood. Studies have shown that probiotic bacteria can modulate the host immune response, enhance the function of the intestinal epithelial barrier or produce antimicrobial compounds than can inhibit pathogenic bacteria. The mechanism of action of probiotic bacteria will be discussed in this chapter.

2. Mode of action of probiotic bacteria

2.1. Immunomodulation of the host

One of the putative effects of probiotic bacteria is the modulation of the host immune response. Current evidence suggests that probiotic bacteria can exert their immunomodulatory effects by altering specific immune parameters in a strain and dose-dependent manner. The immune system is comprised of two systems which includes the innate (non-specific) and the acquired (specific) immune systems. Both components of the immune system have the ability to work independently or collaboratively through a complex network of cells, tissues and organs to defend the host by generating an immune response. The immunomodulatory effect of probiotics is mainly achieved by mechanisms that involve monocytes, macrophages, epithelial, dendritic, and lymphoid cells.

2.1.1. Effects of probiotics on epithelial cells

The intestinal epithelium has a large surface area (~400 m^2) and is known to be the major port of entry for pathogenic microorganisms. The epithelial tissue is comprised of clusters of contiguous epithelial cells that form an impermeable layer which lines the intestinal mucosa and thus provides the first line of defense against invading pathogenic microorganisms. Epithelial cells are important components of the innate immune system and have the ability to produce compounds such as cytokines, chemokines and antimicrobial peptides. The innate immune system differentiates commensal bacteria from microbial pathogens through pattern-recognition receptors (PRRs) that are possibly

located on the epithelial cells (Ng *et al*. 2009). A group of PPRS, Toll-like receptors (TLRs), are embedded in the membranes of the innate immune system cells. These proteinaceous structures recognize microbial components (lipopolysaccharides (LPS), flagella, or bacterial DNA/RNA) of invading microorganisms via pathogen associated molecular patterns (PAMPs) (Takeda and Akira, 2005). Stimulation of TLRs by these microbial components can induce different signaling pathways of which, nuclear factor-κB (NF-κB) has been shown to be the major regulatory pathway in response to invading microorganisms (Elewaut *et al*. 1999). NF-κB is usually found in the cytoplasm conjugated to its inhibitory component, I-κB protein. Degradation of I-κB results in the transfer of NF-κB to the nucleus and its activation which ultimately induces the production of pro-inflammatory cytokines (Hegazy and El-Bedwey, 2010). Studies have demonstrated that some probiotic bacteria have the ability to suppress inflammatory signaling pathways generated by the immune system by inhibiting the production of pro-inflammatory cytokine (interleukin-2 [IL-2], IL-4, IL-5, IL-6, IL-8, IL-12 and tumor necrosis factor alpha [TNF-α]) or by inducing the production of protective cytokines (IL-10, IL-32) that cause increased epithelial cell regeneration or reduced epithelial cell apoptosis (Yan and Polk 2002; Tien *et al*. 2006). It has been reported that certain probiotic bacteria can attenuate pro-inflammatory responses by delaying the degradation of I-κB protein which can inhibit or delay the activation of NF-κB pathway (Neish *et al*. 2000). Zhang *et al*. (2005) observed that *Lactobacillus rhamnosus* GG reduced the production of a pro-inflammatory mediator, interleukin-8 (IL-8), induced by the tumor necrosis factor-α (TNF-α), in Caco-2 intestinal epithelial cells via NF-κB/I-κB pathway. Petrof and colleagues demonstrated that VSL#3 probiotic cocktail (a mixture of 8

different strains) inhibited the degradation of I-κB that resulted in the inhibition of NF-κB pathway (2004). It was also shown that the DNA derived from probiotic cocktail VSL#3 delayed the activation of NF-κB pathway and stabilized the I-κB protein levels (Jijon *et al*. 2004). Therefore, the inhibition of NF-kB activation has been reported as one possible mechanism by which probiotic bacteria modulate the immune response.

2.1.2. Effects of probiotics on lymphocytes, dendritic cells and monocytes

Lymphocytes and dendritic cells: Lymphocytes are a group of white blood cells that play a critical role in the regulation of the adaptive immune system. B and T cells are the two distinct classes of lymphocytes that mediate antibody (humoral) and cell-mediated immune responses, respectively.

B cells are produced and matured in the bone marrow and function by producing antibodies (immunoglobulins) that attach to the foreign antigens that had initially triggered their production (Ng *et al*. 2009). Research suggests that some probiotic bacteria confer their immunostimulatory effects through modulating the function of B-cells. In a study conducted by Kaila *et al*. (1992), the administration of *Lactobacillus rhamnosus* GG to young children with acute rotavirus diarrhea resulted in an increase in the production of IgG, IgA and IgM antibodies, which enhanced the humoral immune response and promoted the clinical recovery.

The other major lymphocytes, T cells, are originated in the bone marrow but mature in the thymus. The function of T cells is highly dependent on the dendritic cells

(DCs) that are present throughout the intestine. DCs are antigen-presenting cells that play an important role in early bacterial recognition in both innate and acquired immune systems. Immature DCs exist in small quantities in different tissues in the body but their maturation process takes place in peripheral tissue where they acquire antigens. Matured DCs then migrate to lymphoid organs where they interact with the T cells. DCs are pivotal activators of the naïve T cells (the T cells that have not previously been in contact with antigens) resulting in the development of an immune response (Banchereau and Steinman 1998; Stagg *et al*. 2004). Many experimental models have suggested that probiotic bacteria can stimulate the regulatory T cells which in turn, mediate an anti-inflammatory response (Di *et al.* 2005; Smith *et al*. 2005).

Monocytes: Monocytes are a group of white blood cells that originate from the bone marrow. During an infection or inflammation, these white blood cells leave the blood stream and migrate into the tissues where they differentiate into macrophages and DCs. Monocytes are considered to be the secondary antigen presenter to T cells (Shi and Pamer, 2011). It was observed that probiotic *L. plantarum* was able to increase the synthesis and production of the protective cytokine (IL-10) in inflamed colon which ameliorated the inflammation and increased pathogen tolerance (Pathmakanthan *et al*. 2004).

2.2. Enhancement of epithelial barrier function by probiotics

The gastrointestinal barrier provides the first intestinal line of host defense by protecting the internal environment against potentially harmful agents (Isolauri, 2001). The compromised barrier will allow pathogens and toxins to enter the body therefore,

maintaining the barrier's integrity is crucial. The intestinal barrier is composed of epithelial cells and intracellular junctions such as tight junctions (TJ), adherens junctions, gap junctions and desmosomes. These structures function to eliminate the majority of invading microorganisms and their metabolites from accessing the subepithelial cells (Ukena *et al*. 2007). Disruption of the epithelial barrier function can be observed in patients with different gastrointestinal complications such as inflammatory bowel disease (IBD) or gastrointestinal infections (Schmitz *et al*. 1999, Sakaguchi *et al*. 2002; Wyatt *et al*. 1993). It has been documented that probiotic bacteria have the ability to enhance the function of the intestinal epithelial barrier in a strain and dose-dependent manner. The improvement of the intestinal barrier function can be achieved through several mechanisms such as competitive exclusion and mucus production, enhancing the expression of tight junction proteins, production of cytoprotective molecules and/or inhibition of epithelial cell apoptosis.

2.2.1 Competitive exclusion of pathogens and mucus production

A number of probiotic strains have the ability to eliminate pathogenic microorganisms by either limiting the intestinal epithelial binding sites or by displacing the microbes that are already attached. Research has shown that some strains of *Lactobacillus* express mucus-binding pili, which put this group of bacteria in an advantage for colonization (Kankainen *et al*. 2009). In experimental studies with murine models some *Lactobacillus* strains caused a decrease in the colonization of *Helicobacter pylori*, the causative agent of gastric ulcers (Johnson-Henry *et al*. 2004; Sgouras *et al*. 2004). Another mechanism by which probiotic bacteria improve barrier function may

relate to mucin (mucus) production from epithelial cells. Mucus layer covers the epithelial surface of the gastrointestinal tract and serves as the first barrier that pathogens face (Ohland and MacNaughton, 2010) and prevents the attachment of pathogenic bacteria to the epithelial layer (Mack *et al*. 2003). Some pathogens have the ability to produce enzymes that digest the mucus. Mucus degradation provides carbon and energy sources to pathogens and makes the epithelial cells readily accessible to them (Deplancke and Gaskins, 2001). It is documented that a thinner mucus coat is observed in sites of inflammation caused by disease-causing bacteria (Swidsinski *et al*. 2007). Some probiotic bacteria such as *L. plantarum* 299v or VSL#3 up-regulate the expression of mucin genes (*MUC*) followed by an increase in mucus secretion (Mack *et al*. 1999; Otte and Podolsky 2004) thus preventing the attachment of bacterial pathogens.

2.2.2. Tight junction protein expression enhancement

Tight junctions are structures that play a significant role in the permeability of the intestinal epithelial barrier and are comprised of two major types of transmembrane proteins: occludin and claudin and one major type of cytoplasmic protein: zonula occludens (ZO). The function of occludin in tight junctions is regulated through phosphorylation at specific sites (Andreeva *et al*. 2001). Claudin, the other major protein in tight junctions, is mainly involved in the passage of ions and small molecules across the barrier in a charge and size-dependent manner (Hou *et al*. 2008). The cytoplasmic protein ZO links the transmembrane junctional proteins to the actin-cytoskeleton (Hartsock and Nelson, 2008). Probiotics *L. rhamnosus GG* and *Bifidobacterium lactis* showed an increase in phosphorylation of occuldin and ZO-1 proteins in intestinal Caco-2

cells, which ultimately resulted in an increased epithelial resistance to invading microorganisms (Mathias *et al*. 2010). Another study revealed that probiotic *Bifidobacterium infantis* caused an increase in the expression of occludin and ZO-1 proteins while the expression of claudin-1 protein was reduced. The changes in the expression of tight junction proteins reduced the intestinal barrier permeability and therefore increased the host immunity to pathogens (Ewaschuck *et al*. 2008).

2.2.3. Production of cytoprotective molecules

As mentioned previously, the intestinal epithelial barrier protects against the potentially harmful antigenic or infectious substances that are present in the intestinal lumen. Some bacteria can produce metabolites that decrease the permeability of the epithelial barrier. For example, production of heat shock proteins that occurs after a thermal stress (i.e., fever) has protective effects on epithelial cells against a variety of oxidative, thermal osmotic or inflammatory stresses and ultimately enhances the epithelial barrier function (Parsell and Lindquist, 1993). Toa *et al*. (2006) demonstrated that *L. rhamnosus* GG induces the expression of heat shock proteins by producing soluble bioactive factors that enhance the function of the epithelial barrier and protect the epithelial cells from hostile microorganisms. Another example of microbial metabolites that fortify the integrity of the intestinal barrier is the quorum sensing molecules (QSM) produced by *Bacillus subtilis*. *B. subtilis* is an avian and mammalian commensal bacterium with potential probiotic characteristics (Casula and Cutting, 2002). Fujiya *et al*. reported that QSM produced by *B. subtilis* upregulates the expression of cytoprotective heat shock proteins that strengthen the epithelial barrier functionality (2007).

2.2.4. Effects of probiotic bacteria on epithelial cell apoptosis

The balance between cell proliferation and cell death maintains the epithelial cell homeostasis. Apoptosis or programmed cell death accounts for the majority of cell loss in the gut (Potten, 1997). It was demonstrated that *L. rhamnosus* GG promoted the integrity of the epithelial barrier via the regulation of both anti-apoptotic (activation of Akt or protein kinase B) and pro-apoptotic pathways (inhibition of p38-mitogen activated kinase (MAPK activation) (Yan and Polk, 2002).

2.3. Antimicrobial production by probiotics

Probiotic bacteria produce a number of metabolic byproducts with antimicrobial activity that can inhibit the growth of pathogenic microorganisms. These antimicrobials include organic acids, hydrogen peroxide, biosurfactants, bacteriocins and bacteriocin-like inhibitory substances (BLIS). In vitro studies have demonstrated that more than half of lactobacilli strains produce all three of these defense factors (Aroutcheva *et al*. 2001).

2.3.1. Production of organic acids

A large number of lactobacilli inhibit the growth of pathogenic bacteria by producing organic acids such as lactic and acetic acids that creates a low pH environment for the growth and survival of pathogens (Vandenbergh, 1993). The inhibitory effects of organic acids against pathogens or spoilage bacteria are attributed to their ability to cross the cytoplasmic membrane (in protonated form), decrease the intracellular pH and

ultimately cause cell death by disrupting the critical cell functions (Cherrington *et al*.1991). Two different research groups demonstrated that probiotic *L. lactis, L. casei Shirota and L. acidophilus* strains had inhibitory effects against pathogenic *E. coli* O157:H7 by producing lactic acid (Brasshears *et al*. 1998; Ogawa *et al*. 2001). Some strains of lactobacilli (*L. acidophilus, L. rhamnosus* and *L. bulgaricus*) and bifido bacteria (*B. bifidus*) produce organic acids (lactic and/or acetic acids) that can inhibit *H. pylori* in vitro (Bhatia *et al*. 1989; Midolo *et al*. 1995). It was also noted that some antimicrobial agents were more effective when used in combination with organic acids. For instance, antimicrobial compounds produced by *L. plantarum* were more effective against the test organisms when used in conjunction with lactic acid (Niku-Paavola *et al*. 1999). It has been demonstrated that lactic acid in addition to its antimicrobial activity, can compromise the integrity of the outer membrane (lipopolysaccharide layer) of Gram-negative cells such as *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium and increase the susceptibility of these organisms to other antimicrobial agents (Alakomi *et al*. 2000). Krasner and colleagues (1956) observed the growth inhibition effect of *Candida albicans culture when* mixed with lactobacilli. This inhibitory effect was attributed to lactic acid production that decreased the pH of the medium to 3.7- 4.2. In other studies, conducted by numerous research groups, vaginal lactobacilli has shown to produce lactic acid (by anaerobic metabolism of glycogen) that maintains the normal vaginal pH (< 4.5). Production of lactic acid is considered to be one of the major protection mechanisms of lactobacilli against vaginal infections (Hanna *et al*. 1985; Boris and Barbes, 2000; Graver and Wade, 2011; Kovachev, 2011). Andersch *et*

al. demonstrated that lactic acid can be as effective as the antibiotic metronidazole in the treatment of bacterial vaginosis (1986).

