

THE POTENTIAL OF A PROBIOTIC-DERIVED ANTIMICROBIAL PEPTIDE FOR
THE CONTROL OF VAGINAL PATHOGENS

By

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ABSTRACT OF THE DISSERTATION

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Bacterial vaginosis (BV) is characterized by replacement of vaginal probiotics with several anaerobic pathogens. This condition can lead to numerous complications during pregnancy and labor. It is also a risk factor for HIV acquisition.

Currently-available antibiotic treatments for BV are notorious for having a high (30-40%) rate of reoccurrence; therefore alternative treatments are critically desired. A multiple-hurdle approach involving bacteriocins may provide such an alternative.

Previous studies indicate that lactocin 160, a bacteriocin produced by *Lactobacillus rhamnosus*, a vaginal probiotic, is active against BV - associated *Gardnerella vaginalis* and *Prevotella bivia*.

The objective of the current study is to understand the mode of action of lactocin 160 against *G. vaginalis* and to seek out other natural antimicrobials exhibiting synergistic activity with this bacteriocin. Our results demonstrate that lactocin 160 targets the cytoplasmic membrane of *G. vaginalis*, causing the efflux of ATP molecules and dissipation of both, $\Delta\Psi$ and ΔpH components of proton motive force. Based on these results, we presume that like many other known bacteriocin-like substances, lactocin 160 triggers formation of transient pores in the cytoplasmic membranes of its target cells. The

interactions between lactocin 160 and four other natural antimicrobials in the ability to inhibit *G. vaginalis* were evaluated using the checkerboard assay. Our results indicate that zinc lactate and soapnut extract act synergistically with lactocin 160 against this pathogen and therefore have a potential to be successfully used as the components of the multiple-hurdle antimicrobial formulation for the treatment of BV.

Dedication

I dedicate this work to my parents, Eric and Vera, and to my late grandma, Bela.

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Table of Contents

ABSTRACT OF THE DISSERTATION.....	ii
Dedication.....	iv
Acknowledgements.....	v
Table of Contents.....	vii
List of Figures.....	xi
Chapter I – Literature Review	1
A. The Etiology of Bacterial Vaginosis.....	2
Abstract.....	3
1. Introduction.....	4
2. <i>Gardnerella vaginalis</i>	8
2.1 Structure and physiology.....	9
2.2 <i>Gardnerella vaginalis</i> as the etiological cause of BV.....	16
2.3 Non-existent animal model for <i>G. vaginalis</i> infection.....	21
3. The ‘incompetent’ vaginal lactobacilli theory.....	25
3.1 Establishment of vaginal microbiota.....	25
3.2 The epidemiological findings.....	29
3.3 <i>In vitro</i> studies.....	32
3.4 Clinical trials.....	35
4. Phage theory for the decline of vaginal lactobacilli.....	39
5. The role of intrinsic host factors in etiology of BV.....	42

6. Discussion.....	51
References.....	53
 B Treatment of BV: need for new approaches.....	72
References.....	76
 C Objectives and Hypothesis.....	79
 Chapter II – Determination of the mechanism of action of lactocin 160 against <i>G. vaginalis</i> cells.....	81
 A. Lactocin 160, a bacteriocin produced by vaginal <i>Lactobacillus rhamnosus</i> , targets cytoplasmic membranes of the vaginal pathogen, <i>Gardnerella vaginalis</i>	82
 Abstract.....	83
1. Introduction.....	84
2. Materials and Methods.....	86
Bacterial strains and growth conditions.....	86
Preparation and use of the antimicrobial solutions.....	86
The membrane-disruption (ethidium bromide) assay.....	88
The ATP assay.....	89
Δ pH dissipation assay.....	90
$\Delta\Psi$ dissipation assay.....	91
Statistics.....	92
3. Results and Discussion.....	92

The cytoplasmic membrane damage caused by lactocin 160 in <i>G. vaginalis</i> cells could not be detected using the ethidium bromide assay.....	92
Lactocin 160 induces efflux of ATP in <i>G. vaginalis</i>	93
Lactocin 160 completely collapses both components of the proton motive force in <i>G. vaginalis</i> cells.....	94
4. Conclusion.....	96
Acknowledgements.....	97
References.....	97
B. A short note on method development.....	106
1. $\Delta\Psi$ dissipation assay.....	106
2. ΔpH dissipation assay.....	107
3. The membrane-disruption (ethidium bromide) assay.....	109
4. The ATP assay.....	110
References.....	112
Chapter III – Identification of compounds having synergistic activity with lactocin 160.....	116
A. Zinc lactate and soapnut extract act synergistically with lactocin 160 against <i>Gardnerella vaginalis</i>	117
Abstract.....	118
1. Introduction.....	119

2. Materials and Methods.....	124
Bacterial strains and growth conditions.....	124
Preparation and use of the antimicrobials.....	124
Determination of the minimal inhibitory concentrations (MICs) of the antimicrobials.....	125
The checkerboard assay.....	126
Analysis of the checkerboard assay data.....	127
3. Results.....	127
4. Discussion.....	128
References	132
Figure captions.....	135
Figures.....	136
Chapter IV – Suggestions for the future studies.....	141
References.....	146
Figure captions.....	147
Figures.....	148
Curriculum Vitae.....	152

Table of Figures

Chapter II

A. Fig. 1 Assessment of membrane integrity using the ethidium bromide essay.....	102
Fig. 2 The effect of lactocin160 and nisin on ATP content of <i>G. vaginalis</i> cells.....	103
Fig. 3 Lactocin 160, much like nisin, induces a complete collapse of transmembrane electrical potential ($\Delta\Psi$) in <i>G. vaginalis</i>	104
Fig. 4 Lactocin 160, much like nisin, completely dissipated the transmembrane pH gradient (ΔpH) in <i>G. vaginalis</i>	105
B. Fig. 1 The effect of lactocin160 and nisin on ATP content of <i>G. vaginalis</i> cells.....	114

Chapter III

Fig. 1 Growth kinetics of <i>G. vaginalis</i> in the presence of poly-L-lysine.....	136
Fig. 2 Isobolograms showing interactions between lactocin 160 and four natural antimicrobials against the vaginal pathogen <i>G. vaginalis</i> (a-d).....	137

Chapter IV

Fig. 1 Biofilm of <i>G. vaginalis</i> grown on a glass cover slip.....	148
Fig. 2 Lactocin 160 does not prevent formation of <i>G. vaginalis</i> biofilms (a-c)...	149

CHAPTER I

LITERATURE REVIEW¹

¹The following manuscript (I.A.) is prepared for publication in the Journal of Applied Microbiology (JAM)

Chapter I.A.

THE ETIOLOGY OF BACTERIAL VAGINOSIS

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ABSTRACT

Bacterial vaginosis (BV) is the most common vaginal infection among women of the childbearing age. This condition is notorious for causing severe complications related to reproductive health of women. Five decades of intense research established many risk factors for acquisition of BV, however due to complexity of BV and due to lack of a reliable animal model for this condition, its exact etiology remains elusive. In this manuscript we use a historical perspective to critically review the development of major theories on etiology of BV, ultimately implicating BV-related pathogens, healthy vaginal microbiota, bacteriophages and the immune response of the host. None of these theories on their own can reliably explain the epidemiological data. Instead, BV is caused by a complex interaction of multiple factors, which include the numerous components of vaginal microbial ecosystem and their human host. Many of these factors are yet to be characterized because the clear understanding of their relative contribution to the etiology of BV is pivotal to formulation of effective treatment and prophylaxis of this condition.

Keywords: bacterial vaginosis, etiology, *Gardnerella vaginalis*, causes, immune response, lactobacilli, *Lactobacillus*

1. INTRODUCTION

The healthy microbiota of the lower genital tract in women predominantly consists of *Lactobacillus* spp., with *L. crispatus*, *L. jensenii* and *L. iners* being the most prevalent species (Pavlova *et al.* 2002; Zhou *et al.* 2004; Shi *et al.* 2009). These bacteria grow in biofilms that tightly adhere to the vaginal epithelia, forming the first line of defense against potential pathogens. The symbiotic relationship between vaginal lactobacilli and their human host is modulated by the hormones circulating in a woman's body which stimulate the vaginal epithelia to produce glycogen (Hay 2005). Vaginal lactobacilli metabolize glycogen secreted by the vaginal epithelia, in turn producing lactic acid, which is largely responsible for the normal vaginal pH being acidic (<4.5) (Donati *et al.* 2010). The acidic environment of a healthy vagina is not permissive for growth of many potential pathogens (Aroutcheva *et al.* 2001a; Donati *et al.* 2010). Additionally, vaginal lactobacilli fend off pathogens through competitive exclusion and through the production of antimicrobials such as hydrogen peroxide and bacteriocin-like substances (Aroutcheva *et al.* 2001a).

The most common vaginal infection among women of childbearing age is bacterial vaginosis (BV). This condition is characterized by replacement of vaginal lactobacilli with predominantly anaerobic microorganisms such as *Gardnerella vaginalis* and *Prevotella*, *Peptostreptococcus* and *Bacteroides* spp. (Forsum *et al.* 2005; Larsson and Forsum 2005; Srinivasan and Fredricks 2008; Livengood 2009). *G. vaginalis* is thought to have the leading role in the infection, making the niche suitable for colonization by strict anaerobes that are largely responsible for clinical symptoms of BV (Swidsinski *et al.* 2005; Swidsinski *et al.* 2008; Josey and Schwebke 2008; Harwich, Jr.

et al. 2010). BV is not caused by the mere presence of the potential pathogens (which is common) but rather by their unrestrained increase in number, often reaching cell counts that are 100-1000 fold above the normal bacterial levels of the vagina (Eschenbach 1993; Eschenbach 1994; Forsum *et al.* 2005; St John *et al.* 2007). However, the exact mechanisms and sequences of the infective processes are largely unknown due to lack of a reflective animal model.

Epidemiological studies indicate that the risk of BV is increased in women of African ethnicity (Simhan *et al.* 2008; Cherpes *et al.* 2008; Klatt *et al.* 2010). Other risk factors include cigarette smoking, douching, antibiotic treatment for another condition, young age of coitarche, acquisition of a new sex partner and a recent history of multiple sex partners (Merchant *et al.* 1999; Yen *et al.* 2003; Verstraelen 2008; Cherpes *et al.* 2008; Fethers *et al.* 2009; Verstraelen *et al.* 2010). The fact that many of the high risk behaviors are also well-established risk factors for acquisition of common sexually transmitted infections (STIs) suggests that BV could be transmitted sexually (Gardner and Dukes 1955; Criswell *et al.* 1969; Gardner 1980; Verstraelen 2008). However, unlike a typical STI with a single etiological agent and clear routes of infection, BV involves multiple pathogens, a great majority of which are also frequently detected (albeit in low numbers) in the vaginas of BV-free and sexually inexperienced women. The fact that there is no evidence for a decrease in the rates of BV recurrence following antibiotic treatment of men sexually involved with affected women is another distinction between BV and the common STIs (Verstraelen *et al.* 2010). In fact, many researchers prefer to view BV not as an infection but as a complex microbial imbalance, with a significant role

played by the indigenous vaginal lactobacilli (Guise *et al.* 2001; Hay 2005; Schwartz *et al.* 2006).

The diagnosis of BV in clinical settings is usually based on fulfillment of three out of four clinical criteria described by Amsel *et al.* (1983). Amsel's criteria include 1) elevated vaginal pH (>4.5), 2) presence of white adherent discharge that contains 3) numerous exfoliated epithelial cells with bacteria (Gram-variable polymorphic rods) attached to their surface (clue cells) and 4) has a characteristic fishy odor especially when 10% KOH is added (whiff test). However, BV can be asymptomatic in about 50 % of women, and for that reason microbiological diagnostic methods, such as Nugent's scoring system (Nugent *et al.* 1991), are preferred in the scientific community (Schwartz *et al.* 2006).

Aside from causing unpleasant symptoms, BV is notorious for setting off an entire array of serious gynecological and obstetric complications. BV is a risk factor for the development of postpartum and post-abortion endometritis and pelvic infection following gynecologic surgery (Watts *et al.* 1990; Lin *et al.* 1999). In pregnant women, BV has been connected to premature labor and preterm delivery that leads to high prenatal mortality (Leitich *et al.* 2003; Romero *et al.* 2004; Oakeshott *et al.* 2004; Stevens *et al.* 2004). *G. vaginalis* and *Prevotella* spp. are high risk factors for intra-amniotic infections (Goldenberg *et al.* 2000; Hashemi *et al.* 2000). BV-associated microorganisms and their toxins capable of crossing placenta are among the major causes of brain injury for fetuses. BV is traditionally considered a risk factor for long-term neurological consequences in children, such as hyperactivity, academic difficulties in

school, and severe handicaps such as cerebral palsy and pre ventricular leukomalacia (Eschenbach 1997; Grether and Nelson 2000; Ling *et al.* 2004). High concentrations of lipopolysaccharides (LPS) were found in the vaginas of women with BV (Platz-Christensen *et al.* 1993). These LPS induce damage in the dopaminergic system in neonates (Ling *et al.* 2004).

Additionally, the physiology of the female genital tract makes women twice as sensitive to HIV as men. A disturbed vaginal ecology caused by BV creates a more permissive environment for acquiring HIV (Schmid *et al.* 2000; Schwebke 2003; Sha *et al.* 2005; Watts *et al.* 2005). Major BV-associated organisms directly up-regulate HIV replication (Al-Harthi *et al.* 1999; Hashemi *et al.* 2000; Simoes *et al.* 2001; Zariffard *et al.* 2005). *G. vaginalis* was found at high concentrations in 60% of HIV positive women (Mascellino *et al.*, 1991). *G. vaginalis* also increased the production of HIV by HIV-infected monocytoïd cells and in certain T cells by as much as 77-fold (Hashemi *et al.* 1999). *P. bivia* and *Pep. assaccharolyticus* also induce HIV expression (Hashemi *et al.* 2000). Moreover, an elevated pH resulting from *Lactobacillus* spp. replacement with BV-associated microorganisms makes the vaginal environment more favorable for HIV proliferation (Taha *et al.* 1998). In addition, a recent study showed that BV increased the risk of viral shedding of genital herpes (HSV-2) by a factor of 2.3 (Cherpes *et al.* 2005).

Conventional treatment of BV with metronidazole and clindamycin (Paavonen *et al.* 2000; Sobel *et al.* 2001), as recommended by the Centers for Disease Control and Prevention, does not eradicate all BV-associated bacteria (Flores Rivera *et al.*, 1997). After treatment for BV, many women remained colonized by *G. vaginalis* or other BV-

associated anaerobes (Ferris *et al.* 1995; Boris *et al.* 1997). These treatments are effective in only 60% of all cases, contributing to the BV recurrence rate of 30-40% (Colli *et al.* 1997; Bannatyne and Smith 1998; Paavonen *et al.* 2000; Eriksson *et al.* 2005). In addition, these treatments play a significant role in the expansion of drug resistance in *G. vaginalis* and *Prevotella*, *Bacteroides*, and *Peptostreptococcus* spp. (Lubbe *et al.* 1999; Bryskier 2001; Liebetrau *et al.* 2003).

One of the major obstacles to discovery of effective treatment and prophylaxis of BV is our limited understanding of this condition, particularly its etiology, which remains enigmatic despite decades of research (Forsum *et al.* 2005; Larsson and Forsum 2005; Larsson *et al.* 2005). In this manuscript, we review the major theories on causes of BV, critically evaluating evidence for the role of *G. vaginalis*, endogenous vaginal lactobacilli, bacteriophages and the genetic predispositions of the host in the etiology of this condition.

2. GARDNERELLA VAGINALIS

Bacterial vaginosis was initially thought to be a sexually transmitted infection propagated by a bacterium that is now known as *Gardnerella vaginalis*. Ever since the discovery of this pathogen in the mid-1950s, its history has been intriguing and full of controversies. The elusive nature of this microorganism is even revealed by the fact that it has been renamed several times, mainly because of its unique cell wall structure and nutritional requirements. As the polymicrobial nature of BV became evident, the role of *G. vaginalis* in the etiology of this condition became less clear. As a result, general

interest in *G. vaginalis* declined in the late 1980s, only to reemerge in recent years as the relationship of this microorganism to BV was reevaluated. Consequently, *G. vaginalis*' cell wall was thoroughly investigated for decades while little is known about the microorganism's physiology and virtually no research was conducted on genetics of *G. vaginalis* until very recently.

2.1 Structure and physiology

The original discovery of *G. vaginalis* was made by Leopold (1953), who described this microorganism as a novel “*Haemophilus*-like” species associated with prostatitis and cervicitis (Catlin 1992). Gardner and Dukes (1955) were the first to describe the microorganism in relation to BV. The bacterial cell's morphology, apparent negative reaction to Gram staining and inability to grow on agar media lacking blood convinced these researchers that they were dealing with a new *Haemophilus* species which they named, based on its origin, *Haemophilus vaginalis*. Eventually it became apparent that, unlike other members of the *Haemophilus* genus, ‘*Haemophilus vaginalis*’ occasionally had a positive reaction to Gram staining and did not require either hemin or NAD for its growth. The microorganism was then temporarily placed into the *Corynebacterium* genus, and for some time was referred to as *Corynebacterium vaginale* (Dunkelberg, Jr. *et al.* 1970; Deane *et al.* 1972). However, the bacterium did not fit into the description of *Corynebacterium* genus because it was catalase-negative and because it lacked arabinose in its cell wall (Catlin 1992). Finally, two large taxonomic studies evaluating multiple criteria revealed the lack of similarities between “*Haemophilus vaginalis*” and other established genera (Piot *et al.* 1980; Greenwood and Pickett 1980).

As a result, a new genus named *Gardnerella* was proposed, with *Gardnerella vaginalis* being the only species in it.

The uncertainties in the taxonomic status of *G. vaginalis* fueled an almost half century long debate about the structure of the microorganism's cell wall. The fundamental difference in the chemical structure and molecular architecture between the two types of bacterial cell walls can generally be revealed by a simple Gram staining technique. Typical Gram-negative cell walls have a complex, multilayered structure with a thin layer of peptidoglycan and an outer membrane largely composed of lipopolysaccharide. In contrast, peptidoglycan arranged into a thick amorphous matrix is the predominant component of a Gram-positive cell wall (Silhavy *et al.* 2010). *G. vaginalis*, however, is commonly described as a Gram-variable or Gram-uncertain microorganism, meaning that its reaction to Gram staining can vary from negative to positive (Catlin 1992). For instance, the cells of *G. vaginalis* can have both a Gram-negative and Gram-positive appearance in a stained preparation of a vaginal smear. Similarly, a pure culture grown on a medium containing starch appeared as Gram-variable. In contrast, the cells grown on vaginalis agar (V-agar) predominantly stained as Gram-negative, while the early exponential phase cells grown on inspissated serum medium (Zinnemann and Turner 1963; Piot *et al.* 1980) mostly stained as Gram-positive (Zinnemann and Turner 1963; Piot *et al.* 1980), indicating that the age of the culture and its growth conditions may influence the reaction to Gram staining.

Numerous attempts to study the biochemistry and ultrastructure of *G. vaginalis* cell wall have led to some conflicting results. The electron micrographs published by

Reyn *et al.* (1966) revealed a single-layered but relatively thin murium which was in close association with the cytoplasmic membrane. Formation of a well-defined septum between dividing cells was clearly seen in a longitudinal section, further indicating the Gram-positive nature of the cell wall.

In contrast, Criswell *et al.* (1971) and Criswell *et al.* (1972) reported that, much like the reference strain of the Gram-negative bacterium *E. coli*, *G. vaginalis* has a multilayered cell wall containing low (20%) peptidoglycan content. These multiple laminations, which are typical for cell walls of Gram-negative organisms, were also reported by Greenwood and Pickett (1980), swaying the scientific community towards the view that *G. vaginalis* has cell wall characteristics reminiscent of a Gram-negative microorganism.

The initial chemical analysis of the peptidoglycan conducted by Criswell *et al.* (1971) showed that although the matrix lacks diaminopimelate, it has a diverse amino acid profile common to Gram-negatives. Additionally, ribitol teichoic acid, which is almost universal to Gram-positive cell walls, was not detected in *G. vaginalis* (Criswell *et al.* 1971). Furthermore, Greenwood and Pickett (1980) reported that *G. vaginalis*' cell wall material obtained by a hot aqueous phenol extraction gave a positive reaction in a limulus amoebocyte lysate (LAL) assay, suggesting the presence of an LPS-like substance. The amino acid profile reported by Criswell *et al.* (1971) was disputed in later publications (Greenwood and Pickett 1980; Sadhu *et al.* 1989). Even more significantly, the detailed chemical analysis of the lipid extract involving tests for LPS-specific components such as heptose and hydroxy fatty acids failed to identify typical

lipopolysaccharides in the cell wall of *G. vaginalis* (Greenwood and Pickett 1980; O'Donnell *et al.* 1984). Moreover, LPS could be detected by neither silver staining on a SDS-PAGE gel or LAL assay (Sadhu *et al.* 1989). Sadhu *et al.* (1989) proposed that the previously reported positive reaction to LPS was induced by lipoteichoic acid, since the extract samples were used in very high concentrations.

At the same time, electron micrographs of ruthenium red stained cells published by Sadhu *et al.* (1989) revealed that the oblique angle of sectioning is likely responsible for the previously reported lamellar appearance of the cell wall. The absence of the outer membrane was clearly observed in images of cells sectioned at the right angle.

Additionally, the absence of an outer cleavage phase in freeze-etched *G. vaginalis* cells once again demonstrated the lack of outer membrane. Although thick (up to 50 nm) peripheral cell walls were visible in a minority of cells, in most cells the cell wall appeared fibrillar and unstructured with thicknesses ranging between 8-12 nm, similar to what has been previously reported (Sadhu *et al.* 1989; Catlin 1992). Sadhu *et al.* (1989) proposed that the fluctuation in thickness of the peptidoglycan layer is responsible for the variable reaction to Gram-staining. The peptidoglycan layer in *G. vaginalis* cells is thought to become thinner as the culture ages, and the relatively thin peptidoglycan matrix cannot effectively retain the crystal violet-iodine complex which typically serves as an indicator in a Gram-staining reaction (Sadhu *et al.* 1989; Catlin 1992).

Muli *et al.* (1999) once again examined the ultrastructure of *G. vaginalis* cells grown in both conventional and biofilm systems and essentially confirmed the Gram-positive nature of the cell walls. This group described the *G. vaginalis* cell wall as a

relatively thin (8-12 nm) but homogeneous fibrillar structure. Interestingly, Muli *et al.* (1999) noticed a group of uncommon cell wall particles seen in cross-section as a set of seven circles (18-20 nm in diameter) which were predominately observed in the biofilm-associated cells. It has been proposed that these fiber-like structures may function as a part of the mesosomal system or as a precursor to a developing septum in some Gram-positive bacteria (Vaniterson 1961).

Electron microscopy has also revealed fimbriae (pili) which are 3 to 7.5 nm in diameter covering the cellular surface (Scott *et al.* 1989). Ultrastructural investigation conducted by Scott *et al.* (1989) indicated that the outer fibrillar coat was predominantly responsible for the attachment of *G. vaginalis* to exfoliated vaginal epithelial cells (clue cells); conversely, fimbriae were involved in attachment of the pathogen to human red blood cells. Both fimbriae and exopolysaccharides are thought to be involved in attachment of *G. vaginalis* to the vaginal epithelium *in vivo* (Scott *et al.* 1989; Catlin 1992). Electron microscopy has also revealed that the cells are non-sporulating, they lack flagella, and they do not possess a typical capsule (Greenwood and Pickett 1980).

Overall, the cells of *G. vaginalis* are small, pleomorphic rods having average dimensions of 0.4 by 1.0-1.5 μm (Edmunds 1960; Catlin 1992); however, the length of some cells may reach up to 2-3 μm (Greenwood and Pickett 1980; Taylor-Robinson 1984). The cell size and morphology largely depend on their growth conditions and on their physiological state (Piot *et al.* 1980; Jolly 1983).

This bacterium is immotile, with the cells frequently occurring in clumps in vaginal smears and when grown in liquid media (Greenwood and Pickett 1980; Taylor-

Robinson 1984). Strands of exopolysaccharide produced by cells can be visualized using electron microscopy and detected using ruthenium red staining (Greenwood and Pickett 1980; Greenwood 1983; van der Meijden *et al.* 1988). They are presumed to be responsible for the cell clumping effect (Catlin 1992).

Until 2010, virtually nothing was known about *G. vaginalis*' genetics. Early studies conducted using a variety of techniques indicated that the genome of *G. vaginalis* has a low (42-43.5 %) GC content (Piot *et al.* 1980; Greenwood and Pickett 1980; Vandamme *et al.* 1991). Additionally, Lim *et al.* (1994) digested chromosomal DNA of four *G. vaginalis* strains with rare restriction enzymes and analyzed the fragments using pulsed-field gel electrophoresis (PFGE), eventually concluding that the size of the *G. vaginalis* genome ranges between 1.67 Mb and 1.72 Mb. Finally, various attempts at genotyping *G. vaginalis*, as described in the next section, revealed an incredible diversity between the genomes of different isolates. Presumably, the difficulty of lysing *G. vaginalis* hindered early genetic explorations (Greenwood and Pickett 1980; Piot *et al.* 1980; Vandamme *et al.* 1991); concurrently, the interest in *G. vaginalis* faded in the 1980s-90s, right when techniques in molecular genetics were rapidly advancing.

Recently, the genomes of several *G. vaginalis* strains were sequenced, providing a plethora of information about the microorganism. These studies estimated the genome of *G. vaginalis* to be 1.62-1.67 Mb with a low GC content (41-42%), thus confirming the earlier findings (Yeoman *et al.* 2010). Sequence analysis of *G. vaginalis*' 16 S rRNA conducted by Yeoman *et al.* (2010) indicated that the bacterium is most closely related to *Bifidobacterium coryneforme* and *Bifidobacterium minimum* (Gram-positives).

Interestingly, the analysis of *G. vaginalis*' genome revealed that, aside from simple conversions, the microorganism lacks enzymes in biochemical pathways involved in amino acid synthesis. It was predicted that *G. vaginalis* can synthesize some but not all purine and pyrimidine bases (Harwich, Jr. *et al.* 2010). The researchers also did not find genes coding for phosphofructokinase and fructose-bisphosphate aldolase, the two enzymes essential for glycolysis; however, enzymes responsible for portions of the pentose phosphate pathway were identified. The authors suggested that the pentose phosphate pathway could potentially compensate for the deficiency in the glycolysis pathway. Finally, Yeoman *et al.* (2010) reported that most of the genes coding for enzymes involved in the TCA cycle are missing from the *G. vaginalis* genome. These recent findings explained at least some of the complex nutritional requirements that are needed for *in vitro* growth of *G. vaginalis*; they are hardly surprising, since the fastidious nature of this microorganism has been apparent from its very discovery (Gardner and Dukes 1955). The bacterium's relatively small genome size and its deficiencies in important biochemical pathways are consistent with the parasitic lifestyle of this microorganism.

Biochemical tests revealed that *G. vaginalis* is catalase-, oxidase- and β -glucosidase-negative. It can ferment starch, dextrin, sucrose, glucose, fructose, ribose, maltose and raffinose. Some strains can also ferment xylose and trehalose. Conversely, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol, and sorbitol (Harwich, Jr. *et al.* 2010). Additionally, *G. vaginalis* can hydrolyse hippurate but not gelatin or esculin. This microorganism is also positive for α -glucosidase activity and for β -hemolysis on human blood, but not sheep's blood

The hemolytic activity associated with *G. vaginalis* is thought to be mainly due to secretion of vaginolysin (VLY), a cholesterol-dependent cytolysin. Vaginolysin recognizes complement regulatory molecule CD59 on the surface of its target cells, which explains this toxin's specificity towards human erythrocytes. *In vivo*, the cytolytic activity of vaginolysin is thought to increase the nutrient availability for its producer strain (Yeoman *et al.* 2010).

2.2 *Gardnerella vaginalis* as the etiological cause of BV

Gardner and Dukes (1955), who were the first ones to link *G. vaginalis* to BV, reported the microorganism being isolated from the lower genital tract of BV-affected women in 92% of cases, compared to a 0% isolation rate from healthy women. In the questionable experiments that followed, Gardner and Dukes (1955) intravaginally inoculated 13 BV-free women with a pure *G. vaginalis* culture. The vaginas of two women got colonized by the microorganism but had no clinical signs of BV, while one woman (8%) developed a symptomatic BV. In a separate experiment, Gardner and Dukes (1955) used vaginal secretions from BV-affected women to inoculate the vaginas of 15 BV-free volunteers. Eleven of these volunteers (73%) developed symptomatic BV that would not resolve spontaneously within four months. Additionally, *G. vaginalis* was isolated from the urethra of 45 out of 47 (96%) men sexually involved with the BV-positive women (Gardner and Dukes 1955). These observations led the researchers to the conclusion that *G. vaginalis* is the sole etiological cause of BV (Eschenbach *et al.* 1989). Years later, the same group of researchers repeated their experiments on human

volunteers, this time managing to induce colonization in 7 out of 29 pregnant women vaginally inoculated with the pure *G. vaginalis* culture (Criswell *et al.* 1969).

Eventually, advances in the formulation of media selective for *G. vaginalis* allowed for the detection of this bacterium when present in low numbers. Subsequent studies found *G. vaginalis* in the vaginas of 14-69% of BV-free women, an incidence that is considerably higher than the 0% originally reported by Gardner and Dukes (1955) (Gardner and Dukes 1955; Totten *et al.* 1982; Hill *et al.* 1984; Masfari *et al.* 1986; Eschenbach *et al.* 1988; Fredricsson *et al.* 1989; Cristiano *et al.* 1989; Mikamo *et al.* 2000).