2.3.2. Production of hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced by bacteria that can reduce oxygen to H_2O_2 or water in aerobic conditions. It has been suggested that production of H_2O_2 in lactic acid bacteria is catalyzed by flavoprotein oxidases that are contained by these cells (Whittenbury 1964). Some lactobacilli probiotics can produce sufficient quantities of H_2O_2 to inhibit bacteria such as *Pseudomonas* spp., *Bacillus* spp., and *Proteus* spp., *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium* (Dahiya and Speck, 1968; Watson and Schubert, 1969; Price and Lee, 1970; Tharrington and Sorells, 1992). The antimicrobial activity of H_2O_2 against bacteria is due to the generation of oxidizing metabolites such as the radical OH^- that can cause damage to the cellular DNA (Zalan *et al*. 2005). The H_2O_2 producing probiotic strains are the predominant microorganisms in the vaginal tract of healthy women (McGroarty, 1993) whereas their absence is associated with increased bacterial infection during pregnancy (Hillier *et al*. 1993*).* In a study conducted by Pashaian and Oganesian (2011), it was observed that *L. delbrueckii* MH-10 isolated from the vagina of healthy female subjects was a potent H_2O_2 producer with a wide spectrum of antimicrobial activity against different bacterial cells.

2.3.3. Production of biosurfactants

Some probiotic microorganisms have the ability to produce biosurfactants, which are amphipathic, surface-active compounds with an inclination to accumulate at interfaces, in particular, liquid-air interfaces. Biosurfactants can facilitate the uptake of water-immiscible substrates by lowering the surface tension at the phase boundary or work as emulsifying agents. Glycolipids are the most extensively studied group of biosurfactants (Fiechter, 1992). Production of biosurfactants from various probiotic strains is widely reported (Walencka *et al*. 2008; Rivardo *et al*. 2009; Gudina *et al*. 2010). These biosurfactants exert their activity by exhibiting antimicrobial and/or anti-adhesion effects. For instance, probiotic *Bacillus subtilis* and *Bacillus lichenformis* produced biosurfactants with anti-adhesin properties that inhibited the biofilm formation of pathogenic bacteria such as *E.coli* and *Staphylococcus aureus* (Rivardo *et al*. 2009). Walencka and colleagues (2008) reported on a biosurfactant produced by *L. acidophilus* that reduced the biofilm formation by *S. aureus* and *S. epidermidis.*

2.3.4. Production of bacteriocins

In the recent years antimicrobial proteins produced by probiotic bacteria have gained a great deal of interest due to problems associated with antibiotic resistance. A number of pathogenic bacteria that are resistant to conventional antibiotics show susceptibility to these natural antimicrobial proteins (Moll *et al*. 1999). Bacteriocins are ribosomally synthesized proteinaceous antimicrobial compounds produced by a large number of microorganisms. Production of bacteriocins is a defense mechanism for bacteria to protect their ecological niche from invading microorganisms (Nes *et al*. 1996).

These antimicrobials inhibit the growth of closely related species (Tagg *et al*. 1976; Cleveland *et al*. 2001) while having no effect on the producer strain (De Vuyst and Leroy 2007). Bacteriocins are usually positively charged peptides (cationic) with hydrophobic patches that kill sensitive cells, in most cases, by forming pores in their membrane thereby causing leakage of low molecular weight intracellular compounds (Fig. 1) and depletion of proton motive force (PMF) and perturbing the membrane of sensitive cells (Chung *et al*. 2000).

The majority of lactic acid bacteria such as *Lactococcus* spp., *Lactobacillus* spp. or *Pediococcus* spp. have the ability to produce bacteriocins and/or BLIS (Stile and Hasting, 1991). Some of the known and well characterized bacteriocins are commonly used for different commercial applications. For example, nisin is a bacteriocin produced by *Lactococcus lactis* with generally recognized as safe (GRAS) status for use in certain food products. Nisin has successfully been used in over forty countries worldwide for food preservation purposes and has been shown to be effective against some Gram-positive foodborne pathogens such as *L. monocytogenes* (Cleveland *et al*. 2001). It should be noted that bacteriocins are differentiated from antibiotics based on their synthesis, mode of action, toxicity, and resistance mechanisms, but they show antimicrobial activity against various pathogens in a manner similar to antibiotics (Cleveland *et al*. 2001).

2.3.4.1. Classification of bacteriocins

Bacteriocins are classified into three distinctive classes; class I, class II, and class III. Class I bacteriocins or lantibiotics are small (<5 kDa) cationic peptides with a minimum of 19 and a maximum of 38 amino acids (Sahl and Bierbaum, 1998).

Lantibiotic bacteriocins undergo a post translational modification and contain unusual amino acid residues such as dehydroalanine, dehydrobutrine, lanthionine and β-methyl lanthionine (Abee *et al*. 1995). A large number of Gram-positive bacteria produce lantibiotics. Currently about 40 lantibiotics with different structures and mechanisms of action have been identified (Chatterjee *et al*. 2005). Class I is further divided into two subclasses: class Ia and class Ib. Class Ia bacteriocins are hydrophobic and cationic peptides that confer their antimicrobial activity by forming a pore in the membrane of sensitive cells. Nisin, the most studied bacteriocin, belongs to the class Ia family and is a 34-amino acid peptide with a molecular mass of 3.5 kDa (Cleveland *et al*. 2002). Class Ib bacteriocins is comprised of globular peptides that carry no net charge with a more rigid structure compared to class Ia (Altena *et al*. 2000).

Class II bacteriocins are small (<10 kDa), heat stable and membrane active peptides that are not modified. This class of bacteriocins is divided into three subclasses; class IIa (pediocin-like bacteriocins), class IIb (two-peptide bacteriocins), and IIc (other none pediocin like bacteriocins). Class IIa bacteriocins are peptides with positive net charge that normally exhibit antimicrobial activity against *L. monocytogenes*. These bacteriocins contain 37 to 48 amino acids, which make up a highly conserved hydrophilic N-terminal sequence and a hydrophobic C-terminal portion (Drider *et al*. 2006). Class IIb bacteriocins are two-peptide bacteriocins that require the complimentary action of both components for full activity. In some instances no activity is observed for individual components and in other cases the two components enhance the activity of one another. The examples of some of the characterized two-peptide bacteriocins include enterocin L50, lactacin F, lactococcin, mutacin IV and plantaricin (Garneau *et al*. 2002). Class IIc

bacteriocins are *sec*-dependent secreted peptides such as enterocin P (Parente and Ricciardi, 1999).

Class III bacteriocins are large (>30 kDa), heat labile peptides. So far only a few class III bacteriocins have been characterized. Helveticin J from *L. helveticus* and enterolysin A from *Enterococcus faecalis* are some examples of class III bacteriocins (Nigutova *et al.* 2008).

3. Spore-forming probiotics

Although lactic acid bacteria are the most commonly used probiotics, in the last couple of decades some spore-forming bacteria have been exploited as probiotics due to their unique properties. The genus *Bacillus* is the most extensively studied group of spore-forming probiotics. Other spore-formers being used as probiotic bacteria are *Paenibacillus polymyxa* and *Brevibacillus laterosporus* that were initially classified as *Bacillus* species (Hyronimus *et al*. 2000; Hong *et al*. 2005; Cutting, 2011). There are several advantages of using spores over other non-spore forming bacteria. Spores are heat resistant and can survive harsh conditions during production and storage processes. They are also able to withstand the extreme physiological conditions such as low pH of the gastrointestinal tract, bile salts and enzymes (Spinosa *et al*. 2000; Hong *et al*. 2005; Cutting, 2011). *B. coagulans, B. subtilis* are two of the main species that are used in commercial probiotic products. *B. coagulans* probiotic supplements are often marketed as *Lactobacillus sporogenes*, which is not a valid species name according to Bergey's Manual of Determinative Bacteriology (Cheng, 1975). *B. coagulans* produces different antimicrobial proteins such as coagulin and lactosporin that exhibit antimicrobial activity

against different Gram positive bacteria (Hyronimus *et al*. 1998; Riazi *et al*. 2007). Several commercially available products claim to contain *B. subtilis* spores as their predominant probiotic strain (Biostart®, Liqualife®, Promarine® and BaoZyme-Aqua) (Cutting, 2011). A popular Japanese staple, Natto, is produced through fermentation of soybeans using *B. subtilis*. This food product contains approximately 10^8 spores per gram and is claimed to exert health benefits by enhanceing the immune system and by reducing blood clotting (Sumi *et al*. 1987; Hosoi and Kiuchi, 2004). It has also been shown that different *B. subtilis* strains produce several antimicrobial proteins such as subtilin and subtilosin that show significant inhibitory activity against some pathogenic microorganisms (Stein *et al*. 2004; Stein, 2005; Kabore *et al*. 2012).

4. Selection criteria for probiotics and their safety

Several research groups have suggested criteria to evaluate potential probiotic strains. Current criteria require probiotic strains to be of human origin if the probiotics are intended for human use, be resistant to gastric acidity and bile toxicity, adhere to epithelial cells and colonize the gut, produce antimicrobial substance and be able to modulate the immune system of the host (Lee and Salminen, 1995). The safety is of pivotal importance when selecting for a probiotic strain. The safety of probiotics can be evaluated by conducting appropriate studies to monitor the probiotics/host interactions and to understand the intrinsic properties of that strain (Saarela *et al*. 2000). While determining the safety of a probiotic, it is crucial to consider the potential vulnerability of the host, genetic stability of the probiotic strain over time, potential for pathogenicity and

toxicity and the potential for transfer of the antibiotic resistance genes (Sanders *et al*. 2010).

5. Probiotics and Bacterial Vaginosis

Bacterial vaginosis (BV) is one of the most common infections in women of reproductive age. It usually occurs among sexually active women and approximately 10-15% of the female population is affected by this infection (Persaud *et al*. 2006). BV results from the replacement of healthy vaginal microflora mostly by anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella bivia* and *Peptostreptococcus* spp. (Lin *et al*. 1999). Although it is not a life threatening infection, it increases a woman's susceptibility to HIV infection upon exposure to a partner infected with HIV, so a reduction in the incidence of BV will subsequently reduce the risk of HIV acquisition. The physiology of the female genital tract makes women more susceptible to HIV than men. Changes created by BV in the vaginal ecosystem have been shown to create a favorable environment for HIV acquisition (Shcmid *et al.* 2000; Schwebke 2003; Sha *et al*. 2005; Watts *et al*. 2005). Direct up-regulation of HIV replication by pathogenic bacteria involved in bacterial vaginosis has been reported in many studies (Al-Harthi *et al*. 1999; Hashemi *et al*. 2000). *G. vaginalis,* the predominant microorganism in BV, has been isolated in 98% of cases and has been found in high concentrations (60%) of HIV positive women (Mascellino *et al*., 1991). *G. vaginalis* and *Prevotella* may also contribute to intra-amniotic infections since these pathogenic microorganisms and their toxins are able to cross the placenta, thus causing brain damage in the fetus (Eschenbach 1997). They can also contribute to premature rupture of membranes and preterm labor

(Hillier *et al*. 1995). Conventional treatments of BV with the antibiotics metronidazole and clindamycin, as recommended by the Centers for Disease Control and Prevention, does not inhibit all the microorganisms that are linked to BV and many women remain colonized with *G. vaginalis* and other anaerobes (Ferris *et al*. 1995; Boris *et al*. 1997). These treatments are only effective in 60% of all cases with 30-40% rate of recurrence of BV. Furthermore, the antibiotic therapy of BV has contributed to expansion of the antibiotic resistant *G. vaginalis, P. bivia* and *Peptostreptococcus* spp. (Bannatyne and Smith 1998; Lubbe *et al*. 1999; Liebetrau *et al*. 2003). Effective BV treatment should avoid a negative impact on the growth of healthy vaginal microflora such as *Lactobacillus* spp. However, the inhibition of lactobacilli growth may occur with metronidazole and clindamycin doses that are even lower than those administered topically (Aroutcheva *et al*. 2001; Simoes *et al*. 2001). The high rates of BV recurrence make conventional treatments discouraging. The search for alternative therapies based on the production of natural compounds by lactobacilli, which protect the vagina against exogenous and endogenous infections, may enhance the efficiency of BV therapy and ultimately reduce the risk of HIV infection.

6. Summary

Probiotic bacteria are becoming more fascinating as we further understand the mechanisms by which they confer their health benefits. A large volume of research papers confirm that probiotic bacteria are able to modulate the host immune system, enhance the intestinal barrier function and produce a variety of antimicrobial compounds.

Due to their beneficial health effects, probiotics and/or their antimicrobial metabolites may provide novel approaches for prevention and treatment of different diseases.

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Hypothesis and Objectives

It is hypothesized that the novel antimicrobial protein produced by *Bacillus coagulans* ATCC7050, lactosporin, is an effective and non-cytotoxic compound for control of bacterial vaginosis. The specific aims addressed by this research include:

1. Isolation and characterization of lactosporin
2. Determination of the mechanism of action by which lactosporin inhibits BV associated microorganisms.
3. In vitro evaluation of the bactericidal effect and cytotoxicity of lactosporin

CHAPTER II

ISOLATION AND CHARACTERIZATION OF LACTOSPORIN

The following articles are published in the Journal of Applied Microbiology and Journal of Microbiological Methods

Chapter II.A.

CHARACTERIZATION OF LACTOSPORIN, A NOVEL ANTIMICROBIAL PROTEIN PRODUCED BY *BACILLUS COAGULANS* ATCC 7050

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1. Abstract

Aims: To characterize the antimicrobial protein produced by *Bacillus coagulans* ATCC 7050 used in the probiotic dietary supplement (Lactospore® Probiotic, Sabinsa Corp.).

Methods and Results: *B. coagulans* was grown at 37°C for 18 h. The cell free supernatant was concentrated 10-fold (lactosporin preparation, LP). The antimicrobial activity of LP was confirmed against the indicator microorganism *Micrococcus luteus* ATCC 10420 in a well diffusion assay. The proteinaceous nature of LP was determined following exposure to different enzymes. The activity of LP was pH-dependent, but stable to heat. The isoelectric point (pI) of LP was determined to be 3.5-4 by isoelectric-focusing. PCR analyses showed no similarity between lactosporin and known antimicrobial proteins produced by members of the *Bacillus* spp.

Conclusion: Lactosporin is a novel antimicrobial protein. Initial characterization indicates that it may fall outside of the conventional classification of class I and II bacteriocins. Loss of activity after exposure to a number of proteolytic enzymes and lipase suggest that this antimicrobial may possess a lipid moiety which contributes to its inhibitory activity.

Significance and Impact of Study: The unique characteristics of lactosporin, including its stability to low pH and heat treatment, and its antimicrobial activity against pathogenic microorganisms, indicate that it may have potential for application in foods and personal care products.