In order to explain epidemiological data showing the common occurrence of *G. vaginalis* in healthy women, numerous studies were directed to identify specific virulent sub-types of this organism responsible for BV. Accordingly, Piot *et al.* (1984) originally distinguished between eight *G. vaginalis* biotypes based on lipase, hippurate hydrolysis, and beta-galactosidase reactions. These characteristics were shown to be stable in multiple subcultures and the typing procedure was simple and reproducible. Certain biotypes were more prevalent than others, although their relative distribution was similar among the samples collected from three international cities. Although Piot *et al.* (1984) failed to link any particular *G. vaginalis* biotype to the occurrence of BV, they made other useful observations. For instance, it was shown that some women harbored multiple *G. vaginalis* biotypes. Moreover, the biotypes isolated following a week-long treatment for BV were identical to those isolated prior to the treatment. Finally, the *G. vaginalis* isolated from the vaginas of the participating women were generally of the same biotypes

as the urethral isolates from their male sexual partners, possibly indicating a sexually transmitted nature of this infection.

In contrast, Briselden and Hillier (1990) reported a statistically significant association between all four lipase-positive biotypes and the manifestation of BV, suggesting that the lipase reaction is important for the pathogenesis of *G. vaginalis*. Moreover, the authors concluded that women who acquired BV during the investigation generally also acquired a new biotype of *G. vaginalis*. Some of these results, however, were later disputed because of a flaw in the detection method for lipase activity (Moncla and Pryke 2009). Additionally, the fact that women can harbor multiple biotypes of *G. vaginalis* (also confirmed by Briselden and Hillier (1990)) significantly complicates the analysis, since the apparent acquisition of a new biotype can simply be a reflection of a change in the ratio of existing biotypes (Piot *et al.* 1984; Moncla and Pryke 2009).

Benito *et al.* (1986) expanded the *G. vaginalis* biotyping scheme by incorporating additional tests for the fermentation of arabinose, galactose and xylose into the system. Some of the 17 newly defined biotypes were more prevalent in women with BV, although they were not identical to the BV-associated biotypes reported by Briselden and Hillier (1990), thus creating yet another discrepancy.

Aroutcheva *et al.* (2001b) reported that *G. vaginalis* which is only positive for hippurate hydrolysis (biotype 5) is predominantly isolated from asymptomatic women, allowing the researchers to suggest the possibility of using this biotype as a marker of “normal vaginal microflora”; however, additional research is needed to make any definitive conclusions. To summarize, it is still pretty much unclear whether any of the

biochemical characteristics selected for biotyping *G. vaginalis* are linked to the virulence of the microorganism.

Attempts at serotyping *G. vaginalis* are also reported in the literature. For instance, 50 strains of *G. vaginalis* tested by Edmunds (1962) were differentiated into seven serological groups based on the precipitin assay involving 13 antisera (Catlin 1992). Ison *et al.* (1987) were able to differentiate between 20 *G. vaginalis* serotypes in a dot blotting test using polyclonal antibodies raised in rabbits. Immunoblot analyses of the whole cell lysates separated on SDS-PAGE gels suggested that the specificity of this reaction was determined by various immunodominant proteins and by a carbohydrate. Of the 91 clinical isolates tested by Ison *et al.* (1987), 79 (87%) were successfully typed using this scheme. To the best of our knowledge, however, the use of *G. vaginalis* serotyping has not yet been utilized for epidemiological studies; hence, the link between specific serotypes of *G. vaginalis* and BV is still unknown.

Genetic subtyping is frequently preferred over phenotypic methods; however, due to great variability in the DNA sequence of various *G. vaginalis* isolates, dividing this single species into a limited number of homogeneous genotypes proved to be challenging. For instance, DNA restriction profiles generated by *Bam*HI, *Eco*RI, *Pst*I and other restriction enzymes were considerably different in all 12 examined *G. vaginalis* strains. Moreover, Southern blot analysis of a specific DNA restriction fragment revealed the fragment's length polymorphism among all the evaluated strains (Nath, 1991). Similarly, restriction endonuclease analysis (REA) involving *Bam*HI, *Eco*RI, *Cla*I, *Hae*II,

HindIII, and *MspI* restriction enzymes revealed major differences between DNA fingerprints of 20 *G. vaginalis* biotype 1 isolates (Wu *et al.* 1996).

Ingiani *et al.* (1997) used several ribotyping techniques in an attempt to differentiate between genetic subtypes of 34 *G. vaginalis* strains. DNA profiles produced by classical ribotyping with Southern blot detection were different for all 34 strains. Conversely, the DNA fragment produced by PCR ribotyping, along with the restriction patterns of 16S–23S rRNA intergenic spacer sequences, were identical in all 34 strains. Limited success was achieved by DNA restriction analysis (ARDRA) technique; depending on the utilized restriction endonuclease, 3-4 *G. vaginalis* genotypes were identified using this method (Ingiani *et al.* 1997). However, there was no link between a specific genetic subtype and the presence of BV.

Yeoman *et al.* (2010) compared the genomes of several *G. vaginalis* strains and reported that two strains originating from the vaginas of women with symptomatic BV had the capability to degrade mucins secreted by the vaginal epithelia to form a protective barrier. In contrast, the strain originating from a woman with asymptomatic BV (Nugent score of 9) did not have this capability. Based on this observation, Yeoman *et al.* (2010) proposed that *G. vaginalis*' ability to degrade mucins could be the decisive virulence factor determining the course of infection. This theory is feasible, although many more *G. vaginalis* isolates must be analyzed in order to make any conclusions.

Conversely, Harwich, Jr. *et al.* (2010) proposed that the key difference between virulent and commensal strains of *G. vaginalis* is in their ability to adhere to the vaginal epithelia and to form biofilms. This conclusion was reached based on a series of *in vitro*

assays that compared five strains of *G. vaginalis*, three of which were BV isolates and two that were isolated from the vaginas of healthy women. Subsequent genetic analysis of a single strain from each ‘set’ revealed sequence differences in the gene coding for biofilm associated protein (BAP), which could potentially effect biofilm properties. Once again, although these observations are intriguing, comparison of additional strains is needed for a meaningful analysis.

To summarize, in spite of considerable research efforts, the specific virulent type(s) of *G. vaginalis* responsible for BV is yet to be identified. Alternative theories that may explain why some women who carry *G. vaginalis* develop BV while others do not are explored in the following sections. In any case, despite the plethora of epidemiological data indicating a strong association (93-100%) of *G. vaginalis* with BV, without direct experiments it has been challenging to implicate this microorganism in the etiology of BV (Pheifer *et al.* 1978; Balsdon *et al.* 1980; Symonds and Biswas 1986; Eschenbach *et al.* 1988; Borchardt *et al.* 1989; Hillier *et al.* 1990; Holst 1990; Mikamo *et al.* 2000). The direct experiments on human subjects similar to the ones performed by Gardner and Dukes (1955) and Criswell *et al.* (1969) would be unethical by the current standards outlined in The Declaration of Helsinki (Rikham 1964; Giordano 2010).

2.3 The non-existent animal model for *G. vaginalis* infection

The lack of a reflective animal model for BV is probably the major reason why the role of *G. vaginalis* in the etiology of this condition is still unclear. Several attempts

to construct a reflective animal model for bacterial vaginosis were reported in the 1980s and 1990s, but they had very limited success.

For instance, the study conducted by Johnson *et al.* (1984) investigated the susceptibility of three primate species to vaginal infection with *G. vaginalis*. The researchers inoculated the lower genital tracts of four tamarins, three chimpanzees, and ten pig-tailed macaques with several strains of *G. vaginalis* and tried recovering the microorganism from these animals throughout an observation period. *G. vaginalis* did not colonize the tamarins or the chimpanzees, but did colonize all of the pig-tailed macaques for a period of 11-39 days. However, the infected animals did not display characteristic signs of bacterial vaginosis, such as an elevated vaginal pH, presence of clue cells or an increased succinate to lactate ratio in their vaginal smears. The poor resemblance of the model's condition to BV in humans is likely due to major differences in the microbiota and physiology of lower primate and human female genital tracts.

Mardh *et al.* (1984) attempted to induce BV in grivet monkeys by vaginally inoculating them with several strains of BV-associated pathogens. Thin, grey and translucent vaginal discharge was noticed in monkeys who were simultaneously infected with *G. vaginalis* and a *Mobiluncus* strain (referred to as a long curvy rods) (Catlin 1992). These symptoms appeared 5 days after the inoculation and persisted until the end of the of the 6-week long observation period. Both microorganisms were recovered from the primates' vaginal smears 12 days after the inoculation and the *Mobiluncus* strain persisted at least for an additional 25 days. Interestingly, monkeys infected with only one

of the pathogens did not exhibit any obvious BV-like symptoms, although in one animal *Mobiluncus* spp. persisted for at least 9 months.

During routine prebreeding examinations, Salmon *et al.* (1990) isolated Gram-variable pleomorphic bacilli from the genital tracts of 4 mares. These bacilli were identified as *G. vaginalis* by various tests. A larger study that followed investigated the occurrence of *G. vaginalis* in specimens collected from the genital tracts of 93 mares (Salmon *et al.* 1991). The presence of *G. vaginalis* was reported in at least 31 of these specimens (although mostly at <50 CFU/sample); furthermore, 70 other isolates were identified as *G. vaginalis*-like organisms (GVLO). The researchers did not observe any evidence of disease in these ‘infected’ horses (including abnormal vaginal discharge), which prompted them to suggest that this condition might be similar to asymptomatic BV in humans. The finding of natural *G. vaginalis* infection in mares was originally perceived as very promising for the development of an animal model for BV; after all, this was the first report of *G. vaginalis* isolated from a nonhuman species (Salmon *et al.* , 1990; Caitlin 1991). To the best of our knowledge, however, the equine model for BV has never been developed any further.

Several previously reported studies confirm the rabbit vaginal model as being somewhat satisfactory for studying BV and antimicrobials active against BV-associated microorganisms (i.e. McDuffie and Gibbs 1996; Gibbs *et al.* 2004). However, a closer analysis of the literature leads us to conclude that a fully reliable animal model for studying the etiology, pathogenesis and treatment of BV will not be forthcoming any time soon.

In the paper by McDuffie *et al.* (2002), direct surgical inoculation of *G. vaginalis* into the uterine horns of pregnant rabbits yielded positive cultures in all animal samples on day 1, but only 60% on day 4 and 50% on day 6, suggesting that infection may not have been established. Further, this does not indicate utility for vaginal inoculation or evaluation of protection from infection. The authors quote an earlier paper by Field *et al.* (1993) that used essentially the same model and concluded that *G. vaginalis* is not a maternal pathogen. The latter paper reported negative fetal effects such as 80% (infected) vs. 95% (uninfected) live birth, a 23% reduction in birth weights, and brain injury in 60% of treated fetuses. Four days after inoculation, decidual samples from all rabbits yielded positive cultures of *G. vaginalis*, although titers were not reported. *G. vaginalis*-treated animals had diffuse infiltration of the decidua and subplacental separation zone with polymorphonuclear leukocytes, consistent with histologic deciduitis.

Amniotic fluid was also positive for *G. vaginalis* in 15 of 17 rabbits. Infection was not systemic as indicated by the lack of a positive blood or peritoneal culture. The authors of this paper conclude “*G. vaginalis* produced a clinical picture suggestive of subclinical infection-positive cultures; mild, if any, maternal symptoms”. Infection was not systemic as indicated by the lack of a positive blood or peritoneal culture. The authors of this paper conclude “*G. vaginalis* produced a clinical picture suggestive of subclinical infection-positive cultures; mild, if any, maternal symptoms”. In summary, the use of this model is inherently expensive, requires surgical expertise, does not address vaginal infection and may not be appropriate for non-pregnant animals.

Finally, Yan *et al.* (1996) reported isolating 145 *G. vaginalis* strains from foxes being raised in various fox farms in China. This group later attempted to develop a vaccine against *G. vaginalis* by using cells inactivated by various chemical agents (Yan *et al.* 1997). The aluminum hydroxide gel inactivated vaccine did not induce any adverse effects in foxes and was consequently used to immunize the animals. The vaccination apparently made the animals more resistant to bacterial challenge for the duration of six months. This model is very promising, although it is unclear why such a successful animal model for BV was not developed any further since 1997 and why, at least to the best of our knowledge, it has not been used in any additional BV-related studies.

Overall, Gelber *et al.* (2008) suggested that the failures of animal models for BV could be related to the specificity of vaginolysin produced by *G. vaginalis* (previous section). Accordingly, transgenic animals expressing CD59 would be vulnerable to *G. vaginalis* infection.

3. THE ‘INCOMPETENT’ VAGINAL LACTOBACILLI THEORY

3.1 Establishment of vaginal microbiota

The microbiota of a healthy human vagina undergoes numerous changes throughout the lifetime of a woman. The natural selection of bacterial strains in the vaginal milieu is modulated by numerous factors, including the host’s genetic predispositions, environment and behavior; however, the overall selection process is poorly understood (Spiegel 1991; Forsum *et al.* 2005; Larsson and Forsum 2005).

Nevertheless, some of the changes in the vaginal microbiota are predictable because the composition of this dynamic bacterial community is influenced by levels of circulating estrogens (Robinson and Ridgway 1994; Macsween and Ridgway 1998; Brabin *et al.* 2005). Normal changes in the vaginal bacterial flora associated with different stages of sexual maturity and a menstrual cycle are described in this section.

Normal *in utero* development of a human fetus occurs in a sterile environment. An infant's initial exposure to microorganisms takes place during childbirth. The encountered bacteria come from the mother's birth canal, hands of the caregivers, and the general surroundings (Forsum *et al.* 2005; Penders *et al.* 2006; Adlerberth 2008; Reinhardt *et al.* 2009).

For the first four to six weeks after birth, residual maternal estrogens still have a strong influence on a female infant's vaginal tissues. As a result, the vagina of a newborn child resembles an adult vagina both morphologically and microbiologically. Aside from the observable physical characteristics, the vaginal mucosa of a newborn is relatively thick, with glycogen being secreted in abundance by epithelial cells (Spiegel 1991; Robinson and Ridgway 1994; Brabin *et al.* 2005; Farage and Maibach 2006). Within the first 24 hours of its life, the vagina of a child is normally colonized by lactobacilli (Robinson and Ridgway 1994; Farage and Maibach 2006). These facultative lactobacilli dominate the vaginal microbiota of the child until the maternal estrogens are metabolized.

Depletion of the residual estrogens is followed by significant changes in the vaginal microbiota. This early stage of sexual development is also associated with a neutral to alkaline vaginal pH (Brabin *et al.* 2005; Farage and Maibach 2006). The

microorganisms found on the skin and in the enteric microbiota also dominate the vaginal microbiota of prepubescent girls. At this stage of development, *Staphylococcus epidermidis*, *Escherichia coli*, and various *Enterococci* are among the most frequently identified aerobic microorganisms in a child's vaginal microbiota (Hammerschlag *et al.* 1978b; Robinson and Ridgway 1994; Myhre *et al.* 2002). Anaerobes such as *Bacteroides melaninogenicus*, *Veillonella parvula*, and *Peptococcus*, *Peptostreptococcus* and *Propionibacterium* spp. also account for a significant component of the vaginal microbiota at this stage (Hammerschlag *et al.* 1978a; Gerstner *et al.* 1982; Hill *et al.* 1995).

Puberty is marked by a gradual rise in estrogen levels, leading to thickening of the vaginal mucosa and an increase in glycogen production. Consequently, multiple researchers who investigated the vaginal microbiota of premenarchial girls have noticed an increase in the frequency of vaginal *Lactobacillus* isolation with age, suggesting that a shift in the vaginal microbiota occurs gradually (Hammerschlag *et al.* 1978b). A study conducted by Yamamoto *et al.* (2009) also revealed that the onset of menarche does not trigger significant changes in the vaginal microbiota.

During reproductive years, the healthy vaginal microbiota is dominated by *Lactobacillus* spp. However, the incidence of non-*Lactobacillus* species such as *Gardnerella vaginalis* and *Prevotella bivia* (in low numbers) is also common, as discussed in previous sections. At this stage of sexual development, the relative thickness and glycogen content of the vaginal mucosa is largely influenced by steroid hormone cycling (Farage and Maibach 2006).

The effects of the menstrual cycle on the vaginal microbiota was investigated by Eschenbach *et al.* (2000), who reported that the numbers of vaginal lactobacilli had a tendency to increase over the course of the cycle. Concurrently, the incidence of heavy levels of non-*Lactobacillus* (including *Prevotella* spp.) growth tended to decrease over the cycle ($P = .002$). As a result, Eschenbach *et al.* (2000) concluded that *in vivo* levels of potential vaginal pathogens are highest during menstruation, making this the most vulnerable time period for development of BV. It is likely that this imbalance in the microbiota is brought about by an increase in the vaginal pH due to passage of the menses. Recovery of the *Lactobacillus* population is then facilitated by an estrogen-mediated increase in the thickness and glycogen content of the vaginal epithelia, which reaches its peak mid-cycle (Eschenbach *et al.* 2000).

Menopause is accompanied by a decrease in estrogen secretion, atrophy of the vaginal epithelia, and an elevated vaginal pH (Devillard *et al.* 2004; Farage and Maibach 2006). This final stage of reproductive maturation is also associated with a decline of typical vaginal microbiota (especially lactobacilli) and with an increased prevalence of coliforms in the vaginal microbiota (Hillier and Lau 1997). Interestingly, a hormone replacement therapy was shown to reestablish the dominance of lactobacilli in the vaginal microbiota of post-menopausal women as well as decrease the incidence of strains with pathogenic potential (Heinemann and Reid 2005).

3.2 The epidemiological findings

The role of vaginal lactobacilli as a primary line of defense against various vaginal pathogens has been recognized for decades. Accordingly, researchers hypothesized that women affected by recurring BV are colonized by *Lactobacillus* strains that are not particularly competent as ‘defenders’. The actual evidence supporting this hypothesis, however, was not discovered until the late 1980s. Eschenbach *et al.* (1989) were the first to postulate that H₂O₂ production by vaginal lactobacilli is critical for sustainment of healthy vaginal microbiota.

The original study conducted by Eschenbach *et al.* (1989) included a population of 95 non-pregnant women, 71% of whom had BV and 29% of whom did not. This research group reported that while the vaginas of 96% of healthy women were colonized by H₂O₂-producing lactobacilli (LB+), LB+ were only isolated from 6% of women with BV ($P<0.001$). This correlation has since been confirmed by numerous studies, several of which are described below.

In a cross-sectional study involving 103 non-pregnant women, Nagy *et al.* (1991) tested 47 vaginal *Lactobacillus* strains collected from healthy women and 39 strains from women with BV for production of H₂O₂. This group reported that 79% of the strains isolated from healthy women and only 23% of the strains coming from the BV-affected women were H₂O₂ producers.

A cross-sectional study involving 275 pregnant women in their second trimester revealed that 59% (117 out of 199) of healthy women and only 13% (10 out of 76) of

women with BV were vaginally colonized by H₂O₂ producing *Lactobacillus* spp. (Hillier *et al.* 1992).

In their subsequent study, Hillier *et al.* (1993) enrolled 171 pregnant women in labor at term. The vaginal microbiota of the participants was classified as normal, intermediate or typical of BV. Vaginal H₂O₂ producing *Lactobacillus* spp. were detected in 5% of women with BV, 37 % of women having the intermediate flora, and in 61% of BV-free women. Additionally, the presence of many BV-related pathogens including *G. vaginalis*, *P. bivia*, *Bacteroides* and *Mobiluncus* spp. was inversely related to the presence of H₂O₂ producing *Lactobacillus* spp..

These early studies were conducted in North America and Western Europe and involved participants residing in those areas. More recent studies conducted in South America, Eastern Europe, and Asia showed the same trend, suggesting that the discovered correlation is a worldwide phenomenon (Puapermpoonsiri *et al.* 1996; Mijac *et al.* 2006; Dimitonova *et al.* 2007; Martinez *et al.* 2008).

Nonetheless, some studies failed to show a correlation between lack of vaginal H₂O₂ producing *Lactobacillus* spp. and BV. For instance, Rosenstein *et al.* (1997) analyzed the vaginal flora of 174 pregnant women. Fifty of these women were diagnosed with BV, and out of this group 19 were shown to be vaginally colonized by lactobacilli, with cell counts reaching 10⁵-10⁶ CFU/ml in the vaginal secretions of six women. Rosenstein *et al.* (1997) then randomly selected 12 women from this group of 19 for further analysis. Surprisingly, 11 (92%) of the 12 women with BV harbored vaginal H₂O₂

producing lactobacilli. Based on these results, the authors suggested that in some women the growth of BV-related pathogens can occur prior to the decline of vaginal lactobacilli.

Nevertheless, the great majority of studies established the inverse association between BV and the occurrence of vaginal H₂O₂ producing *Lactobacillus* spp.. The atypical results reported by Rosenstein *et al.* (1997) could alternatively be explained by a sampling error resulting from a small population size (12 study subjects).

The results of the numerous studies described in this section, make it tempting to suggest that BV is caused by the lack of vaginal H₂O₂ producing *Lactobacillus* spp.; however the established correlation may not necessarily indicate causality (Eschenbach *et al.* 1989). Some evidence demonstrating a protective role of vaginal H₂O₂ producing lactobacilli against BV was provided by a two-year long longitudinal study conducted by (Hawes *et al.* 1996). The study enrolled 182 non-pregnant female participants, 50 of whom developed BV during the two-year period. The results of this study revealed that women lacking vaginal lactobacilli had four times greater risk of acquiring BV than the women harboring vaginal H₂O₂ producing lactobacilli ($P < 0.001$). Presence of vaginal lactobacilli incapable of producing H₂O₂ (LB-) and absence of H₂O₂ producing *Lactobacillus* spp. increased the risk of BV acquisition 2.2 folds ($P = .02$). Accordingly, the authors concluded that the production of H₂O₂ by vaginal lactobacilli is protective against BV (Hawes *et al.* 1996; Pybus and Onderdonk 1999).

3.3 *In vitro* studies

Multiple studies attempted to model the antagonism between vaginal H₂O₂ producing *Lactobacillus* spp. and BV-related pathogens *in vitro*. The great majority of these studies demonstrated the inhibitory and/or bactericidal properties of vaginal H₂O₂ producing lactobacilli against pathogens (Atassi *et al.* 2006; Atassi and Servin 2010); however, the relative contribution of the H₂O₂ produced by the *Lactobacillus* spp. to the overall antimicrobial effect is still a matter of debate. For instance, Klebanoff *et al.* (1991) conducted multiple co-culture experiments and reported that within one hour of incubation in liquid suspension, H₂O₂ producing *Lactobacillus* strains totally inhibited *G. vaginalis* and *P. bivia*; conversely, the pathogens were not inhibited by *Lactobacillus* isolates incapable of producing H₂O₂. The inhibitory activity was enhanced by myeloperoxidase and chloride, and it diminished following the treatment with catalase (but not with heat-inactivated catalase), suggesting that the activity was specifically due to H₂O₂. McLean and McGroarty (1996), O'Hanlon *et al.* (2010) and Atassi *et al.* (2006) conducted similar studies using different vaginal isolates of H₂O₂ producing lactobacilli, however the results were the same.

The ability of vaginal H₂O₂ producing *Lactobacillus* strains to inhibit *G. vaginalis* was also demonstrated using a simultaneous antagonism (sandwich plate) assay on solid media. Additionally, the cell-free supernatants of some vaginal H₂O₂ producing *Lactobacillus* strains were able to inhibit *G. vaginalis* and *P. bivia* in deferred antagonism (well-diffusion) assays (Klebanoff *et al.* 1991; McLean and McGroarty 1996; Dimitonova *et al.* 2007). It is worth mentioning that Fontaine *et al.* (1996) reported cell-free supernatants from several H₂O₂ producing *Lactobacillus* strains of vaginal origin had

only a slight inhibitory effect on *G. vaginalis* and *Mobiluncus* spp., whereas *Bact. ureolyticus* and *Prevotella melaninogenica* were not inhibited at all by these supernatants.

Interestingly, there is a strong correlation between the inhibitory activity of the cell-free supernatants and their pH but not their H₂O₂ content (McLean and McGroarty 1996; Strus *et al.* 2006). Moreover, the inhibitory activity of the supernatants against *G. vaginalis* (quantified through their zone of inhibition) decreased 60-95% after neutralization of the pH by sodium hydroxide and only up to 30% after the elimination of H₂O₂ by catalase (McLean and McGroarty 1996).

Some *in vitro* studies quantified the levels of H₂O₂ produced by the vaginal *Lactobacillus* spp. grown in MRS broth. As expected, the concentration of H₂O₂ in these cultures largely depended on oxygen availability. Accordingly, multiple studies reported undetectable levels of H₂O₂ in vaginal *Lactobacillus* cultures grown under anaerobic conditions (McLean and McGroarty 1996; Strus *et al.* 2006; O'Hanlon *et al.* 2010). Strus *et al.* (2006) established that under aerobic conditions, the concentration of H₂O₂ in cultures of various H₂O₂ producing *Lactobacillus* isolates would generally increase throughout the exponential and stationary growth phases, reaching its peak at the onset of death phase. H₂O₂ production varied greatly among vaginally derived *Lactobacillus* strains. The reported H₂O₂ concentrations in static aerobic cultures ranged between 1-1000 µM, reaching up to 1.8 mM under intense aeration (McLean and McGroarty 1996; Strus *et al.* 2006; Aslim and Kilic 2006; O'Hanlon *et al.* 2010).

Strikingly, Nagy *et al.* (1991) reported that commercially available hydrogen peroxide in concentrations 0.882-88.2 mM did not inhibit the growth of *G. vaginalis*,

Bacteroides, *Mobiluncus* and *Peptostreptococcus* spp. on solid media. Similarly, Fontaine *et al.* (1996) reported that *G. vaginalis*, *Mobiluncus* spp. and *Bact. ureolyticus* were not inhibited on solid media by 8.82 mM H₂O₂ (Pybus and Onderdonk 1999). The minimal bacteriocidal concentrations (MBCs) of H₂O₂ in liquid suspensions containing 10⁸ CFU/ml of *P. bivia* and *G. vaginalis* were 1.7 mM and 3.5 mM, respectively (Strus *et al.* 2006). We are aware of only one reported vaginal isolate (*L. delbrueckii*) capable of producing H₂O₂ in concentrations greater than 1.0 mM (in MRS broth with intense aeration). The concentration of H₂O₂ in most reported cultures of vaginally derived H₂O₂ producing *Lactobacillus* strains are within the 4-350 µM range (McLean and McGroarty 1996; Strus *et al.* 2006; Aslim and Kilic 2006). The same strains are expected to produce much lower quantities of H₂O₂ *in vivo*, since the vaginal environment is mostly anaerobic (Strus *et al.* 2006; O'Hanlon *et al.* 2010). In hypoxic conditions, the metabolism of H₂O₂ producing *Lactobacillus* strains shifts away from production of H₂O₂ and towards the production of lactic acid since the enzymes involved in these two metabolic pathways compete for NADH (McLean and McGroarty 1996).

Finally, O'Hanlon *et al.* (2010) modeled H₂O₂ production in the vagina using real cervicovaginal fluids collected from healthy female volunteers. This research group reported that under hypoxic conditions resembling an *in vivo* environment, H₂O₂ production was undetectable after 4 hours of incubation, whereas the maximum H₂O₂ concentration in the aerated samples reached 23 µM ± 5 µM within 4 hours. Commercially available H₂O₂ at twice this concentration (50 µM) was not inhibitory to *G. vaginalis*, *P. bivia*, *Mycoplasma hominis*, *Mobiluncus curtsii*, *Mobiluncus mulieris*, *Peptostreptococcus anaerobius* and *Hemophilus ducrey*. Additionally, O'Hanlon *et al.*

(2010) detected significant H_2O_2 -blocking activity in both cervicovaginal fluids and semen. The H_2O_2 antagonists in these bodily fluids neutralized 1 mM and 10 mM concentrations of H_2O_2 , respectively, to undetectable levels. Moreover, the H_2O_2 -mediated deactivation of the vaginal pathogens by vaginal H_2O_2 producing *Lactobacillus* strains in the liquid suspension (Klebanoff *et al.* 1991) was completely inhibited by the presence of as little as 1% of cervicovaginal fluids, further indicating their antagonistic properties against H_2O_2 .

To summarize, these *in vitro* studies did not provide evidence supporting the significance of H_2O_2 -mediated control of the vaginal microbiota.

3.4 Clinical trials

If BV is truly caused by the lack of H_2O_2 -mediated control of the vaginal microbiota, then topical application of H_2O_2 should be at least somewhat effective in restoring the microbial balance in BV-affected individuals. Early gynecologists commonly practiced intravaginal douching with hydrogen peroxide to treat (with some degree of success) persistent vaginal discharge, which was presumed to be due to trichomonal infections (Wincelous and Calver 1996). Bacterial vaginosis was not recognized as a separate condition at the time; however, a vaginal discharge successfully treated by this method was probably due to BV since trichomonal infections are unresponsive to peroxide (Wincelous and Calver 1996). The discovery made by

Eschenbach *et al.* (1989), linking vaginal H₂O₂ producing lactobacilli to BV prompted a few researchers to reevaluate this long forgotten approach.