2. Introduction

Microorganisms produce a variety of antimicrobial proteins of which bacteriocins are the most studied. Bacteriocins are ribosomally-synthesized antimicrobial compounds of proteinaceous nature, produced by virtually all microorganisms, and act mostly against closely-related species (Klaenhammer, 1993). The ability of lactic acid bacteria (LAB) to produce bacteriocins is well documented (Kelly *et al*. 1996, Ennahar *et al*. 1999, Parente and Ricciardi, 1999, Rodriguez *et al*. 2003). Bacteriocin-producing LAB have been the focus of a large number of research efforts, due to their ability to inhibit foodborne pathogens such as *Listeria monocytogenes*, and their potential application as food preservatives (Cleveland *et al.* 2001) and in consumer health-related products (Aranha *et al.* 2004, Reddy *et al*. 2004). In addition, other microbial-derived proteinaceous antimicrobials have been reported, such as the non-ribosomally synthesized ε-L-poly-lysine (Yoshida and Nagasawa 2003), the cyclopeptide subtilosin (Stein *et al*. 2004), and lipopeptide (Huang *et al*. 2006). Production of bacteriocins or bacteriocin-like inhibitory substances (BLIS) by several species within the *Bacillus* genus has been reported by several research groups. For instance, polyfermenticin SCD is a heat-labile, proteinase K-sensitive bacteriocin produced by *Bacillus polyfermenticus*, which has antagonistic activity against other *Bacillus* spp. (Lee *et al*. 2001). Subtilin is a lantibiotic-type bacteriocin produced by a strain of *Bacillus subtilis* with inhibitory activity against a wide range of Gram-positive bacteria (Chan *et al*. 1993). This bacteriocin may be of interest for the pharmaceutical industry due to its antimicrobial activity against various human pathogens (Burkard *et al*. 2007). Subtilosin A is a ribosomally-synthesized and post-translationally modified antimicrobial peptide

produced by *Bacillus subtilis.* This cyclic bacteriocin is active against a variety of Gram-positive bacteria including the foodborne pathogen *L. monocytogenes* (Shelburne *et al*. 2007). Hyronimus et al (1998) reported a heat-stable BLIS produced by a strain of *Bacillus coagulans*. The amino acid sequencing of this antimicrobial peptide revealed its similarity to pediocin, a bacteriocin produced by *Pediococcus acidilactici*.

In this paper we describe a new antimicrobial compound named lactosporin produced by a strain of *B. coagulans* isolated from a probiotic dietary supplement, Lactospore® Probiotic (Sabinsa Corp., Piscataway, NJ). Lactosporin-producer strain was first isolated in 1933 and described as *Lactobacillus sporogenes*. It was later classified as *B. coagulans* since this strain possesses key features that are identical to the aforementioned spore-former. It should be mentioned that in many cases, commercial products containing *B. coagulans* use the name *L. sporogenes* on the labels for marketing purposes, although it is not an accepted name for this species according to the Bergey's Manual of Determinative Bacteriology (Chen, 1975). Lactosporin exhibits some characteristics similar to bacteriocins; however, it differs from the previously-reported bacteriocins from *Bacillus* spp., such as coagulin produced by *B. coagulans*, and subtilin and subtilosin A produced by *Bacillus subtilis.*

3. Materials and methods

3.1. Bacterial strains and growth condition

Producer strain *B. coagulans* ATCC 7050 was grown aerobically at 37°C in Difco™ Lactobacilli MRS broth (Becton, Dickinson) with aeration for 24 h. The indicator strains *Micrococcus luteus* ATCC 10420, *L. monocytogenes* Scott A, *Escherichia coli*

O157: H7 ATCC 43859 and *Salmonella enterica* serovar Enteritidis were cultured aerobically in tryptic soy broth (Becton, Dickinson) (enriched with 6 g l^{-1} yeast extract and 2.5 g l^{-1} glucose) at 37°C except for *M. luteus* ATCC 10420, which was grown at 30°C. *Micrococcus luteus* is widely used as an indicator microorganism for bacteriocin detection assays (Li *et al*. 2005, Wirawan *et al*. 2006). In addition, nine vaginal *Lactobacillus* strains isolated from the healthy human subjects were tested in the course of this study along with the following pathogens: *Gardnerella vaginalis* ATCC 14018, vancomycin-resistant *Enterococcus faecium*, methicilin-resistant *Staphylococcus aureus*, *Streptococcus agalactiae* (GBS) strains 35 and 749, and *E. coli* (urogenital strain). All of the above-mentioned microorganisms were grown on tryptic soy agar (TSA; Becton-Dickinson) supplemented with 5% (v/v) sheep blood. *G. vaginalis* strain was grown on a selective Human Blood Tween (HBT) bilayer agar medium (Becton-Dickinson). The *Lactobacillus* strains were propagated on MRS agar plates and the vaginal pathogens were grown anaerobically in a glove box (Plas-Labs, Lansing, MI) at 37 °C overnight. The cultures used in this study were maintained as frozen stocks at -80°C in a bio-freezer.

3.2. Production of lactosporin

The production of lactosporin was carried out in a fermenter (ABEC, USA) with a total volume of 150 l. *Bacillus coagulans* inoculum in MRS (5% v/v) was added to the production medium (MRS broth). After 24 h, the culture was centrifuged (10000 *g*) to remove the cells and the supernatant was collected, concentrated 10-fold by lyophilization and filter-sterilized through a 0.45µm filter (Nalgene, Rochester, NY). The cell-free supernatant was named lactosporin preparation (LP). For some experiments LP

was partially purified by dialysis through a 1 kDa-cutoff dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA).

3.3. Inhibitory activity of lactosporin

The antimicrobial activity of LP was determined by a well-diffusion assay as described by Cintas *et al*. (1995) with minor modifications. A 4-ml lawn of soft (0.7% agar) TSA enriched with 6 g l^{-1} yeast extract and 2.5 g l^{-1}glucose, containing 10^6 CFU ml^{-1} of the indicator strain was poured on top of an enriched hard (1.5% agar) TSA layer. LP sample (200 µl) was added to 16-mm wells punched in the solidified bi-layer agar. Plates were kept in the refrigerator overnight to allow the sample to diffuse into the agar and subsequently incubated at 37°C for 18 h. The inhibitory activity of LP was also tested against *L. monocytogenes* Scott A, *E. coli* O157:H7 (ATCC 43859) and *S.* Enteritidis as described above. In this and all other experiments nisin A was used as a positive control. The commercial preparation (Sigma, St. Louis, MO) contains 2.5% nisin A in denatured milk solids and was solubilized using nisin diluent (HCl solution at pH 2.0). This preparation of nisin A was chosen since it is not different from commercially available GRAS (generally recognized as safe) preparation of nisin that is used for food preservation.

3.4. Effect of pH and temperature on activity of lactosporin

The inhibitory activity of LP was estimated at different pH values by adjusting its pH (approx. 5.2) using 3N HCl and 3N NaOH followed by incubation at room temperature for 2 h. Eight aliquots of the LP, representing pH values 3-10 (without

adjusting the pH to the original pH of lactosporin) respectively, were tested for inhibitory activity against indicator microorganism, *M. luteus*, by the well diffusion assay. The thermostability of LP was determined by incubating the LP samples at 30, 37, 50, 60, 80, and 100°C for 30 min. Aliquots (1 ml) of LP were dispensed in microcentrifuge and heated to the desired temperatures in a heating block (dry bath incubator, Fisher Scientific). The temperature was monitored using a thermometer that was installed in the heating block. Heat-treated samples were cooled in the air to room temperature and subsequently tested for activity against *M. luteus* ATCC 10420 in a well diffusion assay.

3.5. Sensitivity of lactosporin to enzymes

The sensitivity of lactosporin to enzymes was tested by incubating the LP samples in the presence of 10 mg ml^{-1} of each enzyme for 2 h at their optimal temperature, having adjusted the LP and the enzyme mixture to the optimal pH for activity of that specific enzyme according to the manufacturer's protocol (Sigma, St. Louis, MO, USA). The enzymes tested and their respective conditions are as follows: (i) proteinase K (37 ºC, pH 7.5); pepsin (37 ºC, pH 2.0); α-chymotrypsin (25 ºC, pH 7.8); (iv) protease (37ºC, pH 7.5); (v) trypsin (25 ºC, pH 7.6); catalase (25 ºC, pH 7.0) and (vi) lipase classes I, II and VII (37 ºC, pH 7.2) (Sigma). After incubation, the pH of the mixture was adjusted to the original pH of the LP (~ 5.0) with 3N HCl or 3N NaOH and the samples were analyzed for antimicrobial activity in the well diffusion assay with *M. luteus* as an indicator. Controls used for this experiment included LP samples treated with specific solvents used for preparation of each enzyme stock solution (LP + enzyme solvents) (Sigma) and MRS medium treated with 10 mg ml^{-1} of each enzyme solution

(MRS + enzymes). . Similarly the pH of controls was first adjusted to the optimum pH for activity of enzymes and after the incubation was adjusted back to the optimal pH for activity of lactosporin. A 10 mg ml^{-1} nisin stock solution was exposed to 10 mg ml^{-1} α-chymotrypsin and was used as a control for this experiment.

3.6. Detection of antimicrobial activity on SDS-PAGE and native-PAGE

Both 5× and 10× concentrated LP samples were used for visualization of the antimicrobial activity using Native PAGE ready gels (10-20% Tris-HCl, Bio-Rad, Hercules, CA). Two duplicate samples were separated on the same gel (100 V). At the end of electrophoresis the gel was cut into two halves, vertically. One part was stained with silver stain (Bio-Rad, Hercules, CA) and the other part was tested for antimicrobial activity in overlay test as described previously (Bhunia *et al* 1987) with *M. luteus* as the reference microorganism.

3.7. Determination of the antimicrobial activity of lactosporin as arbitrary units ml^{-1}

Arbitrary Unit (AU) ml^{-1} is defined as a reciprocal of the highest dilution of an antimicrobial compound which gives a visible zone of inhibition in the overlay assay against the indicator microorganism (Callewaert and De Vuyst, 2000). Eight serial two fold dilutions of a partially purified and 20× concentrated and dialysed LP were prepared. The antimicrobial activity of each dilution was determined in a well diffusion assay against sensitive strain *M. luteus*. Nisin A (10 mg ml^{-1} stock solution) was used as a positive control for this experiment. Two-fold dilutions of Nisin A stock solution were

similarly prepared to a dilution of 2^{-8} and tested for inhibitory activity in the well diffusion assay.

3.8. Determination of the isoelectric point (pI) of lactosporin

The isoelectric point (pI) of lactosporin was determined by using Rotofor® (BioRad, Hercules, CA), an isoelectric focusing apparatus. A 50-ml sample of partially-purified 2× concentrated LP was applied to the Rotofor® unit in an ampholyte preparation with a pH range of 3-10 (BioLyte, BioRad). The separation procedure was performed as described by the supplier. Following separation, 20 fractions were collected, and the pH of all fractions was adjusted to approx. 4.5-5.0 with 3N HCl or 3N NaOH. The activity of each fraction was determined by well diffusion assay against *M. luteus*.

3.9. Polymerase Chain Reaction (PCR) testing

PCR was used to investigate the possible relatedness of the antimicrobial protein produced by *B. coagulans* ATCC 7050 with the known bacteriocins produced by *Bacillus* spp. including coagulin produced by *B. coagulans* I_4 (Hyronimus *et al*. 1998), and subtilin and subtilosin, which are produced by *Bacillus subtilis* (Klein *et al*. 1992, Stein *et al*. 2004). Genomic DNA was extracted from overnight cultures of *B. coagulans* and *B. subtilis* ATCC 6633. Briefly, the cells were harvested by centrifugation at 13,000 *g* for 3 min at room temperature and resuspended in 0.5 M EDTA pH 8.0. The cell suspension was treated with 100 µl of 20 mg ml^{-1} lysozyme, 10 µl of 20 mg ml^{-1} proteinase K and 8 µl of 2.5 U $µl^{-1}$ mutanolysin (all from Sigma, St. Louis, MO, USA) at 37 °C for 60 min. The purification of genomic DNA from the cell lysate was conducted using the Wizard®

SV genomic DNA purification system (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol. *B. coagulans* I_4 plasmid pUC19 containing a 4.9 kb fragment encoding coagulin operon, and genomic DNA were a kind gift from Dr. Maria Urdaci (Enita de Bordeaux, Gradignan Laboratoire de microbiologie, France). Primers specific for the known *Bacillus* bacteriocin structural genes *coaA, spaS,* and *sboA* were designed, and synthesized by Sigma Genosys (Germany) (Table1). PCR-based detection of these genes was accomplished using a master mix containing the appropriate primer pair, dNTPs (10 mmol l^{-1}), buffer, and 0.2 U HotMaster Taq polymerase (all PCR reagents were supplied by Eppendorf, Hamburg, Germany) according to the manufacturer's specifications. PCR was performed using an Applied Biosystems GeneAmp PCR System 2400 apparatus (Applied Biosystems, Foster City, CA) and products were separated on a 1% agarose gel.

4. Results

4.1. Spectrum of antimicrobial activity of lactosporin

Lactosporin was active against the Gram-positive microorganisms *M. luteus* and *L. monocytogenes* when tested in the well diffusion assay, but not against Gram-negative bacteria. LP also did not show inhibitory activity against most of the vaginal lactobacilli tested, in contrast to nisin which inhibited all the vaginal lactobacilli tested (Table 2). The activity of LP was 16 AU ml^{-1} when tested against the indicator strain *M. luteus*.

4.2.Sensitivity of lactosporin to different pH, temperature and enzymes

Testing of LP at a wide range of pH values showed it to be more active at lower pH values with total loss of activity occurring at pH values over 7.0 (Fig. 1). Upon heat-testing, the antimicrobial compound was stable at a temperature range of 30-60 °C after 30 min of incubation, and only a 1mm decrease in the size of inhibition zone occurred when lactosporin was at 80 and 100 °C (Fig. 2). There was no detectable antimicrobial activity following exposure of LP to: (i) the proteolytic enzymes proteinase K, trypsin, α-chymotrypsin and protease, and (ii) lipase classes II and VII (data not shown).

4.3. Determination of isoelectric point (pI) of lactosporin

Isoelectric focusing of partially purified LP with a Rotofor® cell yielded 4 active fractions. The zones of inhibition of tested samples from fractions 18, 19 and 20 were large (5, 7 and 12 mm respectively), without a clearly-defined edge; in contrast, the zone of inhibition of fraction 1 was small with a well-defined edge. Further experiments showed that the antimicrobial activity seen in fractions 18-20 was due to the ampholyte used in the Rotofor system (Riazi *et al*. 2007), and that fraction 1 is the active fraction of the LP. The pI of lactosporin was estimated to be approx. 3.5- 4.0 (Fig. 3).

4.4.Visualization of lactosporin activity by SDS-PAGE and native-PAGE

The antimicrobial activity of LP was detected following analysis by native-PAGE. This was represented by zones of inhibition for both the five and ten fold concentrated LP on the half of the gel overlaid with *M. luteus,* corresponding to the two bands observed on the silver-stained gel portion (Fig. 4). Following SDS-PAGE, a band

was visualized with a molecular mass of 25-30 kDa, however no zone of inhibition was observed on the gel portion overlaid with *M. luteus* (data not shown).

4.5. PCR analysis

PCR using primers specific for the known *Bacillus* bacteriocins structural genes *coaA, spaS* and *sboA* yielded the expected-sized product from *B. coagulans* I_4 (*coaA*) and *B. subtilis* ATCC 6633 (*spaA* and *sboA*), but not from the lactosporin-producer *B. coagulans* ATCC 7050.