The trial conducted by Wincelous and Calver (1996) enrolled 30 women with relapsing BV who fulfilled all four of Amsel's criteria and were positive for mixed vaginal anaerobes. During the procedure, women were placed on the lithotomy couch with their pelvis lifted relative to the upper body. Then, 3% hydrogen peroxide was introduced into their vaginas using a disposable plastic bivalve speculum. After three minutes of exposure, the hydrogen peroxide was drained out by reclining the couch back into the horizontal position. At the follow-up exam three weeks post-treatment, all 23 women who completed the trial tested negative for clue cells and for mixed vaginal anaerobes. Twenty-two women (95%) had a normal vaginal pH (<4.5) and were negative for the amine test. Overall, 18 (78%) women self-reported a complete remission of the symptoms and none of the participants complained about undesirable side effects related to the treatment. Based on the results obtained in this trial, the success rate and the acceptability of the H₂O₂ treatment for BV is comparable to conventional treatments; the main shortcoming of this study, however, is that it lacked a control group.

Chaithongwongwatthana *et al.* (2003) compared the efficacy of a single H₂O₂ douche for BV treatment to a single oral metronidazole regimen, although the results were not as encouraging as the ones reported by Wincelous and Calver (1996). This randomized, placebo-controlled trial enrolled 142 women with BV diagnosed by Amsel's criteria. The follow-up exam two weeks after treatment revealed that oral metronidazole was more effective in curing BV than a H₂O₂ douche (78.6% vs. 62.5%, $P = 0.036$). The

trial did not have a group treated with placebo only. Therefore, it is difficult to compare a cure rate due to a single H₂O₂ douche to a spontaneous recovery rate.

We also came across a case study describing a 17-year old virginal adolescent with exceptionally persistent BV, which was completely cured by a multisession H₂O₂ douching. The patient was seeking medical help due to “extraordinarily malodorous” vaginal discharge; however, her symptoms were unresponsive to conventional antibiotic treatments during the six months preceding the experimental trial. Further examination by Papanikolaou *et al.* (2002) revealed *G. vaginalis* and other anaerobes in the girl’s vaginal secretions. The secretions were also positive for clue cells, an elevated pH>4.5, and the amine ‘whiff’ test. Following yet another unsuccessful metronidazole regimen, the patient was given a douche containing a 3% H₂O₂/15% NaCl/10% povidone iodine solution which was administered daily for ten consecutive days. Additionally, the girl’s vaginal walls were cleansed during each session using small gauze, which probably assisted in a removal of pathogenic biofilms. Remission of all the symptoms was documented a month after the treatment and the patient remained symptom-free during the year that followed. Repeated vaginal washouts with H₂O₂ accompanied by a mechanical removal of biofilms may potentially be an effective treatment for BV, which is worth investigating in a well-controlled clinical trial.

In a cohort study conducted by Cardone *et al.* (2003), 3% hydrogen peroxide was vaginally administered to 58 non-pregnant women with recurrent BV on a daily basis for a duration of one week. Three months after the treatment, a great majority of the participants was no longer affected by clinical symptoms of BV: 98% had vaginal

pH<4.5, 89% were free of malodorous leucorrhoea, 97.8% had a negative amine test and 100% were negative for clue cells. Additionally, H₂O₂ producing *Lactobacillus* spp., but not the typical anaerobic strains, were isolated from vaginal smears of all participants. Accordingly, the authors concluded that H₂O₂ treatment for BV is inexpensive, fully acceptable and at least as effective as conventional antibiotic treatments.

Intravaginal administration of hydrogen peroxide is a promising treatment for BV; however, additional clinical trials are needed to evaluate the efficacy of this approach. So far, two out of the three trials evaluating this remedy produced very encouraging results, providing further evidence that H₂O₂-mediated control is significant for maintaining microbial balance in the vaginal milieu. The concentration of H₂O₂ (3%) used in these trials was much higher than the H₂O₂ concentrations expected to be produced by vaginal lactobacilli *in vivo*. However, in the healthy human vagina, H₂O₂ works in conjunction with other antimicrobials (lactic acid, bacteriocins, etc.) produced by the native vaginal microbiota. Moreover, the local concentrations of H₂O₂ at the frontline where lactobacilli ‘hold their positions’ against pathogens can be much higher than the total H₂O₂ concentrations measured *in vitro*. Finally, peroxidases, in combination with halide ions found in normal vaginal secretions, greatly enhance the toxicity of hydrogen peroxide to anaerobic species (Klebanoff *et al.* 1991; Tomas *et al.* 2004). Therefore, in a healthy vaginal environment this antimicrobial could be effective at much lower concentrations.

In vitro studies failed to show the significance of H₂O₂, on its own, for control of vaginal microbiota, although they confirmed its role as an integral part of the natural defenses produced by vaginal lactobacilli (Atassi and Servin 2010). Conversely, the expected levels of H₂O₂ production in the vaginal environment, which is hypoxic, are very low. Therefore, the role of this antimicrobial *in vivo* is still unclear and the causal relationship between vaginal H₂O₂ producing *Lactobacillus* spp. and BV is yet to be established.

4. PHAGE THEORY FOR THE DECLINE OF VAGINAL LACTOBACILLI

The healthy vaginal environment created by lactobacilli through the production of lactic acid, hydrogen peroxide and bacteriocins is thought to be hostile for proliferation of *G. vaginalis* and other BV-associated pathogens (such as strict anaerobes). Some theories state that the overgrowth of pathogens characteristic to BV has to be preceded by a major disturbance within the *Lactobacillus* population (Pavlova *et al.* 1997; Blackwell 1999). The resultant decline of lactobacilli, allows for a shift in the vaginal microbiota, which is similar to the ‘meteor theory of dinosaur extinction’ explaining how mammals became the dominant class of vertebrates on Earth. It is well accepted that excessive douching and/or use of spermicidal agents, and the use of antibiotics can cause a disturbance within the vaginal *Lactobacillus* population (Tao *et al.* 1997). Alternatively, Pavlova *et al.* (1997) proposed that bacteriophages could cause a decline in vaginal lactobacilli. The involvement of bacteriophages in the etiology of BV would explain why this condition is epidemiologically similar to STIs, and yet the rate of its recurrence in women is

unaffected by an antibiotic treatment of their male partners (Blackwell 1999). In their study, Pavlova *et al.* (1997) collected vaginal specimens from 37 participants, 16 of which were diagnosed with BV based on Amsel's criteria. Among the 37 *Lactobacillus* strains isolated from these specimens, seven (19%) were identified as phage carriers (lysogens). The proportion of lysogens was presumably higher among the strains derived from BV-affected women, although the number of these strains was too low to establish statistical significance. Further *in vitro* investigation revealed that some of these phages were able to infect a broad range of *Lactobacillus* species originating from different women.

A very similar but much larger study with 209 participants in USA and Turkey was conducted by Kilic *et al.* (2001). This study confirmed the previous finding; sixty-seven (32%) out of 209 tested vaginal *Lactobacillus* strains were induced to release phages. Most importantly, the presence of lysogens was more common among women with BV ($P<0.05$). Once again, all the isolated phages were infective against a wide range of vaginal *Lactobacillus* strains, including the ones that were collected on a different continent.

All the phages identified in these two studies were temperate, i.e. they would only lyse a small portion of the infected bacteria unless induced. However, once released from a bacterial cell, some of these phages would undergo a lytic cycle in a different bacterial strain (Pavlova *et al.* 1997; Kilic *et al.* 2001). Accordingly, a lysogen or a phage itself may potentially be introduced into a healthy vagina through sexual activity, causing lysis

of the native *Lactobacillus* strains (Pavlova *et al.* 1997; Blackwell 1999; Pavlova and Tao 2000; Kilic *et al.* 2001).

Another interesting finding was reported by Tao *et al.* (1997), who sampled multiple commercially available probiotic products for phages capable of infecting vaginal lactobacilli. A number of such phages were isolated from yogurts. All the phages were temperate and only 20% of the tested vaginal *Lactobacillus* strains were susceptible to their infection. Nonetheless, the presence of these phages in commonly consumed foods suggests an additional route of phage infection for vaginal lactobacilli. Some scientists, however, believe that if vaginal *Lactobacillus* populations were dramatically affected by phages derived from commercial foods, BV would be far more prevalent (Blackwell 1999). Additionally, the lack of BV-related complaints from women who regularly consume yogurts indicates that there is no obvious link between ingestion of fermented milk and BV.

It has been proposed that, *in vivo*, some external factors can induce these normally temperate phages into a lytic cycle (Blackwell 1999; Kilic *et al.* 2001). Interestingly, a study conducted by Pavlova and Tao (2000) revealed that the lysogenic *Lactobacillus* strains of vaginal origin can be induced to release phages by benzo[α]pyrene diol epoxide, a metabolically activated form of benzo[α]pyrene found in a cigarette smoke. The phage theory of the lactobacilli decline, along with this finding, may explain why cigarette smoking is a significant risk factor for bacterial vaginosis. Accordingly, the inhaled benzo[α]pyrene is metabolically converted to its activated form in the liver and is eventually secreted into the vagina. Previous studies reported detectable levels of

cigarette smoke chemicals in the cervico-vaginal mucus of women who smoke (Pavlova and Tao 2000). Although the expected secretion levels of benzo[*a*]pyrene diol epoxide would be insufficient to directly kill bacteria, this known mutagen can cause bacterial cell lysis by inducing phages (Pavlova and Tao 2000). Additional external factors capable of inducing phages within the infected vaginal lactobacilli will likely be identified in the future. We speculate that these factors may include stressors produced by the host immune response.

Moreover, if bacteriophages truly play a significant role in the etiology of BV, then intravaginal installation of phage-resistant probiotic strains could be effective for treatment and prophylaxis of this condition (Blackwell 1999). We speculate that phage-mediated selective pressures would give an advantage to these extraneous resistant *Lactobacillus* strains over the endogenous vaginal lactobacilli, allowing for the highly desirable long-term colonization (strain replacement therapy).

5. THE ROLE OF INTRINSIC HOST FACTORS IN ETIOLOGY OF BV

As mentioned in previous sections, BV-associated pathogens can frequently be detected in the lower genital tract of asymptomatic women. At least some of these pathogens are thought to originate from the GI tract, which is considered their natural habitat (Witkin *et al.* 2007). It remains unclear why certain women are unaffected by these pathogens while the others develop BV. Furthermore, the specific factors determining the severe BV-related complications in some women but not in others are

also poorly understood (Witkin *et al.* 2007). Currently, many researchers are leaning towards the idea that the host immunity is a decisive factor in the equation determining initial development and further course of BV (Forsum *et al.* 2005; St John *et al.* 2007; Witkin *et al.* 2007). Several approaches are being used to evaluate the implication of the host's immune response in the etiology of BV. For instance, multiple studies attempt to compare vaginal concentrations of various immune mediators in women with and without BV (Mattsby-Baltzer *et al.* 1998; Genc *et al.* 2004b; St John *et al.* 2007). This methodology can establish a correlation between the signs of BV and the immune response, but not necessarily a cause-effect relationship. Therefore, *in vitro* models can additionally be used, to assess the expression levels of immune mediators of cervico-vaginal epithelia in response to BV microbiota. However, to the best of our knowledge, much like with the animal models, a reliable *in vitro* model for BV is yet to be developed. That is why in the current section we mainly concentrate on epidemiological studies exploring hereditary predisposition towards bacterial vaginosis. The main purpose of these studies is to relate the etiology of both BV and the associated complications to genetic variabilities (gene polymorphisms) occurring within a population. So far, research in this area primarily targeted genes coding for components of the innate immune system, because of their prominent role in other infectious conditions (Genc and Schantz-Dunn 2007; Misch and Hawn 2008).

Innate immune response is triggered when pathogen-associated molecular patterns (PAMPs) are recognized by host cells. The PAMPs are biologically important and therefore largely invariant components of many microbial pathogens (Aderem and Ulevitch 2000; Ozinsky *et al.* 2000). These potential threats act as ligands for Toll-like

receptors (TLRs), which belong to a family of transmembrane proteins expressed by various immune and tissue cells of the host. So far, 10 TLRs have been identified in humans (St John *et al.* 2007; Misch and Hawn 2008). Possession of multiple receptors allows the host to distinguish between major groups of pathogens and to react accordingly. For instance, double-stranded viral RNA is a ligand for TLR3, while bacterial flagellen is recognized by a TLR6 (Witkin *et al.* 2007). Certain components of microbial cell walls such as peptidoglycan from Gram-positive bacteria and mannan from yeast are recognized by a combination of multiple TLRs (TLR2 in conjunction with either TLR1 or TLR6 (Witkin *et al.* 2007)). TLR activation triggers expression of pro-inflammatory mediators, such as cytokines and chemokines. These molecular signals have multiple functions, including activation and recruitment of certain immune cells, such as neutrophils, to the site of a potential infection (Forsum *et al.* 2005). The submucosal localization of immune cells is thought to be partially responsible for the observed rise of vaginal HIV concentration among the BV-affected individuals (Spear *et al.* 1997; Hillier 1998; Zariffard *et al.* 2005). The TLR-mediated signaling cascade, initiated in response to BV, is also thought to directly induce expression of HIV virus and further down the line, a premature myometrial contraction (Al-Harthi *et al.* 1998; Al-Harthi *et al.* 1999; Genc and Schantz-Dunn 2007).

It has been proposed that the various effector molecules such as bacterial proteases and toxins, produced by BV-related pathogens, might inactivate TLRs on cervico-vaginal epithelia. These compounds can inactivate local immune response through a direct degradation of TLRs, by interference with TLR-ligand recognition, or alternatively, by inducing anti-inflammatory cytokines such as IL-10 (Witkin *et al.* 2007).

It is known, for instance, that unsaturated fatty acids, commonly accumulated in the lower genital tract of BV-affected individuals, inhibit activation of TLR2 and TLR4 in murine monocytic cell line (Lee *et al.* 2003). The inactivation of the innate immune response, in theory, would allow unrestrained multiplication of pathogens, manifested as BV. Most importantly, it has been suggested that certain polymorphisms in genes coding for innate immune system components (i.e. TLRs) would make women susceptible to these bacterially produced mediator molecules and therefore vulnerable to BV (Witkin *et al.* 2007).

An intriguing finding supporting the polymorphisms theory was published by Genc *et al.* (2004b). This group reported a *TLR4* variant (*TLR4* 4785A>G polymorphism) associated with rise in vaginal pH ($P = 0.05$), and with at least a tenfold increase in cell numbers of *G. vaginalis* ($P < 0.0001$) and anaerobic Gram-negative rods ($P = 0.08$). Additionally, increased vaginal levels of IL-1 β were detected among 896A homozygotes who were vaginally colonized by *G. vaginalis* and anaerobic Gram-negative rods, but not among *TLR4* 896G allele carriers, colonized with the same microorganisms. This observation suggested that *TLR4* 4785A>G polymorphism reduced the host's immunological responsiveness to BV-related pathogens (Genc *et al.* 2004b; Genc and Schantz-Dunn 2007). Two hundred and thirty eight pregnant women at 18-22 weeks gestation participated in the study; the *TLR4* 4785G allele, associated with the increased numbers of vaginal pathogens and decreased immune-responsiveness, was identified in 10.3% of the population. Surprisingly, however, there was no significant association between *TLR4* 4785A>G polymorphism and the incidence of BV as diagnosed by Nugent's score.