5. Discussion

In this study, lactosporin, an antimicrobial compound produced by the probiotic-derived *B. coagulans* ATCC 7050 strain, has been isolated and partially characterized. Its spectrum of activity included the food-pathogen *L. monocytogenes*, but like most other bacteriocins derived from Gram-positive bacteria (Abee *et al*. 1995), it did not inhibit any of the Gram-negative bacteria tested. The activity of this antimicrobial substance is remarkably pH-dependent, but is not affected by a wide range of temperature. Furthermore, the activity of this antimicrobial compound was no longer detected after exposure to a number of proteolytic enzymes such as proteinase K and protease, thus indicating the proteinaceous nature of this inhibitory substance. Loss of activity following exposure to lipase suggests that this antimicrobial compound may be a protein with an associated lipid moiety which appears to be required for activity similar to class IV bacteriocins (Klaenhammer 1993). The most recent classifications of bacteriocins exclude class IV, which leaves no room for our new BLIS to be placed in. Of the

remaining 3 classes, only class III gathers large size molecules such as zoocin A from *Streptococcus equi* subsp. *zooepidemicus*, millericin B from *Streptococcus milleri*, streptococcin A-M57 from *Streptococcus pyogenes* and dysgalacticin from *Streptococcus dysgalactiae* (Simmonds *et al.* 1996; Beukes *et al.* 2000; Heng *et al.* 2004, Heng *et al*. 2006). However, these bacteriocins are heat-labile substances from various species of *Streptococcus* whereas lactosporin is produced by the *Bacillus* Spp. and is heat-stable. Therefore, it is highly possible that the newly isolated lactosporin belongs to antimicrobial lipopetides or surfactin-like molecules (Yu *et al*. 2002, Stein *et al*. 2005).

A protein band with a molecular weight of about 25-30 kDa was visualized on the stained portion of a gel following SDS-PAGE. However, no corresponding antimicrobial activity of this protein was detected on the portion of the gel overlaid with the indicator microorganism. The loss activity might be due to the irreversible denaturation of this protein by SDS or reduction of disulfide bridges that might be present in the structure of this antimicrobial protein with 2-β-mercaptoethanol. On the other hand, antimicrobial activity of lactosporin was detected on an overlaid portion of a gel following native-PAGE. Although the band with molecular weight of approx. 37 kDa corresponding to this zone of inhibition was visualized on the stained portion of the gel, conclusions regarding the exact molecular weight of this protein cannot be made due to the fact that proteins in native electrophoresis systems migrate according to their charge, and not their weight. Hyronimus *et al*. (1998) and Le Marrec *et al*. (2000) have conducted comprehensive studies on coagulin, the BLIS produced by *B. coagulans* I_4. Unlike lactosporin, the activity of which is highly pH-dependent, the activity of coagulin was retained at a wide range of pH (3-8). It has been reported that coagulin belongs to the

group of pediocin-like bacteriocins (class IIa), with slight differences in structure when compared to pediocin PA-1 and AcH. In general, class IIa bacteriocins are characterized as small (<10-kDa) molecules with a net positive charge and basic pI values (8-10) (Drider *et al*., 2006). The results of the present study indicate that lactosporin is an anionic protein with a pI value of 3.5-4 which is in contrast to the characteristics of class IIa bacteriocins. Genetic analysis through PCR-screening also demonstrated no relatedness between the antimicrobial protein produced by *B. coagulans* ATCC 7050, and coagulin produced by *B. coagulans* I_4, or to subtilin and subtilosin produced by *B. subtilis*. These data collectively suggest that lactosporin is a novel antimicrobial protein with characteristics different to these previously-described bacteriocins produced by *Bacillus* spp.

Antimicrobial proteins that are naturally produced by LAB are currently receiving well-deserved attention due to the potential health problems associated with the use of chemical antimicrobial agents in foods and personal care products, and because of the rapidly growing microbial resistance to conventional antibiotics (Abee *et al*. 1995; Cleveland *et al.* 2001). These issues have created a demand for the use of safe and natural antimicrobial compounds in foods and personal care products, for preservation and medical applications. Previous studies have demonstrated that a large number of antimicrobial substances produced by bacteria, such as bacteriocins and BLIS, are inhibitory to some pathogenic microorganisms such as *L. monocytogenes*. Nisin is the most studied bacteriocin with FDA-approved GRAS status for application in certain food products (U.S. Food and Drug Adminstration 1988). Produced by *Lactococcus lactis* ssp. *lactis,* it belongs to the lantibiotic class of bacteriocins and shows activity against a large

number of Gram-positive bacteria (Cleveland *et al.* 2001). The heat stability, increased antimicrobial activity at low pH values, along with its activity against foodborne pathogens and microorganisms associated with bacterial vaginosis, are the very important factors that make lactosporin, too, a great potential candidate for food and medical/personal care applications.

6. Acknowlegements

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7. References

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8. Table and figure legends

Table 1. Inhibitory activity of lactosporin preparation against different bacteria.

Table 2. Primers specific for the structural genes of coagulin, subtilin and subtilosin used in this study.

Fig. 1. The pH-dependent activity of lactosporin preparation. Higher activity was retained at lower pH values and total loss of antimicrobial activity occurred at pH values of 7 and above.

Fig. 2. Temperature-stability of lactosporin preparation. After 30 min of treatment at 100°C, the preparation retained around 80% of its initial activity when tested against *M. lutues*.

Fig. 3. Isoelectric focusing of lactosporin preparation using Rotofor® cell. The antimicrobial activity of each fraction was tested against *M. luteus* in a well-diffusion assay. Bars with asterisks indicate fractions with antimicrobial activity due to the activity of the ampholyte used in isoelectric focusing (Riazi *et al*. 2007).

Fig 4. Native-PAGE visualization of 5× and 10× concentrated lactosporin preparation. A band was visualized for each of the samples used on the stained portion of the gel (A) corresponding to zones of inhibition on the portion of the gel overlaid with the sensitive strain *M. luteus* (B).

9. Tables and Figures

Table 1.

Microorganism	**Zone of Inhibition (mm)**
M. luteus	5
Lactobacillus 710	0
Lactobacillus 711	0
Lactobacillus 748	0
Lactobacillus 735	0
Lactobacillus 701	0
Lactobacillus 807	0
Lactobacillus 618	0
Lactobacillus 757	3
Lactobacillus 758	4
G. vaginalis	8
GBS 35	0
GBS 749	0
VRE	0
MRSA	0
E. coli (Urogenital)	0
E. coli O157:H7	0
S. Enteritidis	0
L. monocytogenes	4

Table 2.

Bacteriocin	Producing Organism	Primer	Sequence (5' to 3')	Reference
Coagulin[1]	*B. coagulans*	CoaAFwd	5'-GGTGGTAAATACTACGGTAATGGGGT-3'	(Le Marrec *et al*. 2000)
		CoaDRev	5'-GTGTCTAAATTACTGGTTGATTCGT-3'	
Subtilin[2]	*B. subtilis*	SpaSFwd	5'-CAAAGTTCGATGATTTCGATTTGGATGT-3'	(Klein *et al*. 1992)
		SpaSRev	5'-GCAGTTACAAGTTAGTGTTTGAAGGAA-3'	
Subtilosin[2]	*B. subtilis*	SboAFwd	5'-CGCGCAAGTAGTCGATTTCTAACA-3'	(Stein *et al*. 2004)
		SboARev	5'-CGCGCAAGTAGTCGATTTCTAACA-3'	

[1] Primer sequence based on GenBank accession number AF300457
[2] Primer sequence based on GenBank accession number AJ430547

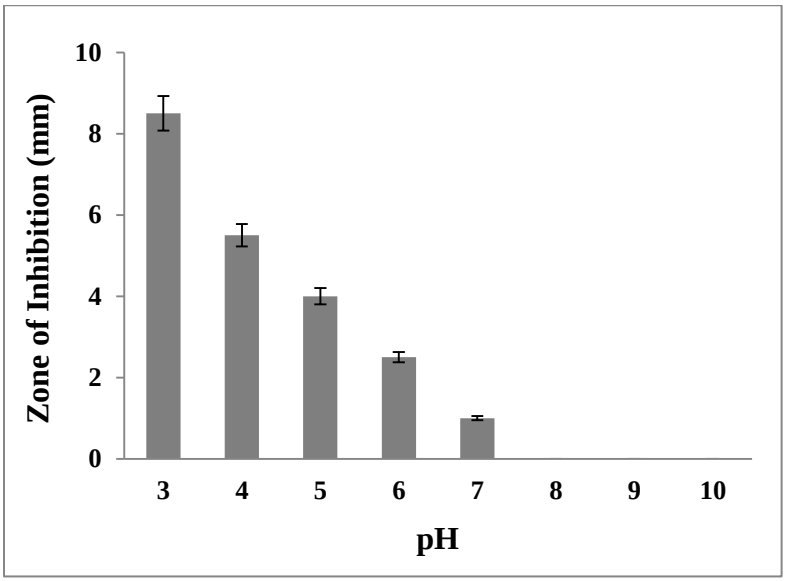

Fig. 1.

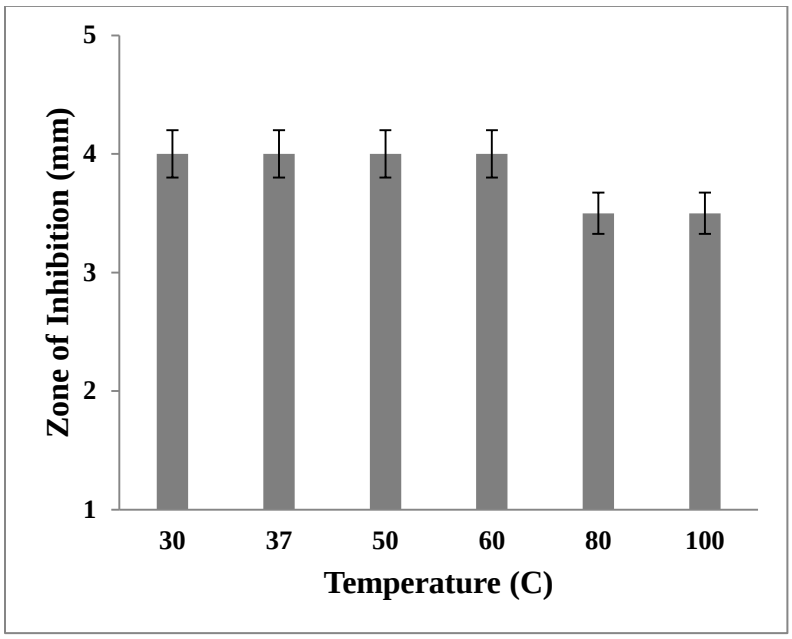

Fig. 2.

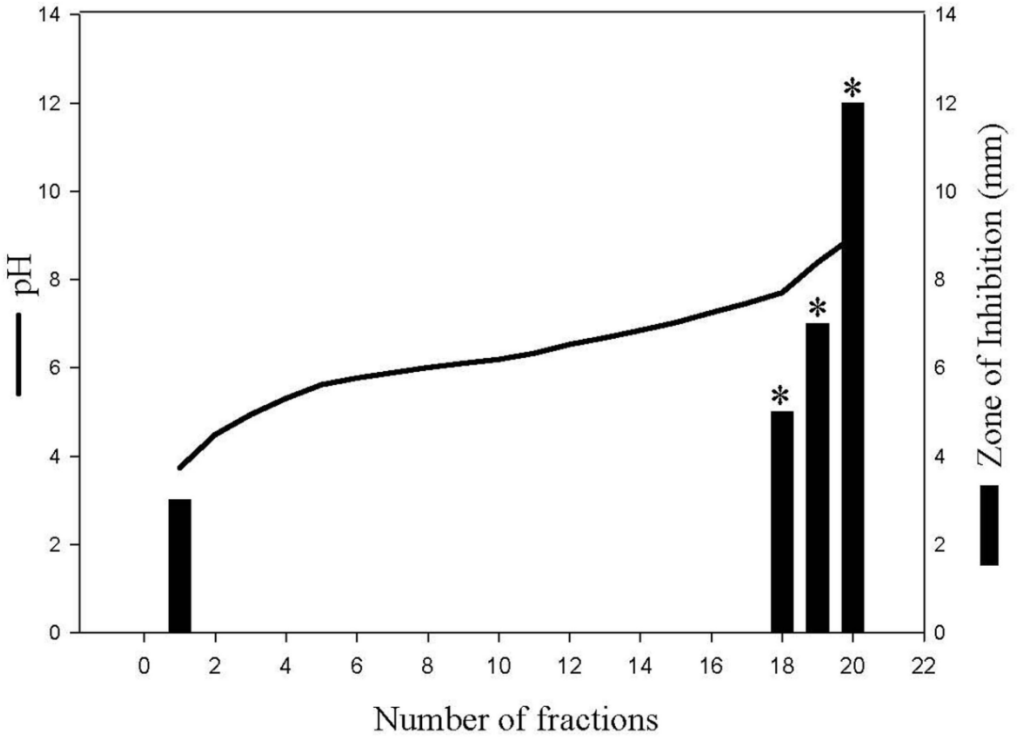
pH
Zone of Inhibition (mm)
Number of fractions
0
2
4
6
8
10
12
14
16
18
20
22
*
*
*

Fig. 3.

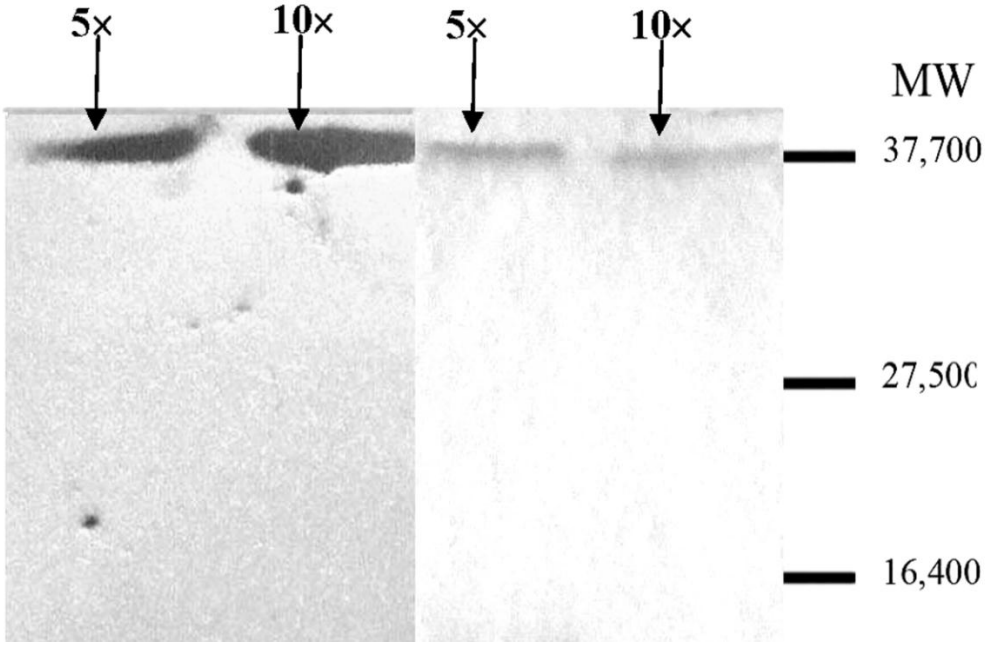

Fig. 4.