Two later studies involving 885 and 144 women in mid-pregnancy also could not reveal a link between the *TLR4* 4785A>G polymorphism and BV (Goepfert *et al.* 2005; Verstraelen *et al.* 2009). The association between BV and two other *TLR4* polymorphisms, *TLR4* 5095C>T and -2026A>G (the promoter region), has not been established either. Although, the univariate analysis conducted by Goepfert *et al.* (2005) suggested that women with BV were less likely to have a polymorphism at *TLR4* 5095 loci, the association lost its significance when the race variable was taken into account since the participants were predominantly African-American women. Indeed, if the structurally Gram-positive *G. vaginalis* is the ‘pioneer colonizer’ of the niche, leading the way for the other BV-related pathogens, then it is not surprising that TLR4, an endotoxin receptor, is not the key player in BV etiology (Verstraelen *et al.* 2009).

It is worth mentioning that multiple studies assessed the implication of *TLR4* 896A >G polymorphism in premature deliveries among pregnant women; however the results were conflicting, as such association was reported in one study (Lorenz *et al.* 2002), but not in two others (Ferrand *et al.* 2002; Hartel *et al.* 2004).

Goepfert *et al.* (2005) also looked for associations between BV occurring in mid-pregnancy and polymorphisms in various genes coding for cytokines, including interleukins (ILs) and tumor necrosis factor- α (TNF- α). Multivariate analysis, controlled for maternal race, revealed that both IL8 -845 T>C and IL1 β Exon 5 +3954 T>C polymorphisms were protective against BV, while IL6 -174 G>C polymorphism increased the risk of developing this condition. The significance of these findings is yet to be established.

Increased vaginal levels of pro-inflammatory cytokine, interleukin-1 β (IL-1 β), have been associated with BV by numerous studies (Mattsby-Baltzer *et al.* 1998; Genc *et al.* 2004b; St John *et al.* 2007). The interleukin-1 receptor antagonist (IL-1ra) acts as a competitive inhibitor of IL-1 by binding the IL-1 receptor without initiating a signaling cascade (Azevedo *et al.* 2010). Differences within a microsatellite region in intron 2 of *IL-1RN*, the gene coding for IL-1ra, give rise to five alleles. Three of these alleles (variants 3, 4 and 5) occur rarely, accounting for <5% of allele's frequency (Genc and Schantz-Dunn 2007). Of the remaining two alleles, *IL-1RN**2 has been associated with the elevated expression of IL-1ra and simultaneously with a decreased expression of IL-1 β by human monocytes *in vitro* (Danis *et al.* 1995; Vamvakopoulos *et al.* 2002). The same *IL-1RN**2 allele has also been linked to multiple chronic inflammatory conditions; therefore it was logical to suspect the involvement of this polymorphism in BV.

Genc *et al.* (2004a) reported that among African-American women, but not among other ethnicities, BV based on Nugent's score was more prevalent in *IL-1RN**2 carriers than in non-carriers (60% vs. 15%, $P=.04$). Additionally, Genc *et al.* (2004a) reported association of this allele with many-fold increase in numbers of anaerobic Gram-negative rods, mycoplasma, peptostreptococci ($P<0.05$), and with the decrease in numbers of lactobacilli ($P<0.001$) in the lower genital tract of African-American women. Surprisingly, the lack of this allele was associated with the higher rate of *G. vaginalis* and *Peptostreptococcus spp.* isolation ($P=.02$) in Hispanic women, and this allele did not have any effect on vaginal flora in Caucasian women. Within the entire study population (212 pregnant women in their mid-trimester) this polymorphism was also linked to elevated vaginal pH ($P<0.006$) and to a decreased IL-1 β response to a few BV-associated

pathogens, including *G. vaginalis* and some anaerobic Gram-negative rods. Conversely, Genc *et al.* (2004a) reported exaggerated local IL-1 β response to BV-associated pathogens among the *IL1RN**1 homozygotes, who were also at a significantly-higher risk of giving preterm birth. Accordingly, Genc and Schantz-Dunn, (2007) proposed that IL-1 β hypo-responders (*IL1RN**2 carriers) are at higher risk of developing BV because of their inability to mount an appropriate immune response to a potential threat. In contrast, IL-1 β hyper-responders (*IL1RN**1 homozygotes) are less predisposed to BV, however, they are more likely to develop obstetric complications due to this condition. The theory proposed by Genc and Schantz-Dunn, (2007) is supported by the fact that previous studies (Genc *et al.* 2004c; Genc and Schantz-Dunn 2007) linked the elevated vaginal levels of IL-1 β measured during pregnancy to subsequent spontaneous preterm birth.

Cauci *et al.* (2007) evaluated the same *IL-1RN* polymorphisms in relation to BV in a cohort consisting of 570 non-pregnant Italian women, thereby eliminating the race factor. In this study, women with BV were recruited by Amsel's criteria, with the condition also being confirmed by a Nugent's score. Three out of the five *IL-1RN* variants were observed within the study population, and only the rare *IL-1RN**3 allele had a tendency to be protective against BV ($P = 0.049$). The authors pointed out that the distribution of this rare allele somehow deviated from a Hardy–Weinberg equilibrium, therefore questioning their own results. It is worth noting that Cauci *et al.* (2007) did not evaluate isolation rates and quantities of BV-related pathogens. Additionally, all the participants were Caucasian; therefore it is difficult to make a comparison between these results and those obtained by Genc *et al.* (2004a).

Genc *et al.* (2007) also conducted an epidemiological study involving 203 pregnant women at 18 to 22 weeks gestation, to assess a contribution of *TNFA* -308G>A polymorphism (promoter region of the gene) to BV etiology. *TNFA* codes for Tumor Necrosis Factor- α (TNF- α), a pro-inflammatory cytokine with numerous regulatory functions. Previous studies revealed that among the two alleles, *TNFA* -308A had a higher transcriptional activity. Genc *et al.* (2007), however, reported that the *TNFA* 308G>A polymorphism was not linked to bacterial vaginosis as diagnosed by Nugent's score. There was also no association between this polymorphism with either vaginal presence or quantity of BV-associated pathogens nor with the elevation of vaginal pH. The same conclusion was also reached by Goepfert *et al.* (2005) in an even larger study involving 946 pregnant women with various ethnic backgrounds, suggesting that this polymorphism has no influence on rate of BV. Interestingly, Genc *et al.* (2007) reported that bacterial vaginosis induced a more than six fold increase ($P=.02$) in vaginal TNF- α levels among the *TNFA*-308A carriers, but not among the *TNFA*-308G homozygotes. This amplified TNF- α response may explain certain obstetric complications caused by BV in some women. Accordingly, Genc *et al.* (2007) reported that pregnant women with BV who carry this hyperexpressive *TNFA*-308A allele, have a six-fold higher risk to deliver prematurely than the *TNFA*-308G homozygotes with BV. Strikingly, Macones *et al.* (2004) reported that among pregnant women with BV, *TNFA*-308A carriers (hyper-responders), had a six-fold higher risk to deliver prematurely than *TNFA*-308G homozygotes. It is worth noting that the same study also reported that symptomatic BV (diagnosed by Amsel's criteria), on its own, did not increase a risk for preterm delivery when other known risk factors such as ethnicity, genital infections and *TNFA* genotype

were taken into account. These results, however, were met with criticism because clinical criteria as opposed to microbiological criteria were used to diagnose BV. Genc and Schantz-Dunn, (2007) provides a detailed review on genetic predispositions to premature birth.

Mannose Binding Lectin (MBL) is another component of innate immunity suspected in being involved in BV etiology. This protein, shown to be localized in vaginal mucosa, binds carbohydrate moieties on cell surfaces of invading microbial agents, thereby activating complement system. MBL insufficiency has been linked to some infectious conditions. Moreover, SNPs in the first exon (at codons 52, 54, and 57) of the gene coding for MBL (*MBL2*), were correlated to low serum levels of this protein (Eisen and Minchinton 2003). However, the epidemiological study involving 322 Caucasian Italian women failed to link any of the three *MBL2* alleles along with the MBL deficiency to the recurrent BV, diagnosed by a Nugent's score (Milanese *et al.* 2008). Lack of association between the same *MBL2* polymorphisms and BV was also observed in a cohort containing 201 Caucasian Italian women.

To summarize, the evidence linking specific genetic polymorphisms to BV is scarce, and it has not been always consistent across the various studies. As a result, the role of intrinsic host factors in etiology of BV is still unclear, requiring more research to be conducted. Additionally, the research in this area so far mainly targeted the components of the non-specific immune response. Aside from the immune mediators, other hereditary host factors such as the ones responsible for selection of healthy vaginal microbiota should also be considered.

With that said, most of the inconsistencies can be resolved by considering a larger and more homogenous study population. Stringent control for ethnic background and other known risk factors would eliminate many variables (Cauci *et al.* 2007). It is also likely that any particular allele has only a minor effect on vaginal microbiota that can only be revealed within a large cohort. Ultimately, predisposition to BV is probably determined not by a single allele but by a specific allelic combination known as a haplotype (Cauci *et al.* 2007). We anticipate that future research exploring specific human haplotypes in relation to BV will bring promising results. Nevertheless, it is also important to bear in mind that although heredity may predispose a woman towards BV, the condition itself is caused by interaction of intrinsic host factors with the environment.

6. DISCUSSION

Our interaction with microorganisms begins at birth, and continues throughout our lives and even after death as our bodies decompose. Numerous factors, including our genetic make-ups and our behaviors, facilitate these dynamic interactions. It has been widely accepted that symbiosis between vaginal lactobacilli and their human hosts are imperative for human reproductive health. The importance of this symbiotic relationship is reflected by the fact that bacterial vaginosis, a condition resulting from disturbances in healthy vaginal microbiota, have serious gynecological and obstetric complications.

In this article we critically evaluated four major theories explaining etiology of bacterial vaginosis. The epidemiological findings along with numerous *in vitro* studies

indicate that the symptoms of BV and the related complications arise from drastic increase in numbers of vaginal anaerobes with *G. vaginalis* leading the way. However, BV is not a typical sexually transmitted infection even though various sexual activities are well-known risk factors for acquisition of this condition. Instead, BV is a microbial imbalance among the constituents of vaginal microbiota. Most likely this imbalance has a complex etiology which involves interactions between pathogenic species, endogenous vaginal microbiota, the host and possibly bacteriophages. The relative contributions of these factors are unknown. Additionally, the interactions between these factors are modulated by a woman's behavior and her environment further complicating the overall 'equation'. This inherent complexity along with the lack of a reliable animal model is the likely reason why etiology of BV remains a mystery after decades of research. Nonetheless, the clear understanding of the pivotal factors involved in the etiology of BV along with their interactions is imperative to finding an effective treatment for this condition.

Topical application of the purified antimicrobials produced by vaginal lactobacilli can potentially be used to restore microbial balance in vaginal ecosystem through a selective inhibition of BV-related pathogens (Klebanoff *et al.* 1991; Wineslaus and Calver 1996; Papanikolaou *et al.* 2002; Cardone *et al.* 2003; Dover *et al.* 2008). Moreover, immuno-modulators could potentially be used to compensate for the hereditary deficiencies that make some women vulnerable to BV. Finally, if bacteriophages play a significant role in etiology of BV, then bacterial strain replacement therapy using phage-resistant *Lactobacillus* strains could potentially be effective for prevention and treatment of this condition.

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Chapter I.B.

TREATMENT OF BV: NEED FOR NEW APPROACHES

Conventional treatment for BV with metronidazole and clindamycin (Paavonen *et al.* 2000; Sobel *et al.* 2001), as recommended by the Centers for Disease Control and Prevention, does not eradicate all of the BV-associated bacteria (Flores Rivera *et al.*, 1997). After a successful treatment for BV, many women remain colonized with *G. vaginalis* and other BV-related pathogens without having any apparent symptoms (Boris *et al.* 1997; Swidsinski *et al.* 2005; Swidsinski *et al.* 2008). Additionally, some *in vitro* studies demonstrate that metronidazole and clindamycin, used in concentrations recommended for the topical treatment of BV, can induce a total inhibition of vaginal lactobacilli (Simoes *et al.* 2001; Aroutcheva *et al.* 2001b). Due to emergence of the antibiotic-resistant forms of the vaginal pathogens and due to the antibiotic-mediated irradiation of healthy vaginal microbiota, the antibiotics are only effective in 60% of all BV cases with the BV recurrence rates reaching 30-40% within the first two months from the ‘successful’ treatment (Colli *et al.* 1997; Bannatyne and Smith 1998; Paavonen *et al.* 2000; Beigi *et al.* 2004; Eriksson *et al.* 2005; Austin *et al.* 2005; Nagaraja 2008).

Poor efficacy of the conventional treatment poses a critical need for discovery of novel antimicrobials that would inhibit BV-related pathogens without having a negative impact on vaginal lactobacilli. Bacteriocins produced by vaginal *Lactobacillus* spp. are promising alternatives to antibiotics. These peptides can potentially be used to restore vaginal microbial balance in women with BV (Dover *et al.* 2008).

Bacteriocins are defined as ribosomally-produced proteinaceous substances of bacterial origin that exhibit antimicrobial activity (Nissen-Meyer and Nes 1997). Many microorganisms produce bacteriocins to protect their ecological niche from bacteria competing for the same environment (Nes *et al.* 1996; Riley and Wertz 2002). Bacteriocins usually consist of 15-50 amino acids (Ennahar *et al.* 2000). Many of them are positively charged molecules with hydrophobic patches. These peptides kill sensitive cells by forming pores in their cytoplasmic membranes causing leakage of cellular materials, and the depletion of the transmembrane electric potential ($\Delta\Psi$) and/or of the pH gradient (ΔpH) (Chung *et al.* 2000). The initial binding of many bacteriocins to the cytoplasmic membrane of a target cell is facilitated by the electrostatic interactions with the negatively charged phosphate groups on the cell's surface (Lins *et al.* 1999). Accordingly, phospholipid composition of a target bacterial membrane, the presence of docking molecules on the membrane, and the extracellular pH influence bacteriocin's activity (Chen and Montville 1995; Chen *et al.* 1997; Breukink *et al.* 1999).

Bacteriocins produced by vaginal lactobacilli were shown to be effective against some vaginal pathogens. For instance, the peptide extracted from a vaginal isolate, *L. salivarius*, inhibited the growth of *Enterococcus* spp. and *Neisseria gonorrhoeae* (Ocana *et al.* 1999; Juarez Tomas *et al.* 2002). Pentocin, isolated from *L. rhamnosus* had a fungistatic effect on *Candida albicans* (Okkers *et al.* 1999). Among 22 studied vaginal *Lactobacillus* strains, 77.3% exhibited bacteriocin-like activity against *G. vaginalis* (Aroutcheva *et al.* 2001a). McLean and McGroaty (1996) showed that human *G. vaginalis* are highly sensitive to lactobacilli. Lactic acid and low pH levels work

synergistically with bacteriocins and may be more important than hydrogen peroxide for inhibiting the growth of *G. vaginalis* (Dembale *et al.* 1998).

Lactocin 160 was discovered by our collaborators in Rush-Presbyterian-St. Luke's Medical Center in Chicago, Illinois (Aroutcheva *et al.* 2001c). These researchers characterized 22 *Lactobacillus* strains isolated from vaginas of healthy women. The culture supernatant of the strain later identified as *L. rhamnosus* 160, showed the strongest activity against *G. vaginalis* in a well diffusion assay. As a result, the strain was selected for further studies which indicated that the inhibitory activity of this bacterium's supernatant was not merely due to the produced organic acids. The bacterium was grown anaerobically; therefore the antimicrobial activity due to H₂O₂ was also excluded, suggesting it might be due to a bacteriocin-like substance(s). Aroutcheva *et al.* (2001c) were unable to purify the peptide directly from the culture supernatant of *L. rhamnosus* 160 due to the interference of some components found in the bacterial growth medium. Ultimately, these researchers developed a unique partial purification procedure for lactocin 160 which is described in the MATERIALS AND METHODS section of Chapter II.A. (In the following chapters, we refer to this partially purified preparation as lactocin 160). *In vitro* studies showed that lactocin 160 inhibits the growth of clinical isolates of *G. vaginalis*, *P. bivia* and group B *Streptococcus*. spp. without affecting the growth of vaginal *Lactobacillus* strains (Aroutcheva *et al.* 2001c; Dover *et al.* 2007). The activity of lactocin 160 against *G. vaginalis* was greatly enhanced by the acidic conditions which is typical of many bacteriocins. Conversely, this antimicrobial activity was completely inhibited by treatment of the lactocin 160 preparation with proteinase K,

indicating that the active component has a proteinaceous nature (Aroutcheva *et al.* 2001, personal communication).

Lactocin 160's selective inhibitory properties against BV-related pathogens and its natural origin make this peptide a promising alternative to antibiotics. Moreover, the safety studies conducted by Dover *et al.* (2007) indicate that intra-vaginal application of lactocin 160 does not induce irritation in rabbits. Additionally Dover *et al.* (2007) reported that, *in vitro*, lactocin 160 induces only a slight irritation in human vaginal ectocervical cells as compared to common spermicidal agents, suggesting that this antimicrobial is safe for topical application.

Our collaborators at Rush-Presbyterian-St. Luke's Medical Center under the leadership of Dr. Alla Aroutcheva (co-PI of the project) are currently in the process of determining the amino acid sequence as well as other characteristics of lactocin 160. In the following chapters we describe a model study characterizing mode of action of lactocin 160 against the target microorganism, *G. vaginalis*. Additionally, we identify two natural antimicrobials which act synergistically with lactocin 160 against this vaginal pathogen.

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Chapter I.C.

OBJECTIVES AND HYPOTHESIS

1. OBJECTIVES

The primary objective of this study is the elucidation of the molecular mechanism of action of lactocin 160 against *G. vaginalis*. This objective can be further divided into sub-objectives which will determine the effect of lactocin 160 on the common targets of the antimicrobial proteins, such as:

- the membrane integrity
- the cellular ATP content
- the trans-membrane electric potential ($\Delta\Psi$)
- the trans-membrane pH gradient (ΔpH)

of *G. vaginalis*.

The secondary objective of the study is the investigation of possible synergies between the activity of lactocin 160 and that of other natural antimicrobials.

Before any of these objectives can be addressed, the methods used in the investigation have to be optimized for the use with *G. vaginalis* utilizing nisin as a reference bacteriocin.

2. HYPOTHESIS

We hypothesize that lactocin 160 induces damage in the cytoplasmic membranes of the target cells (*G. vaginalis*) causing dissipation of the proton motive force and/or depletion of the cellular ATP content.

CHAPTER II

DETERMINATION OF THE MECHANISM OF ACTION OF LACTOCIN 160 AGAINST *G. VAGINALIS* CELLS²

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Chapter II.A.

LACTOCIN 160, A BACTERIOCIN PRODUCED BY VAGINAL *LACTOBACILLUS RHAMNOSUS*, TARGETS CYTOPLASMIC MEMBRANES OF THE VAGINAL PATHOGEN, *GARDNERELLA VAGINALIS*.

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ABSTRACT

Bacterial vaginosis (BV) is a commonly occurring vaginal infection that is associated with a variety of serious risks related to the reproductive health of women. Conventional antibiotic treatment for this condition is frequently ineffective because the antibiotics tend to inhibit healthy vaginal microflora along with the pathogens. Lactocin 160, a putative bacteriocin produced by healthy vaginal lactobacilli, is a promising alternative to antibiotics; this compound specifically inhibits the BV-associated vaginal pathogens such as *Gardnerella vaginalis* and *Prevotella bivia* without affecting the healthy microflora. This study investigates the molecular mechanism of action for lactocin 160 and reveals that this compound targets the cytoplasmic membrane of *G. vaginalis*, causing the efflux of ATP molecules and dissipation of the proton motive force.

Key words: probiotics, lactocin 160, bacterial vaginosis, bacteriocin, mechanism, mode of action

1. INTRODUCTION

Bacterial vaginosis (BV) is characterized by replacement of healthy vaginal microflora, which predominantly consists of *Lactobacilli* spp., by a variety of potentially pathogenic species such as *Gardnerella vaginalis*, *Prevotella*, *Bacteroides*, *Peptostreptococcus*, and *Mobiluncus* (Falagas *et al.* 2007; St John *et al.* 2007). Although BV is not a life-threatening condition, it has been linked to numerous complications related to the reproductive health of women. BV clearly elevates the risk of an infection following gynecological surgery, such as an abortion (Larsson *et al.* 2005). In pregnant women, BV may lead to intra-amniotic infections that can cause serious brain damage in the developing fetus (Goldenberg *et al.* 2000; Newton *et al.* 1997). Women affected by BV during their pregnancy are also at risk of giving birth prematurely, resulting in a high rate of infant death (Leitich *et al.* 2003; Oakeshott *et al.* 2004). Finally, BV is recognized as a major risk factor for transmission and acquisition of certain STIs, including genital herpes and HIV infection (Cherpes *et al.* 2003; Cherpes *et al.* 2005; Sewankambo *et al.* 1997). In particular, the BV-associated pathogens *G. vaginalis* and *P. bivia* were shown to directly induce replication of the HIV virus in several cell lines (Hashemi *et al.* 1999; Hashemi *et al.* 2000). Furthermore, the study conducted by Cherpes *et al.* (2005) suggests that genital tract shedding of HSV-2 is amplified by BV.

Conventional treatment of BV is by administration of the antibiotics clindamycin and metranidazole. The problem with these antibiotics is that they tend to inhibit native vaginal microflora along with the pathogens, thus contributing to a high (20 %) rate of BV reoccurrence within one month of the therapy (Weir 2004). Many researchers now perceive and treat BV as an microecological imbalance rather than an infection (Hay

2005). Accordingly, the effective treatment for this condition should selectively target the pathogenic microorganisms, while allowing the healthy vaginal microflora to recover. The bacteriocins produced by vaginal lactobacilli have recently attracted the attention of the scientific community as a possible remedy against BV (Aroutcheva *et al.* 2001a; Barberis *et al.* 1997; Simoes *et al.* 2001). Bacteriocins are defined as ribosomally synthesized antimicrobial peptides which are generally active against microorganisms closely related to the producer strain (Nissen-Meyer and Nes 1997). Lactocin 160 is a putative bacteriocin produced by a clinical strain of *Lactobacillus rhamnosus* isolated from a healthy human vagina. This antimicrobial was shown to selectively inhibit clinical strains of *G. vaginalis* and *P. bivia* but not the healthy vaginal isolates (Aroutcheva *et al.* 2001b), which makes it a promising alternative to antibiotics for prophylaxis and treatment of BV (Aroutcheva *et al.* 2001b; Li *et al.* 2005). Furthermore, both in-vitro and in-vivo models show that the topical application of lactocin 160 does not induce irritation of vaginal epithelia, indicating that it is safe for intravaginal applications (Dover *et al.* 2007).

The molecular mechanism of action of lactocin 160 has been investigated against *Micrococcus luteus* 10420, a model microorganism commonly used to study bacteriocins (Li *et al.* 2005). It was determined that lactocin 160 causes an efflux of ATP and dissipation of the transmembrane electric potential in *M. luteus* cells. In this study we investigate the mechanism of action of lactocin 160 against *G. vaginalis*, one of the key pathogens involved in BV. Nisin was used as a model bacteriocin because the molecular mechanisms of action of this antimicrobial have been studied in great detail against a variety of bacterial species. Bacteriocins have been previously evaluated as a treatment

for BV (Barberis *et al.* 1997; Simoes *et al.* 2001). However, to the best of our knowledge, this is the first report on the molecular mechanism of action of a bacteriocin against the BV-associated organism *G. vaginalis*. The apparent lack of research exploring the mode of action of antimicrobial peptides against this vaginal pathogen may be explained by the fact that *G. vaginalis* is an extremely fastidious organism, thus requiring every assay to be specifically tailored to its ideal growth or survival conditions.

2. MATERIALS AND METHODS

Bacterial strains and growth conditions

Frozen stock of *G. vaginalis* ATCC 14018 and *L. rhamnosus* 160 cultures were kept at -80° C in their appropriate growth medium supplemented with 15% glycerol. Brain Heart Infusion (BHI) broth (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) was used for the proliferation of *Gardnerella vaginalis*, while MRS broth (Difco) was used to grow *L. rhamnosus* 160. The cells were grown under anaerobic conditions at 37° C without agitation. Both microorganisms were subcultured multiple times prior to being used in the experimental procedures.

Preparation and use of the antimicrobial solutions

The partially purified preparation of lactocin 160 was obtained using the method previously described by Aroutcheva *et al.* (2001b); Dover *et al.* (2007); Li *et al.* (2005). The protocol was adapted for large-scale fermentation at the Cell Production and Recovery Facility (Waksman Institute, Rutgers University, NJ) and used to produce 50 liters of *L. rhamnosus* 160 culture. All the purification procedures were followed as

described by Aroutcheva *et al.* (2001b), leading to the production of 11.38 g of lyophilized, partially purified preparation which was subsequently used in the experimental procedures. The 10 AU ml⁻¹ stock solution of lactocin 160 was prepared by dissolving 300 mg of the powder in 1 ml of distilled water and contained 960 µg of protein as quantified using Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). The solution was then filter-sterilized through a 0.2 µm syringe filter (NALGENE, Rochester, NY) and used within four hours.

The 100 AU ml⁻¹ stock solution of nisin was prepared by dissolving 10 mg of 2.5% commercial nisin preparation (Sigma-Aldrich) in 1 ml of nisin diluent (0.02 mol l⁻¹ hydrochloric acid solution, pH 1.7). The solution was then filter-sterilized with a 0.2 µm filter (NALGENE) and used within four hours.

The activity of the lactocin 160 preparation, as expressed in arbitrary units (AU), was determined using the 96-well plate method described by Naghmouchi *et al.* (2007b). Briefly, the serial dilutions of lactocin 160 were prepared from the stock solution using sterile distilled water. Each well of the 96-well plate contained 100 µl of the lactocin 160 solution (various concentrations), 10 µl of the overnight *G. vaginalis* culture and 90 µl of the bacterium's growth medium. The plate was incubated anaerobically at 37 ° C for 72 hours, and the lowest dilution of the lactocin 160 preparation that gave the full inhibition of the microbial growth was assigned 1 AU. The activity of the nisin preparation against *G. vaginalis* was assessed in a similar way.

Data presented in the Results and Discussion section illustrates the mode of action of lactocin 160 and nisin at 1 AU ml⁻¹ so that a comparison between these two antimicrobials could be drawn. Higher concentrations of the antimicrobials were also

evaluated with equivalent results (data not shown). The 4.44 mmol l⁻¹ aqueous solution of lactic acid was used as a negative control for lactocin 160, as this is the lactic acid concentration previously reported for this partially-purified antimicrobial preparation (Dover *et al.* 2007). Lactic acid has some antimicrobial properties which had to be accounted for using the control (Aroutcheva *et al.* 2001a). Similarly, nisin diluent (20 mmol l⁻¹ aqueous solution of hydrochloric acid) was used as a negative control for nisin.

The membrane-disruption (ethidium bromide) assay

The cytoplasmic membrane integrity of the *G. vaginalis* cells was assessed using the method described by Benito *et al.* (1999). Briefly, the culture of *G. vaginalis* was grown anaerobically at 37° C to an OD₆₀₀ of 0.6 and then aliquoted into microcentrifuge tubes. Ethidium bromide (Sigma-Aldrich, St. Louis, MO) was added to each tube for a final concentration of 100 µmol l⁻¹. Immediately after the addition of ethidium bromide, cells were treated for five minutes with either 1 AU ml⁻¹ lactocin 160 or the corresponding controls. Following the treatment, cells were washed twice with the equivalent volume of PBS so that any unbound ethidium bromide was eluted. Bound ethidium bromide was quantified via its fluorescence using a PerkinElmer LS-50B spectrofluorometer (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). The fluorescence measurements were taken at 22° C using quartz cuvettes (10 mm light path) with excitation and emission wavelengths of 493 and 610 nm, respectively, and with a slit width of 10 nm. Heat treatment (100° C for 10 minutes) was used as a positive control for membrane damage. Fluorescence of the experimental samples was normalized to the

positive control with the assumption that those cells lysed by the heat experienced 100% membrane damage.

The ATP assay

The effect of lactocin 160 on the cellular ATP content of *G. vaginalis* was elucidated using the method previously described by Li *et al.* (2005). Briefly, *G. vaginalis* was grown anaerobically at 37° C to an OD₆₀₀ of 0.6. The cells were washed once with an equivalent volume of 50 mmol l⁻¹ MES buffer (pH 6.5) and then re-suspended in 50 mmol l⁻¹ MES at half the volume of the original culture. Subsequently, the cell suspension was energized with 0.2 % glucose for 20 minutes, aliquoted into 1.5 ml microcentrifuge tubes and then treated for five minutes with either 1 AU ml⁻¹ of lactocin 160 or the corresponding controls. To assess the total ATP levels of the treated cell suspension, 20 µl was mixed with 80 µl of DMSO (FisherBiotech, Fair Lawn, NJ) and then diluted with 4.9 ml of cold deionized water (4° C). DMSO lyses cells, hence inducing the release of intracellular ATP into the supernatant. For assessment of the extracellular ATP levels, 100 µl of the treated cell suspension was diluted with 4.9 ml of 50 mmol l⁻¹ MES buffer (pH 6.5). ATP contents of the prepared solutions were determined using the commercially available ATP Bioluminescent Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. The total ATP levels of cell suspensions treated with the negative controls were very consistent; thus, their average was used to normalize all results and express them as percentage values.