Chapter II.B.

Commercial ampholytes used for isoelectric focusing may interfere with bioactivity based purification of antimicrobial peptides.

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Keywords: Ampholytes, Rotofor, isoelectric focusing, bacteriocin

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1. Abstract

BioRad's Rotofor® system has been frequently used for the purification of proteins and smaller peptides such as bacteriocins. In this study, we report that some commercially available ampholytes used with the Rotofor® isoelectric focusing system possess antimicrobial activity, which may interfere with the purification of bacteriocins and bacteriocin-like substances.

Rotofor® is an isoelectric focusing apparatus which has been used for more than a decade for the purpose of protein purification (Ayala *et al*. 1998). Preservation of the biological activity may be crucial when biologically active proteins and peptides are studied using this purification method. Little attention is given, however, to the fact that the ampholytes used for the isoelectric focusing may actually interfere with some proteins' biological activity or with the activity detection assays.

Bacteriocins are small antimicrobial peptides that are synthesized ribosomally by virtually all bacteria (Cleveland *et al*. 2001). Bacteriocins of lactic acid bacteria have attracted much attention due to their food-related, personal care and medical applications. Purification of these antimicrobials using isoelectric focusing has been reported by several research groups (i.e., Green *et al*. 1997; Venema *et al*. 1997).

In Rotofor®, the separation of proteins is caused by formation of a gradually increasing pH gradient. This pH gradient is formed by ampholytes, zwitterionic molecules with low molecular weight, as the electric field is applied. The ampholyte systems used for the isoelectric focusing commonly contain a mixture of compounds with different isoelectric point (pI) values. When the electric field is applied, ampholyte molecules tend to migrate according to their net charge and then align themselves in a specific position between anode and cathode based on their isoelectric point (Stoyanov *et al.* 2005). In this study, we investigated the antimicrobial activity of the commercially available ampholytes commonly used for purification of the small antimicrobial peptides.

The ampholytes used in the study were Bio-Lyte® pH 3-10 (BioRad, Hercules, CA), Pharmalyte pH 3-10 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and Ampholine pH 3.5-9.5 (Amersham Biosciences AB, Uppsala, Sweden).

The activity of ampholytes was tested against *Micrococcus luteus* ATCC 10420, *Escherichia coli* O157:H7 ATCC 43859, *Listeria monocytogenes* Scott A, *Salmonella enterica* serovar Typhimurium UMR1, *Lactobacillus rhamnosus* 160 (L 160), *Lactobacillus* 710 (L 710), *Lactobacillus lactis* NCK 401 (NCK 401), *Pediococcus pentosaceus* ATCC 43200, and *Lactobacillus casei* Strain Shirota (LcS) in well diffusion assay as described by Lyon and Glatz (Lyon and Glatz, 1992). All assays were performed in duplicate. Nisin (10 mg/ml) and nisin diluent (hydrochloric acid solution, pH 1.7) were used as positive and negative controls for this experiment. Bio-Lyte®, Ampholine™ and Pharmalyte™ total samples were active against *L. monocytogenes*, *P. pentosaceus*, *M. luteus*, *L. casei* Shirota, *L. rhamnosus* 160, *L. lactis* NCK 401, *L. casei* Shirota, and *Lactobacillus* 710. All tested ampholytes were inhibitory to the Gram-positive bacteria used in this experiment and non-inhibitory to Gram-negative strains (Table 1). The inhibitory effects of ampholytes on Gram-positive and Gram-negative bacteria are similar to those of bacteriocins (Green *et al.* 1997). In Gram-negative bacteria, the outer membrane, which consists of phospholipids, proteins and lipopolysaccharides (LPS), protects the cell from antimicrobial action of bacteriocins and other small antimicrobial peptides (Abee *et al*. 1995).

The sample for Rotofor® was prepared by adding 2% (w/v) of ampholyte, 10% (v/v) glycerol (FisherChemicals, Fairlawn, NJ) and glycine (Merck, Darmstadt, Germany) at a final concentration of 5% (w/v) to 50 ml deionized water. The sample was loaded into the Rotofor® cell. After the procedure was completed the fractions were harvested and the pH of each fraction was measured and adjusted to approximately 5.0. The activity of each fraction was tested in well-diffusion assay using *M. luteus* ATCC 10420 as an indicator microorganism (Venema *et al*. 1997). The investigated electrolyte samples seemed to display a common trend in antimicrobial activity against *M. luteus;* the most noticeable inhibitory effect was exhibited by the fractions with higher isoelectric points. For the Bio-Lyte® brand, progressively increasing antimicrobial activity was observed in the fractions 15 through 20 (Fig 1.A.). Similarly, the Pharmalyte™ displayed increasing antimicrobial activity in fractions 18 through 20. Interestingly, for this brand, some antimicrobial activity was also noticeable in fraction 1 (Fig 1.C.). In the Ampholine™ sample, only fractions 18, 19, and 20 were inhibitory to the indicator microorganism, and though fractions 18 and 19 are the most inhibitory to the indicator microorganism (Fig 1.B.), the zone of inhibition in these fractions was not sharp. Fraction 14 to 20 of the nisin sample (10mg/ml) subjected to isoelectric focusing caused very sharp inhibition zones when used with the Bio-Lyte® ampholyte compared to the Bio-Lyte® sample alone (Fig 1.A.). Interestingly, the total sample of Ampholine™, when tested against *M. luteus*, showed a much larger zone of inhibition (12 mm) than the fraction 20 (pI 9-10) that was separated through the Rotofor®.

The commercially available ampholyte systems used in this study contain mixtures of short polypeptide molecules. Each of these molecules has a distinct pI point. There are only two amino acid residues (Lysine and Tyrosine) with the pK_a of their side chains in the range between 9 and 10. The polypeptide molecules with the pI point in the range 9-10 are likely to be rich in Lysine and/or Tyrosine. The antimicrobial effect of the ampholyte mixture can be due to various poly-lysines that are well known for their antimicrobial properties (Liang and Kim 1999, Yoshida and Nagasawa 2003). The presence of poly-lysine in the ampholyte mixture is purely speculative, since the specific composition of the commercial ampholytes from Amersham and Bio-Rad is not disclosed by the manufacturers (Stoyanov *et al.* 2005). Ultimately, however, these commercial ampholyte systems will have to be reformulated if they are to be used for purification of antimicrobial proteins based on the function. Finally, our observations suggest that of all ampholytes tested, Ampholine™ could be the most favorable choice for use in the Rotofor® system to purify antimicrobial peptides.

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3. Table and Figure Legends

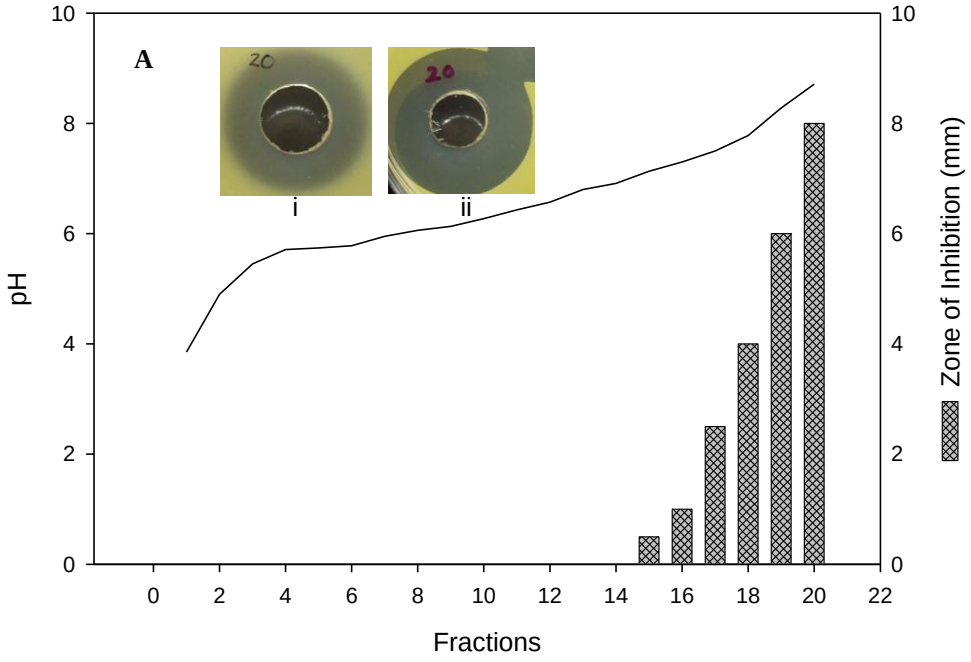
Table 1. Zones of inhibition (mm) caused by Bio-Lyte®, Ampholine™ and Pharmalyte™ against different bacterial strains. The inhibitory activity of ampholytes was determined by well diffusion assay.

Fig. 1. Antimicrobial activity distribution of Bio-Lyte® **(A)**, Ampholine™ **(B)**, and Pharmalyte™ **(C)** ampholytes after preparative isoelectric focusing. The inhibitory activity was determined by well diffusion assay using *M. luteus* ATCC 10420 as the indicator strain. Figure inserts iA and iiA show inhibition zones caused by Bio-Lyte® and nisin (10 mg/ml), respectively, in well diffusion assay against *M. luteus*. Similarly figure inserts iB and iC are visual representation of zones of inhibition caused by Ampholine™ and Pharmalyte™, respectively, after being fractioned through Rotofor®.

4. Tables and Figures

Table 1.

	Ampholyte			Controls	
Bacteria	Bio-Lyte®	Ampholine™	Pharmalyte™	Nisin (10mg/ml)	Nisin diluent
E. coli O157: H7	0	0	0	0	0
S. Typhimurium	0	0	0	0	0
L. monocytogenes	1	1	2	7	0
P. pentosaceus	6	2	4	15	0
M. luteus	18	12	17.5	15	0
L. lactis NCK 401	2	0	2	2	0
L. casei Shirota	3	2	2	10	0
L. rhamnosus 160	1	1	1	16	0
Lactobacillus 710	3	2	4	15	0


A
20
20
i
ii
pH
Zone of Inhibition (mm)
Fractions
0
2
4
6
8
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12
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20
22

Fig. 1.A.

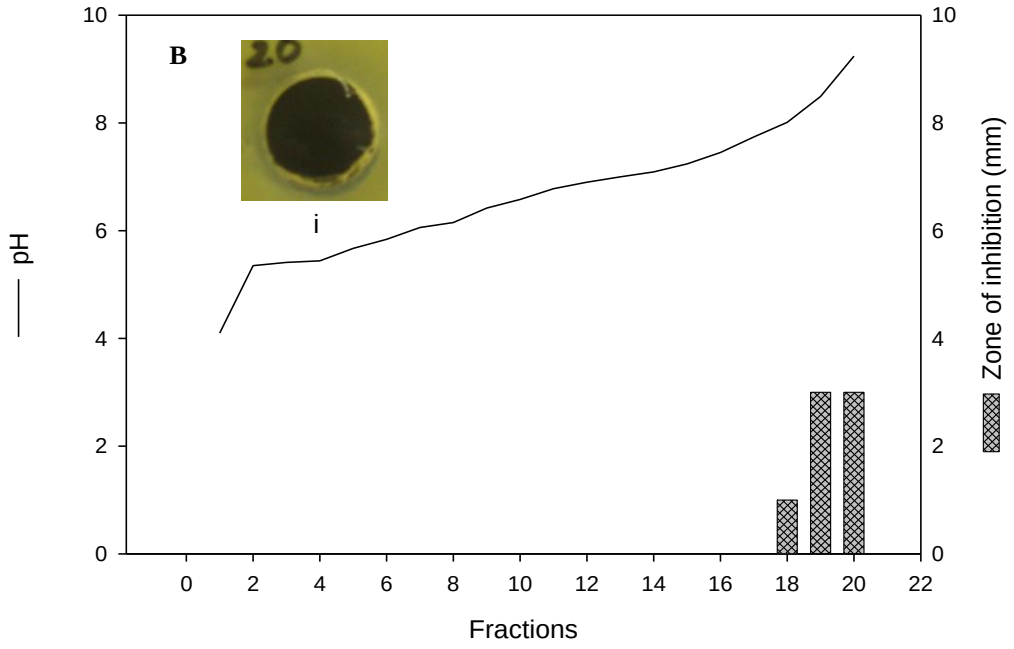
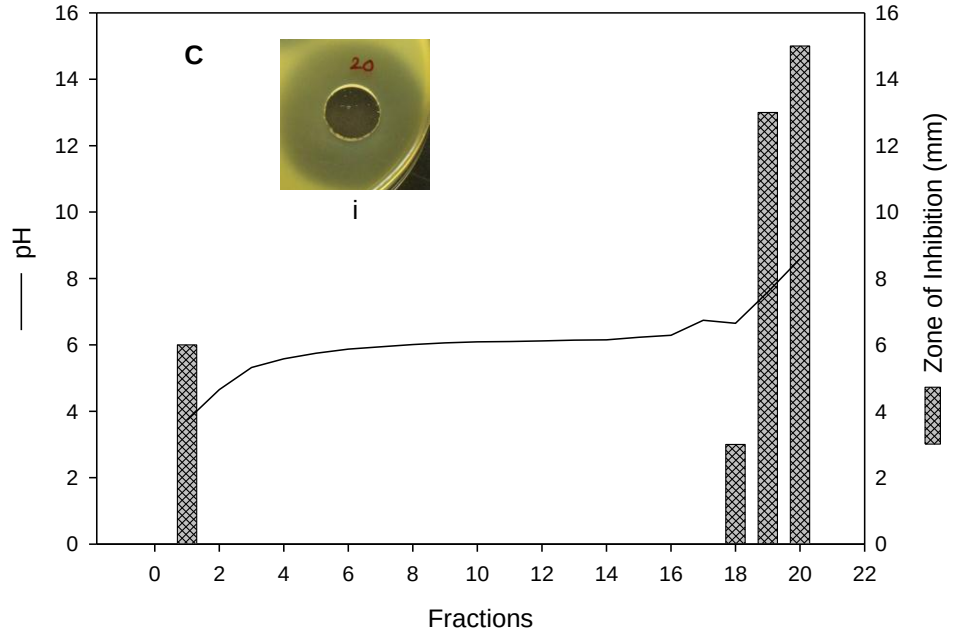

B
i
pH
Zone of inhibition (mm)
Fractions
0
2
4
6
8
10
12
14
16
18
20
22

Fig. 1.B.


C
20
i
pH
Zone of Inhibition (mm)
Fractions
0
2
4
6
8
10
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14
16
18
20
22

Fig. 1.C.

CHAPTER III.

DETERMINATION OF MODE OF ACTION AND SAFETY OF LACTOSPORIN

The following article is submitted for publication to the Journal of Applied Microbiology

Chapter III.

Determination of mode of action and safety of lactosporin, a novel antimicrobial protein produced by *Bacillus coagulans* ATCC 7050.

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1. Abstract

Aims: to determine the mechanism of action of antimicrobial protein, lactosporin, against *Gardnerella vaginalis* and to evaluate its safety in-vitro.

Methods and Results: *Bacillus coagulans* ATCC 7050 was grown at 37 °C for 18 hours. The cell free supernatant was concentrated 10-fold and screened for antimicrobial activity against indicator strain *Micrococcus luteus*. The mode of action of lactosporin was determined by measuring the potassium release and monitoring the changes in transmembrane potential ($\Delta\psi$) and transmembrane pH (ΔpH) of the sensitive cells. Lactosporin caused efflux of potassium ions from *M. luteus* cells and dissipation of ΔpH in *G. vaginalis* while it had no effect on the $\Delta\psi$. The safety of lactosporin was evaluated by using EpiVaginal™ ectocervical (VEC-100) tissue model. Over 80% of the cells in the vaginal tissue remained viable after exposure to lactosporin for 24 hours.

Conclusions: Lactosporin potentially exerts its antimicrobial activity by selective dissipation of ΔpH and/or by causing leakage of ions from the sensitive cells. Safety studies suggest that lactosporin is a non-cytotoxic antimicrobial for vaginal application.

Significance and Impact of the Study: This study revealed that lactosporin is an effective and safe antimicrobial preparation with potential application for control of bacterial vaginosis.

2. Introduction

Bacteriocins are ribosomally-synthesized antimicrobial compounds of proteinaceous nature produced by virtually all microorganisms that act against closely related species (Klaenhammer 1993). Bacteriocins produced by lactic acid bacteria (LAB) have received great attention in the past decade due to their potential applications as food preservatives (Cleveland *et al*. 2001). Production of bacteriocins and bacteriocin-like inhibitory substances (BLIS) from the *Bacillus* genus have been studied by several research groups (e.g., Babasaki *et al*., 1985; Stein *et al*. 2004; Shelburne *et al*. 2007; Sutyak *et al*. 2007; Sutyak Noll *et al*. 2011). Many bacteriocins and BLIS kill sensitive cells by a common mechanism of action through the formation of a transient pore in the cytoplasmic membrane resulted in the leakage of small intracellular compounds and dissipation of proton motive force (PMF) component(s). PMF is an electrochemical proton gradient across the cytoplasmic membrane which is composed of two components; membrane potential ($\Delta\psi$) and pH gradient (ΔpH). PMF is directly involved in a number of biological processes such as ATP synthesis, active ion transport, protein phosphorylation and bacterial motility (Montville and Bruno, 1994; Montville and Chen, 1998). Antimicrobial proteins may deplete either or both components of PMF and cause intracellular ATP depletion by either efflux or intracellular ATP hydrolysis (Chen and Montville, 1995; Montville *et al*. 1995).

Lactosporin is a novel antimicrobial protein produced by a strain of *B. coagulans* isolated from a dietary supplement, Lactospore® Probiotic (Sabinsa Corp., East Windsor, NJ, USA). Previous work has studied the characterization of

this antimicrobial including inhibitory spectrum, pH, temperature and enzyme sensitivity, isoelectric point determination, antimicrobial activity visualization by SDS and native-PAGE and PCR analysis. Lactosporin may have applications in feminine care products due to its antimicrobial activity against *G. vaginalis* (Riazi *et al*. 2009), one of the most frequent causative organisms in bacterial vaginosis (BV) (Turovskiy *et al*. 2011). BV is one the most common infections in women of reproductive age and approximately 10-15% of the female population are affected by this infection (Persaud *et al*. 2006). Effective BV treatment should avoid a negative impact on the healthy vaginal microflora such as *Lactobacillus* spp. Conventional treatments of BV with antibiotics such as metronidazole and clindamycin may have negative impact on the growth of vaginal lactobacilli (Aroutcheva *et al*. 2001) while lactosporin has no inhibitory effects against the healthy vaginal microflora (Riazi *et al*. 2009).

The aim of this research was to study the mechanism of action of lactosporin against *G. vaginalis* and to evaluate the safety of lactosporin in-vitro using the EpiVaginal tissue model from MatTek Corporation (Ashland, MA, USA).

3. Materials and Methods

3.1. Bacterial strains and growth condition

Producer strain *B. coagulans* ATCC 7050 was grown aerobically at 37 ºC in Difco Lactobacilli MRS broth (Becton, Dickinson) with aeration for 24 hours. The indicator strains *Micrococcus luteus* ATCC 10420 was cultured aerobically in tryptic soy broth (Becton, Dickinson) (enriched with 6 g l^{-1} yeast extract and 2.5 g

l^{-1} glucose) at 30 ºC. *M. luteus* is frequently used as an indicator microorganism in bacteriocin detection assay (Li *et al*. 2005; Wirawan *et al*. 2006). *G. vaginalis* ATCC 14018 was grown on a selective human blood Tween bilayer agar medium at 37 ºC, anaerobically. The cultures used in this study were maintained as frozen stocks at -80 ºC in a bio-freezer.

3.2. Production and inhibitory activity of lactosporin

The production of lactosporin was carried out as described by Riazi *et al*. (2009). For the mode of action and safety assays reported in this paper, the same batch (lot# 2/3) of lactosporin preparation was used (previously used for lactosporin characterization studies). Lactosporin samples were further concentrated by lyophilization (Freeze-dryer 4.5, Labconco) for both mode of action and safety experiments. The antimicrobial activity of lactosporin was confirmed by a well diffusion assay against *M. luteus* and *G. vaginalis*, as described by Cintas *et al*. (1995). In all experiments nisin A was used as a positive control. The commercial preparation (Sigma, St Louis, MO, USA) contains 2.5% nisin A in denatured milk solids and was solubilized using nisin diluent (HCl solution at pH 1.7).

3.3. Measurement of arbitrary units of activity (AU)

An arbitrary unit is defined as the reciprocal of the highest dilution showing inhibition against the indicator microorganism (Kojic *et al*. 1991). Ten 2-fold dilutions of lactosporin were made and tested for activity in well diffusion

assay against *M. luteus*. AU ml^{-1} was calculated based on the highest dilution with antimicrobial activity.

3.4. Measurement of potassium release

The measurement of potassium efflux was performed as described by Orlov *et al*. (2002) with some modifications. A potassium-selective electrode (Phoenix Electrode Company, TX, USA) was used to measure the K^+ ion release from the *M. luteus* ATCC 10420 after treatment with lactosporin or nisin. *M. luteus* was grown in trypticase soy broth enriched with 2.5 g l^{-1} glucose and 6 g l^{-1} yeast extract. Twenty ml culture was incubated aerobically at 37 °C until an optical density (OD_{600}) of 1-1.2 was reached. The cells were washed three times (5000 *g*, 10 min, 4 °C) with 20 ml of a 10 mM Tris acetate buffer solution containing 100 mM NaCl, pH 7.4. The washed cells were resuspended in 5 ml of the same buffer and kept on ice until use. The electrode was calibrated with 0.01, 0.1 and 1 mM solutions of KCl in 100 mM NaCl before each experiment and the measurements were done in small (10 ml) Pyrex beakers (Fisher Scientific) that contained a small magnetic stirrer. A 500 µl of a 20 times concentrated (320 AU) and dialyzed lactosporin or a 500 µl of a 10 mg ml^{-1} nisin A solution was added to each beaker containing 5 ml of the cells and the potassium release was measured every minute for 5 minutes. Nisin diluent and MRS medium were used as controls for this experiment.

3.5. Dissipation of transmembrane potential (Δψ)

The changes in transmembrane potential (Δψ) of intact *G. vaginalis* cells were determined according to the protocol outlined by Sims et al., (1974) and modified by Turovskiy *et al*. (2009). Briefly, *G. vaginalis* cells were grown anaerobically at 37 ºC in BHI medium supplemented with 3% horse serum to an OD_{600} of 0.6. Twenty ml of the culture were harvested by centrifugation (5000 *g*,10 min, 22 °C) followed by a wash step with 20 ml of fresh growth medium and resuspension in 200 µl of the same medium at room temperature. A Perkin Elmer LS-50B spectrofluorometer (Perkin Elmer Inc, Boston, MA) was utilized to evaluate the changes in the Δψ of the cells with excitation and emission wavelengths of 643 and 666 nm, respectively, and a slit width of 10 nm.

In quartz cuvettes with a 10 mm light path, 5 µl of the fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3$ (5)) (Molecular Probes, Eugene, OR) was added to 2 ml of fresh BHI broth supplemented with 3% horse serum followed by addition of 20 µl of the cell suspension, which caused a substantial decrease in fluorescence intensity. After the signal was stabilized, 2 µl of 5 mM nigericin (Sigma) was added to convert the ΔpH to Δψ. After the signal was equilibrated, either lactosporin, lactosporin diluent (MRS medium) (negative control), nisin (positive control) or nisin diluent (negative control) were added to each cuvette. This step was followed by an addition of 2 µl of 2 mM valinomycin (Sigma) to dissipate the remaining Δψ.

3.6. Dissipation of the pH gradient (ΔpH)

The changes in transmembrane pH (ΔpH) of *G. vaginalis* cells caused by lactosporin were studied according to the protocol outlined by Molenaar *et al.* (1991) and the modifications made by Turovskiy *et al*. (2009). Briefly, *G. vaginalis* was inoculated in 20 ml of fresh BHI medium supplemented with 3% horse serum and grown anaerobically at 37 ºC to an OD_{600} of 0.6. The cells were harvested by centrifugation (5000 *g*, 10 min, 22 °C), washed twice with 50 mM potassium phosphate buffer (PPB, pH 6.0) and resuspended in 200 µl of PPB. BCECF-AM (MP Biomedicals, Inc., Solon, OH), a pH sensitive fluorescent probe was added to the cell suspension for 5 minutes at room temperature. After the probe was incorporated into the membrane, the cells were washed twice with 1 ml of 50 mM phosphate buffer saline (PBS, pH 6.0) and resuspended in 200 µl of the same buffer. To monitor the dissipation of ΔpH, 2 ml of PPB (pH 7.0) and 10 µl of the cell suspension were added to quartz cuvettes and the fluorescence was measured in a Perkin Elmer LS-50B spectrofluorometer with slit widths of 5 nm for excitation and 15 nm for emission, and wavelengths of 502 and 525 nm, respectively. Once the signal had stabilized, the cells were energized with 4 µl of 2.2 mM glucose. The addition of glucose caused an increase in intracellular pH, therefore a noticeable and gradual increase in the fluorescence intensity was observed. After the signal stabilized, 2 µl of 5 µM valinomycin was added to convert the Δψ to ΔpH. After the addition of valinomycin, the cells were treated with either lactosporin, lactosporin diluent (negative control), nisin (positive

control) or nisin diluent (negative control). The last step included adding 2 µl of 2 µM nigericin to dissipate the remaining ΔpH.

3.7. Determination of safety of lactosporin using EpiVaginal™ ectocervical tissue model

The EpiVaginal™ (VEC-100) ectocervical tissue model (MatTek Corporation, Ashland, MA, USA) was utilized to evaluate the safety of lactosporin for vaginal application. To determine the cytotoxic effects of lactosporin on EpiVaginal™ tissue, the protocol outlined by MatTek Corp and Dover (2007) was followed. Briefly, tissues were placed in a 6-well plate containing 900 µl of DMEM-based DC-100 MM medium (MatTek Corporation) prior to exposure to the test compounds. The plate containing the tissues was placed in an incubator with 5% CO_2 for 1 hour at 37 °C to pre-equilibrate the tissues. After incubation for 1 hour, the media was removed and replaced with 900 µl of VEC-100-MM medium and then 83µl of lactosporin was applied on VEC-100 tissues topically in triplicate for 24, 36 and 48 hours. For the 48 hour exposure time, two washers were used to airlift the tissue inserts and 5 ml of the assay medium was placed in each well. Two negative controls were used for the cytotoxicity assay; sterile distilled water and an antifungal cream containing 4% miconazole nitrate (Monistat-3, Ortho McNeil Pharmaceutical, Inc., Raritan, NJ, USA) that is known to be a nontoxic preparation for vaginal application (Sawyer *et al*. 1975; Ozyurt *et al*. 2001; Ayehunie *et al*. 2006). The viability measurements

for these two negative controls were performed at 24, 36 and 48 hours similar to lactosporin.

A spermicidal cream containing 4% Nonoxynol-9 (Ortho Options CONCEPTROL vaginal Contraceptive Gel, Advanced Care Products, Skillman, NJ, USA) was used as the positive control due to its known toxicity to vaginal tissues for 2, 4 and 6 hours. At the end of each exposure time, tissue viability was determined using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The effective time for each product to reduce the tissue viability to 50% (ET-50) was then calculated.

The viability of ectocervical cells was determined by measuring the breakdown of the yellow tetrazolium to purple formazan as only viable cells are able to carry out this reaction (Mosmann, 1983). After completion of each exposure time for lactosporin and the controls, the liquid in the inserts was removed and the tissues were washed with Dulbeccos phosphate buffer saline solution (D-PBS). Test compounds were removed by gently swabbing the surface of the tissue with a sterile polyester fiber tipped swab (Thermo-Fisher, Waltham, MA, USA). Tissues were placed in a 24-well plate and each well was filled with 300 µl of MTT solution that was prepared in culture medium (1 mg ml^{-1}). After 3 hour incubation at 37 °C with 5% CO_2, the tissue inserts were removed and placed in a new 24-well plate and 1660 µl isopropanol (MatTek Corporation) was added to each well to extract the formazan. The plate was incubated at room temperature in the dark for 24 hours. Then, 200 µl of this mixture was transferred to a 96-well plate and measured spectrophotometrically in triplicates at 570 nm

using a plate reader (MRX revelation, Dynex Technologies, VA, USA). The percentage of tissue viability was calculated using the following equation; %viability = OD_{570} (treated tissue)/OD_{570} (control tissue).

4. Results

4.1. Antimicrobial activity of lactosporin

Lactosporin showed antimicrobial activity against *M. lutues* (indicator strain) and *G. vaginalis* when tested in a well diffusion assay.

4.2. Measurement of potassium ion release

We evaluated the pore-forming activity of lactosporin against selected indicator strain *M. luteus* using a potassium-sensitive electrode. Figure 1 demonstrates the effect of antimicrobials used in our experiment on potassium release from *M. luteus* cells. Both lactosporin and nisin caused rapid efflux of K^+ ions from the membrane of the indicator organism. After addition of MRS medium, one of our negative controls, a slight raise in K^+ ion concentration was observed that can be attributed to the presence of potassium in the formulation of this medium.

4.3. Lactosporin does not dissipate the transmembrane potential (Δψ) of *G. vaginalis.*

The fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3$ (5)) was used to qualitatively assess the changes occurred by lactosporin on the transmembrane potential (Δψ) of the *G. vaginalis* cells.

An increase in the fluorescent signal of the probe after addition of the antimicrobial is indicative of the transmembrane potential collapse. The increase in the fluorescent signal is due to the depolarization of the cell membrane by the antimicrobial compound. In contrast to nisin that caused an immediate collapse of $\Delta\psi$, lactosporin did not dissipate this component of the PMF (Fig 2.A.). The ionophore valinomycin was subsequently added to the cells to deplete the remaining transmembrane potential. Addition of valinomycin to the cells that were exposed to nisin did not cause any further depletion of the $\Delta\psi$ but caused complete dissipation of $\Delta\psi$ in cells treated with lactosporin, nisin diluent and lactosporin diluent (Fig 2.A. and Fig 2.B.). It can be concluded that nisin causes an instantaneous collapse of the $\Delta\psi$ in *G. vaginalis* cells unlike lactosporin that has no effect on this component of the PMF.

4.4. Lactosporin causes dissipation of the transmembrane pH (ΔpH).

The pH sensitive fluorescent probe BCECF-AM was used to track the changes in the transmembrane pH of the *G. vaginalis* cells. Ionophore valinomycin was utilized to convert the transmembrane potential to transmembrane pH before addition of the antimicrobial compounds. Lactosporin and nisin both caused an immediate decrease in the fluorescent signal of the BCECF-AM probe indicating a decrease in the internal pH of the cells. In contrast, nisin diluent and lactosporin diluent did not have such an effect on the interacellular pH of *G. vaginalis* cells. Nigericin was added to deplete any remaining ΔpH in the cells. Addition of this ionophore to lactosporin and nisin

treated cells indicated that almost all ΔpH was already depleted and did not cause any further dissipation of this component of the PMF (Fig 3.A. and Fig 3.B.),

4.5. Lactosporin is a safe and non-cytotxic compound for vaginal application.

The EpiVaginal™ ectocervical tissues were utilized to study the cytotoxic effects of lactosporin on vaginal tissues. The tissue viability was approximately 66% after a 48 hour exposure to lactosporin (Fig. 4). The viability of the tissues treated with the negative control, 4% miconazole nitrate, after 48 hours was about 41% while the positive control, Nonoxynol-9, reduced the viability to 28% in 6 hours. The ET-50 value for lactosporin could not be calculated since the tissue viability was not less than 50% at any of the time points used in this experiment. The calculated ET-50 values for Nonoxynol 9 and miconazole were approximately 4 and 23 hours, respectively.

5. Discussion

In this study, we investigated the mechanism of action of lactosporin against *G. vaginalis* cells in order to determine whether this antimicrobial preparation was safe for vaginal application. Our mode of action data indicated that lactosporin instantaneously dissipated the transmembrane pH gradient (ΔpH) of the *G. vaginalis* cells while it had no effect on the transmembrane electric potential ($\Delta\psi$), in contrast to nisin that fully depleted both components of the PMF. Similar to lactosporin, the selective dissipation of one of the portions of the PMF has been

reported for a number of other bacteriocins. For instance, Herranz *et al*. (2001) indicated that enterocin P did not cause any changes in the pH gradient of the *Enterococcus faecium* T136 cells while it caused full depletion of the transmembrane electric potential and the release of intracellular ATP from the target cells. Subtilosin, a bacteriocin produced by *Bacillus amyloliquefaciens*, acts similar to lactosporin by depleting the transmembrane pH gradient and has no effect on the transmembrane electric potential of the *G. vaginalis* cells (Sutyak Noll *et al*. 2011). Majority of bacteriocins have amphiphilic and cationic characteristics. They act by permeabilization of the membrane of the sensitive cells, forming transient pores and the leakage of intracellular materials such as small ions, amino acids or ATP and dissipation of proton motive force that may result in secondary effects such as inhibition of protein, DNA, RNA and peptidoglycan synthesis (Montville and Bruno, 1994). Our previous research indicated that lactosporin is an anionic antimicrobial protein that potentially targets the membrane of sensitive cells (Riazi *et al*. 2009).The ability of lactosporin to form pores in sensitive cells of *M. luteus* was observed by rapid K^+ efflux from these cells. The K^+ assay requires bacterial cells to be washed with Tris acetate buffer solution and kept on ice throughout the experiment to slow down the cells metabolism. These conditions worked well for the indicator organism *M. luteus* but the fastidious *G. vaginalis* could not survive the assay's condition. Therefore, only the K^+ efflux results for *M. luteus* cells are reported.

Together, PMF and K^+ leakage data suggest that lactosporin may exert its antimicrobial activity by targeting the cytoplasmic membrane of sensitive cells

and forming transient pores followed by leakage of small ions and dissipation of proton motive force. Our previous data indicated that lactosporin had antimicrobial activity against the vaginal pathogen *G. vaginalis* but did not have any effects on the healthy vaginal lactobacilli (Riazi *et al*. 2009). The MatTek EpiVaginal™ tissue model was utilized to evaluate the cytotoxic effects of lactosporin on vaginal and ectocervical epithelial cells. Our data revealed that lactosporin is a safe preparation for vaginal application to control bacterial vaginosis. The EpiVaginal™ in vitro test is gradually replacing the traditional in vivo models to study vaginal irritation due to its higher reliability and reproducibility. A prior study conducted in our laboratory evaluated the effects of an antimicrobial peptide, lactocin 160, on vaginal tissues using the EpiVaginal™ in vitro model and the in vivo rabbit vaginal irritation (RVI) system (Dover *et al*. 2007). This study confirmed that the used in vitro model is a reliable alternative for animal testing and provides higher reproducibility. In a different study, EpiVaginal™ tissue system was used to evaluate safety of an antimicrobial peptide subtilosin (Sutyak Noll *et al*. 2011).

Natural antimicrobial proteins produced by friendly bacteria have received a great deal of attention in the last decade due to the potential health problems associated with the use of chemical antimicrobial agents in foods and personal care products, and because of the rapidly growing microbial resistance to conventional antibiotics (Abee *et al*. 1995; Cleveland *et al*. 2001). Antimicrobial resistance has become a major problem that is associated with increased negative impacts on human health and healthcare costs (Cohen, 1992). The bacterial

resistance issue can be addressed by using a multiple-hurdle approach. In hurdle technology different synergistic antimicrobial agents with different mechanisms of action are utilized to more efficiently inhibit the growth of target microorganisms (Cleveland McEntire *et al*. 2003). In our future research we will explore the synergistic activity of lactosporin with other natural antimicrobial agents against *G. vaginalis*. This information will be used to design and formulate more effective products for control of bacterial vaginosis.

6. Acknowledgements

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8. Figures legends

Fig. 1. Impact of lactosporin and nisin on the integrity of the cytoplasmic membrane of *Micrococcus luteus* was evaluated by monitoring the efflux of K^+ ion after addition of lactosporin, nisin and the negative controls. Antimicrobials were added after 20 seconds after the first mesurement, and potassium release was monitored with a potassium-sensitive electrode. Both nisin (—◆—) and lactosporin (—▲—) caused leakage of intracellualar potassium ions. The experiment was performed twice creating two replicates. The values represented in this figure are the calculated means of the two replicates.

Fig. 2. Lactosporin (A) did not cause any changes in the fluorescent signal indicating that it does not dissipate transmembrane electric potential ($\Delta\psi$) in *G. vaginalis* cells. Addition of nisin (B) caused an increase in the intensity of the fluorescent signal, due to release of the $DiSC_3(5)$ probe and dissipation of $\Delta\psi$. Addition of valinomycin to dissipate the remaining $\Delta\psi$ did not cause any further increase of fluorescence in nisin-treated sample (B) since nisin caused a total depletion of the $\Delta\psi$. Negative controls in both samples (A, B) had no effects on the $\Delta\psi$ of *G. vaginalis* cells.

Fig. 3. Addition of lactosporin caused an immediate decrease in the fluorescent signal indicating complete dissipation of the transmembrane pH gradient (ΔpH) of *G. vaginalis* cells (A). Nisin caused a more gradual decrease in the fluorescent signal that indicated a slower dissipation of the ΔpH component of the PMF.

There was no additional depletion of the ΔpH after addition of nigericin (A, B). Both negative controls had no effect on the pH gradient of *G. vaginalis* (A, B).

Fig. 4. Lactosporin is a non-cytotoxic antimicrobial for human vaginal cells as tested in EpiVaginal tissue model (VEC-100). This figure presents % viability of vaginal cells at different time points following treatment with nonoxynol 9, miconazole and lactosporin. The experiment was performed twice in triplicate and the error bars represent the standard deviation.

9. Figures

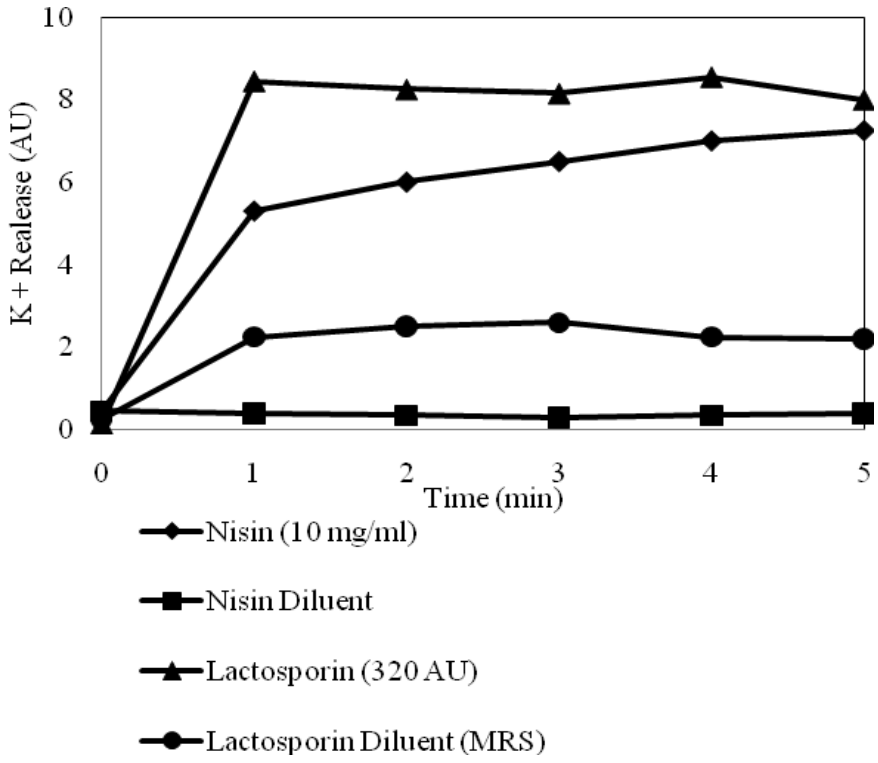

Fig. 1.

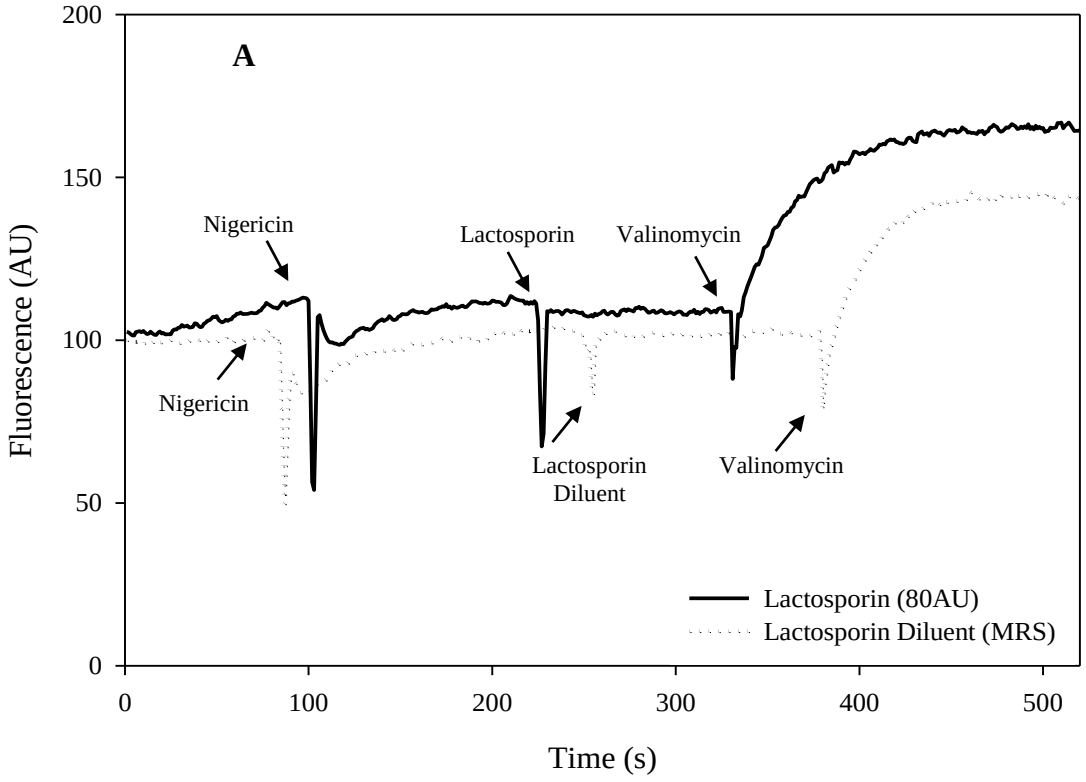
A
Fluorescence (AU)
Time (s)
Nigericin
Lactosporin
Valinomycin
Nigericin
Lactosporin Diluent
Valinomycin
Lactosporin (80AU)
Lactosporin Diluent (MRS)
0
50
100
150
200
0
100
200
300
400
500

Fig. 2.A.

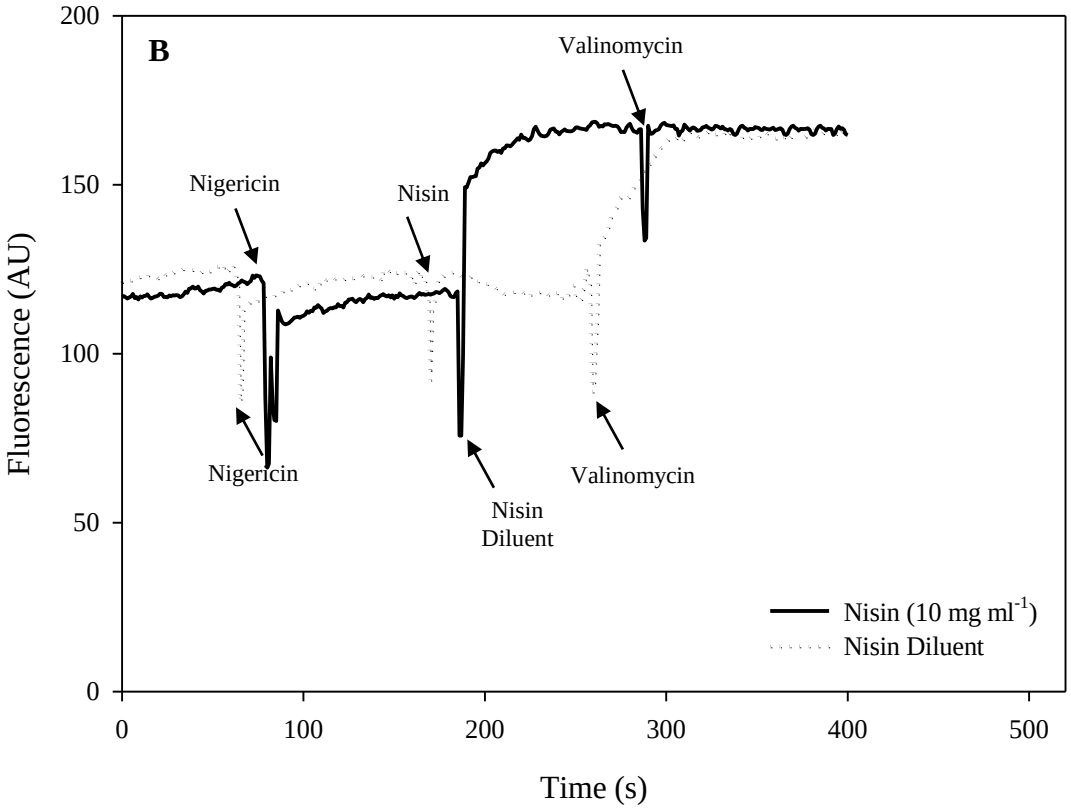
B
Valinomycin
Nigericin
Nisin
Nigericin
Valinomycin
Nisin
Diluent
Nisin (10 mg ml-1)
Nisin Diluent
Fluorescence (AU)
Time (s)
0
50
100
150
200
0
100
200
300
400
500

Fig. 2.B.

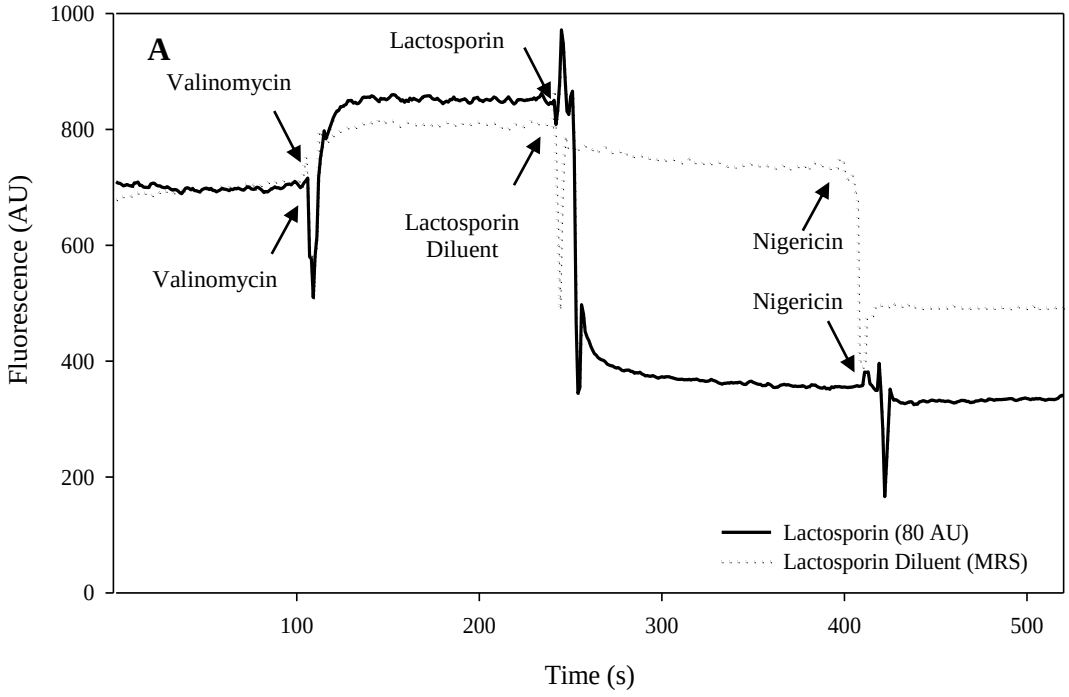
A
Valinomycin
Lactosporin
Valinomycin
Lactosporin
Diluent
Nigericin
Nigericin
Lactosporin (80 AU)
Lactosporin Diluent (MRS)
Fluorescence (AU)
Time (s)
0
200
400
600
800
1000
100
200
300
400
500

Fig. 3.A.

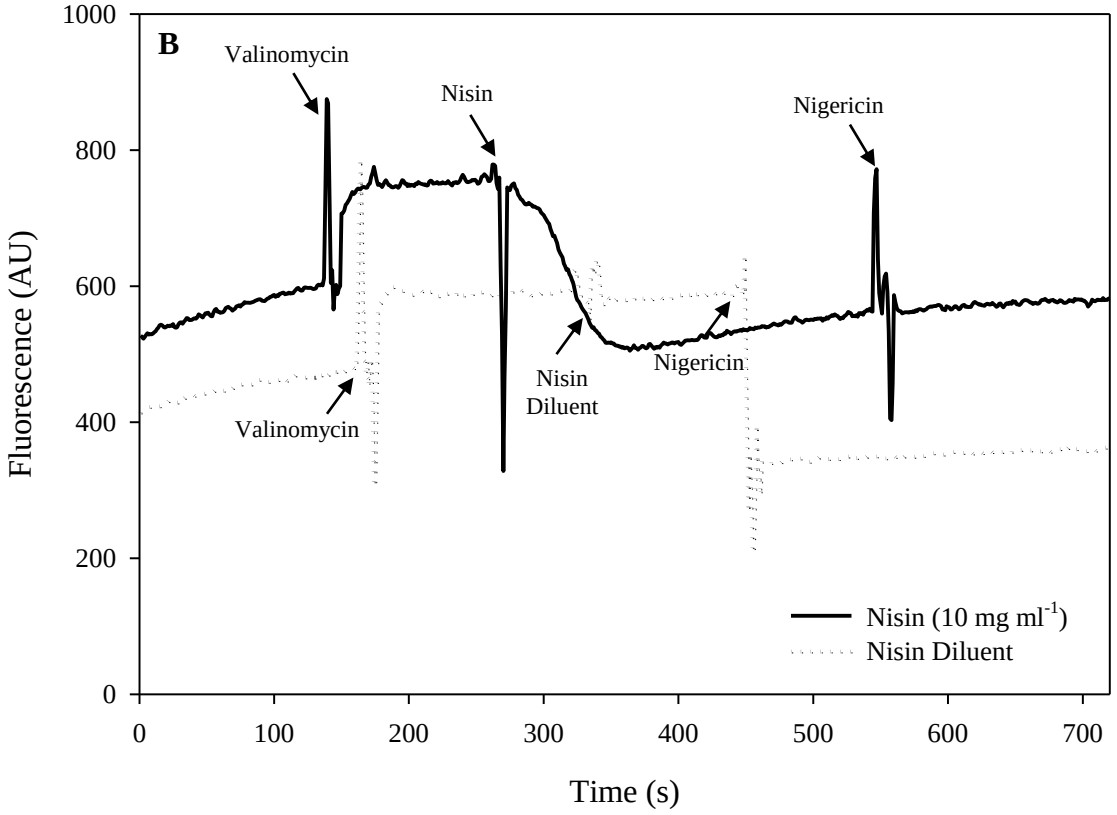
B
Valinomycin
Nisin
Nigericin
Nisin
Diluent
Nigericin
Valinomycin
Nisin (10 mg ml-1)
Nisin Diluent
Fluorescence (AU)
Time (s)
0
200
400
600
800
1000
0
100
200
300
400
500
600
700

Fig. 3.B.

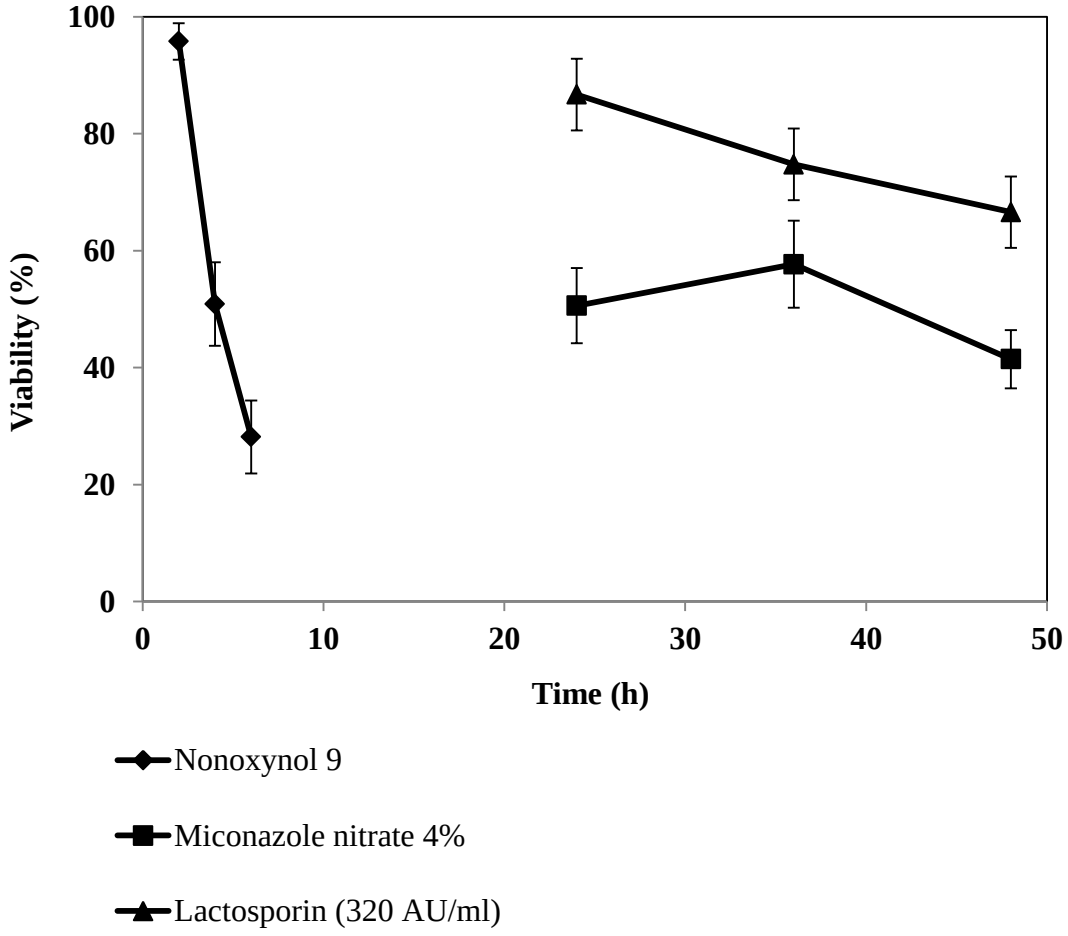
100
80
60
40
20
0
Viability (%)
0
10
20
30
40
50
Time (h)
Nonoxynol 9
Miconazole nitrate 4%
Lactosporin (320 AU/ml)

Fig. 4.

CHAPTER IV.

SUGGESTIONS FOR THE FUTURE RESEARCH

Synergistic activity of lactosporin with other natural antimicrobial compounds

Control of bacterial vaginosis can be achieved more effectively using multiple antimicrobial agents either sequentially or simultaneously. Multiple studies have successfully demonstrated the synergistic effects of bacteriocins and other natural compounds against multiple clinical pathogens (Badaoui Najjar *et al*. 2007). Some of the natural antimicrobials that may have synergistic effect with lactosporin includeε-poly-L-lysine, saponins and lactic acid salts. ε-poly-L-lysine is a homopolymer of lysine with a broad spectrum of activity against different microorganisms. ε-poly-L-lysine has a different mechanism of action than most bacteriocins. It interacts electrostatically with the membrane of the sensitive cells and causes stripping and abnormal distribution of the cytoplasm (Shima *et al*. 1984) as opposed to most bacteriocins which kill sensitive microorganism by forming pore in the membrane of target cell causing leakage of cellular material. Polylysine has been approved by FDA as a GRAS compound for human use (Tarantino 2004).

Saponins are another group of natural antimicrobial compounds that may have potential synergistic activity with lactosporin. Saponins are glycosides of steroids derived from plants, which act as natural surfactants. Most studies suggest that the biological activity of saponins is due to their membrane

permeabilizing activities (Francois *et al*. 2002). Some commercially available preparations of saponins claim to have spermicidal and anti-HIV effect (CONSAP spermicidal cream). These compounds are used to control foodborne pathogens in certain meat products, and in the food industry for preservation of in some ready-to-eat cooked meat products. The antimicrobial activity of some of these lactic acid salts is due to their ability to lower the water activity (Lamkey *et al*. 1991).

Several research groups show that the antimicrobial activity of lactates is due to their ability to cross the membrane of sensitive cells as undissociated compounds. Lactates are generally active at lower pH values in which the relative quantity of undissociated acid is higher. McEntire *et al*. (2003) studied the combined effect of nisin and lactic acid salts to inhibit the foodborne pathogen *Listeria monocytogenes*. They reported a synergistic effect between nisin and zinc lactate while other lactic acid salts such as calcium, potassium and magnesium did not work synergistically with nisin to inhibit *L. monocytogenes*.

The "checkerboard assay" will allow us to look at the effect and interaction of many different substances and concentrations at the same time. This method will be used in order to investigate any possible synergies at a broad range of concentrations of antimicrobials being studied (Yoshida and Nagasawa 2003).

Protein sequencing of lactosporin

In order to determine the protein sequence of lactosporin, a partially purified sample (prepared by isoelectric focusing) will be run through non-denaturing gel electrophoreis (Native-PAGE) to separate the protein with antimicrobial activity. As described earlier in chapter 2, two duplicate samples

will be separated on the same gel and at the end of electrophoresis the gel will be cut into two halves. One part will be stained and the other part will be tested for the antimicrobial activity using the overlay method. The protein band that produces a zone of inhibition on the overlaid half of the gel will be excised and used to determine its sequence using Edman degradation method.

Curriculum Vitae

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