Δ pH dissipation assay

The effect of lactocin 160 on the transmembrane pH gradient of *G. vaginalis* cells (Δ pH) was determined using the method described by Molenaar *et al.* (1991) with some modifications. To prepare the cells, an initial 20 ml culture was incubated anaerobically at 37° C until an OD₆₀₀ of 0.6 was reached. The culture was centrifuged at 5000 g, washed twice with 20 ml of 50 mmol l⁻¹ potassium phosphate buffer (PPB, pH 6.0) and subsequently re-suspended in 200 µl of fresh PPB. The cell suspension was mixed with 20 µl of the pH sensitive probe BCECF-AM (MP Biomedicals, Inc., Solon, OH), and incubated for 5 min at room temperature to allow the diffusion of the probe into the cytoplasm. The cells were then washed twice with 1 ml of 50 mmol l⁻¹ PBS (pH 6.0) and re-suspended in 200 µl of the equivalent buffer. Changes in the intracellular pH of the cells were monitored using a LS-50B spectrofluorometer (PerkinElmer). The fluorescent measurements were taken at 22° C in quartz cuvettes (10 mm light path) with excitation and emission wavelengths of 502 and 525 nm, respectively, and with slit widths of 5 nm for excitation and 15 nm for emission. Each cuvette containing 2 ml of 50 mmol l⁻¹ PPB (pH 7.0) was spiked with 10 µl of the cells loaded with BCECF-AM. Sharp fluctuations in fluorescence intensity are generated when the sample compartment of the spectrofluorimeter is opened; therefore it is always necessary to allow ca. 20 seconds for the signal to equilibrate. As the fluorescence signal equilibrated, the cells were energized with 2.2 mmol l⁻¹ glucose. The resulting increase in the fluorescence of the probe indicates an elevation in the intracellular pH of the cells. Immediately after the signal equilibrated, cells were spiked with 5 µmol l⁻¹ valinomycin (MP Biomedicals, Solon, OH) to convert the $\Delta\Psi$ component of the proton motive force into transmembrane pH

gradient. Once again, the signal was allowed to equilibrate before cells were treated with either 1 AU ml⁻¹ lactocin 160 or the corresponding controls. Finally, 2 µmol l⁻¹ nigericin (MP Biomedicals) was used to completely dissipate any residual ΔpH.

ΔΨ dissipation assay

The effect of lactocin 160 on the transmembrane electrical potential of the cells (ΔΨ) was determined using a modified version of the method described by Sims *et al.* (1974).

Briefly, 20 ml of *G. vaginalis* culture was incubated anaerobically at 37° C until an OD₆₀₀ of 0.6 was reached. The cells were then centrifuged at 5000 g, washed once with 20 ml of fresh growth medium and re-suspended in 200 µl of fresh medium. The fluorescent probe 3, 3' - dipropylthiadicarbocyanine iodide (DiSC₃ (5)) was used to monitor changes in the ΔΨ of the cells. The fluorescence intensity of the probe was measured continuously in quartz cuvettes (10 mm light path) at 22° C using a LS-50B spectrofluorometer (PerkinElmer), with excitation and emission wavelengths of 643 and 666 nm, respectively, and a slit width of 10 nm. Each cuvette contained 2 mL of BHI broth supplemented with 3 % horse serum; DiSC₃ (5) probe was added to a final concentration of 5 µmol l⁻¹. Next, 20 µl of the cell suspension was added to the system, resulting in a noticeable decrease in the fluorescence of the probe. As the signal stabilized, the cells were spiked with 5 µmol l⁻¹ nigericin to convert ΔpH into ΔΨ; equilibration of the signal was followed by addition of either 1 AU ml⁻¹ lactocin 160 or the negative controls. Finally, 2 µmol l⁻¹ valinomycin was used to collapse any remaining ΔΨ.

Statistics

The ethidium bromide and the ATP assay were conducted at least twice, in duplicate. The results were analyzed using the Student's *t*-test ($P \leq 0.01$). Only one replicate was used for the ΔpH and $\Delta\Psi$ dissipation assays, due to the time-sensitive nature of these assays; however each of these experiments was repeated three times producing equivalent results.

3. RESULTS AND DISCUSSION

The cytoplasmic membrane damage caused by lactocin 160 in *G. vaginalis* cells could not be detected using the ethidium bromide assay

The cell membrane is the primary site of action for most reported bacteriocins (Hechard and Sahl 2002; Montville and Bruno 1994). In particular, bacteriocins such as enterocin P and lactococcin G form transient channels in the cytoplasmic membranes of their target cells (Herranz *et al.* 2001; Moll *et al.* 1996), while the activity of others, such as mersacidin and divergicin M35, may even lead to cell lysis (Bauer and Dicks 2005; Brotz *et al.* 1995; Naghmouchi *et al.* 2007a).

The ethidium bromide assay is arguably the simplest way to assess the effect of an antimicrobial on the membrane integrity of a prokaryotic cell. Ethidium bromide is a fluorescent compound with high affinity for DNA molecules. It penetrates bacterial cells via damaged cytoplasmic membranes, and subsequently binds to the intracellular DNA. Alternatively, ethidium bromide may bind extracellular DNA molecules from lysed cells. The unbound, residual ethidium bromide is removed with the washing buffer (Barker and Park 2001; Benito *et al.* 1999).

The results obtained from the ethidium bromide assay indicate that the bacteriocin nisin instigates severe membrane damage in *G. vaginalis* even at 1 AU ml⁻¹ (Fig 1b). In contrast, the membrane damage induced in *G. vaginalis* by lactocin 160 could not be detected using this assay (Fig 1a).

Lactocin 160 induces efflux of ATP in *G. vaginalis*

Bacteriocins frequently deplete the intracellular ATP pool of the cells they target. Efflux of ATP and/or of its precursor molecules (ADP, phosphates) may occur through cellular membranes perturbed by the activity of a bacteriocin (Abee *et al.* 1994; Hechard and Sahl 2002). In aerobic cells, bacteriocins also prevent ATP synthesis through depletion of transmembrane gradients (Montville and Bruno 1994). Finally, the cellular response repertoire to bacteriocins commonly involves expenditure of ATP.

The activity of lactocin 160 induced a transmembrane efflux of ATP in *G. vaginalis* cells (Fig 2a). Nisin, in contrast to lactocin 160, triggered the intracellular hydrolysis of ATP in *G. vaginalis* (Fig 2b). It is important to note that the negative controls, including cells treated with PBS buffer (data not shown), had most of their ATP content externalized, indicating that *G. vaginalis* poorly tolerates the conditions of the assay.

Lactocin 160 has putatively been described as a bacteriocin (Aroutcheva *et al.* 2001b; Li *et al.* 2005), therefore it is likely that, much like many other known bacteriocins, it exerts its effect by formation of transient pores in the cytoplasmic membrane of the target cell (Hechard and Sahl 2002). These transient channels may be ion-specific, as they frequently differ in the size, charge, and in the duration of their

existence (Herranz *et al.* 2001; Moll *et al.* 1996). Hence it is not surprising that lactocin 160 makes the target cytoplasmic membrane permeable to ATP but not to ethidium bromide, as described in the previous section. This further indicates that the antimicrobial activity (at 1 AU ml⁻¹) is not due to cell lysis. The specificity of the channels formed by lactocin 160 can be evaluated further by elucidating the effect of this antimicrobial on various transmembrane potentials in *G. vaginalis*.

Lactocin 160 completely collapses both components of the proton motive force in *G. vaginalis* cells

A common consequence of the disruption of cytoplasmic membranes, caused by bacteriocins, is a collapse of the Proton Motive Force (PMF), the electrochemical gradient essential for numerous cellular processes (Mitchell 1966; Montville and Bruno 1994). Bacteriocins can dissipate both the electrical ($\Delta\Psi$) and chemical (ΔpH) components of the PMF (Hechard and Sahl 2002), although some may specifically target only one of the gradients (Herranz *et al.* 2001; McAuliffe *et al.* 1998).

Lactocin 160, much like nisin, induced complete collapse of the transmembrane electric potential ($\Delta\Psi$) in *G. vaginalis* cells. The assay was conducted using the *G. vaginalis* growth medium; consequently there was no need to energize the cells through supplementation of glucose (Sims *et al.* 1974). Following the addition of nigericin (K^+/H^+ exchanger), cells were treated with either lactocin 160 (Fig 3a) or nisin (Fig 3b), both of which resulted in a sharp increase in fluorescence, while the corresponding negative controls did not produce such an effect. Under the assay conditions, an increase in the fluorescence intensity of the probe is caused by depolarization of the cellular cytoplasmic

membranes. The depletion of $\Delta\Psi$ induced by either lactocin 160 or nisin was total, evident by the fact that final treatment with the ionophore valinomycin (used to dissipate any residual $\Delta\Psi$) did not lead to any additional increase in fluorescence intensity.

Lactocin 160, much like nisin, also induced the collapse of the ΔpH component of the PMF, as evidenced by a sharp decrease in the fluorescence of the energized, valinomycin-treated cells (Fig 4a-b). A drop in the fluorescence intensity of the cells loaded with the pH sensitive probe BCECF-AM, points to a decrease of their intracellular pH. Conditions of the experiment were selected so that the pH of the assay buffer would be lower than the intracellular pH (data not shown). Consequently, a bacteriocin-induced decrease in intracellular pH signifies the ability of the antimicrobial to dissipate the transmembrane pH gradient (ΔpH) of the target cell. As expected, the lactic acid solution used as a negative control for Lac160 slightly acidified cytoplasmic pH in *G. vaginalis* cells (Fig 4a). This mild acidification of the cytoplasm, however, did not cause a complete collapse of ΔpH , since the subsequent addition of nigericin triggered a further decrease of the cellular cytoplasmic pH, ultimately leading to the complete dissipation of ΔpH . In contrast to their corresponding negative controls, both lactocin160 (Fig 4a) and nisin (Fig 4b) completely dissipated ΔpH in *G. vaginalis*; i.e. addition of the ionophore valinomycin did not cause a further fall in the fluorescence intensity of the probe. It is interesting to note that the drop in fluorescence caused by nisin is immediately followed by a minor rise before the signal stabilizes (Fig 4a). This 'secondary' rise in fluorescence was not observed with either lactocin 160 or nigericin, even though the experiment was repeated numerous times. This observed effect may be related to the ability of nisin to trigger intracellular ATP hydrolysis in *G. vaginalis* cells, as illustrated in previous

sections. Most importantly, this discrepancy between the cellular response to nisin and to lactocin 160 further confirms the hypothesis that although both antimicrobials target the cytoplasmic membrane of the pathogen, their precise mechanism of action may still differ.

4. CONCLUSION

This study has revealed that lactocin 160 dissipates both components of the proton motive force ($\Delta\Psi$ and ΔpH), and induces the efflux of ATP in the vaginal pathogen, *G. vaginalis*. The activity of lactocin 160 against *G. vaginalis* is likely to be due to the formation of transient pores in the cytoplasmic membrane of the pathogen. Although the bacteriocin nisin may also dissipate $\Delta\Psi$ and ΔpH by transient pore formation, the intrinsic characteristics of these channels are likely to differ from those formed by lactocin 160.

The safety and narrow spectrum of activity associated with lactocin 160 renders this antimicrobial agent as a promising candidate for the treatment and prophylaxis of bacterial vaginosis. A clear understanding of its mechanism of action, however, is vital for the intelligent design of effective formulations involving multiple hurdles. In general, cells have difficulty adapting to various unrelated stress factors; therefore a combination of antimicrobials with different mechanisms of action is likely to have a synergistic effect against the target microorganism. By the same token, multiple hurdles make it less probable for resistant mutants to arise and be selected for.

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FIGURE CAPTIONS

Fig. 1 Assessment of membrane integrity using the ethidium bromide assay.

Lactocin 160 (Lac160) and the lactic acid solution (Control-) had an equivalent effect on the concentration of cell-bound ethidium bromide, indicating that the cytoplasmic membrane damage induced by lactocin 160 in *G. vaginalis* could not be determined through the ethidium bromide assay (1a). Nisin, in contrast to lactocin 160, causes a detectable level of damage in the cytoplasmic membranes of *G. vaginalis* cells (1b); the cells treated with nisin (Nis) fluoresce with a significantly higher intensity than the cells treated with nisin diluent (Control-).

Fig. 2 The effect of lactocin160 and nisin on ATP content of *G. vaginalis* cells. Open bars represent the level of extracellular ATP, while closed bars represent the total ATP content (extracellular plus the intracellular ATP). Accumulation of extracellular ATP in the *G. vaginalis* culture treated with lactocin 160 (Lac 160) indicates that the antimicrobial induced the efflux of ATP across the cytoplasmic membrane of the cells (2a). In contrast, nisin (Nisin) caused a significant decrease in the total ATP content of the culture, signifying that this bacteriocin triggered the intracellular hydrolysis of ATP in *G. vaginalis* (2b). The corresponding negative controls for Lac 160 and nisin, were the cells treated with the lactic acid solution and nisin diluent respectively (both represented as Control-).

Fig. 3 Lactocin 160, much like nisin, induces a complete collapse of transmembrane electrical potential ($\Delta\Psi$) in *G. vaginalis*. Two $\mu\text{mol l}^{-1}$ nigericin (Nig) was initially used to convert ΔpH component of PMF into $\Delta\Psi$. A sharp increase in the fluorescence of DiSC₃ (5), following the addition of lactocin 160 (Lac160, 3a) and nisin (Nis, 3b), signifies that these antimicrobials dissipate the transmembrane electric potential in *G. vaginalis*. In contrast, the corresponding negative controls, lactic acid solution and nisin diluent, respectively (both designated as ‘Control-‘), did not affect fluorescence of the probe. Two $\mu\text{mol l}^{-1}$ valinomycin (Val) was ultimately used to completely collapse any residual $\Delta\Psi$.

Fig. 4 Lactocin 160, much like nisin, completely dissipated the transmembrane pH gradient (ΔpH) in *G. vaginalis*. Initially, the cells loaded with BCECF-AM were energized with 2.2 mmol l^{-1} glucose (Glu). Two $\mu\text{mol l}^{-1}$ valinomycin (Val) was then used to convert the $\Delta\Psi$ component of the PMF into ΔpH . Decrease in fluorescence intensity following the addition of lactocin 160 (Lac160, 4a), or nisin (Nis, 4b), indicate that these antimicrobials dissipate transmembrane pH gradient in *G. vaginalis*. Nisin diluent and lactic acid solutions, respectively, were used as the corresponding negative controls (Control-). Finally, 2 $\mu\text{mol l}^{-1}$ nigericin (Nig) was used to completely collapse any residual ΔpH .

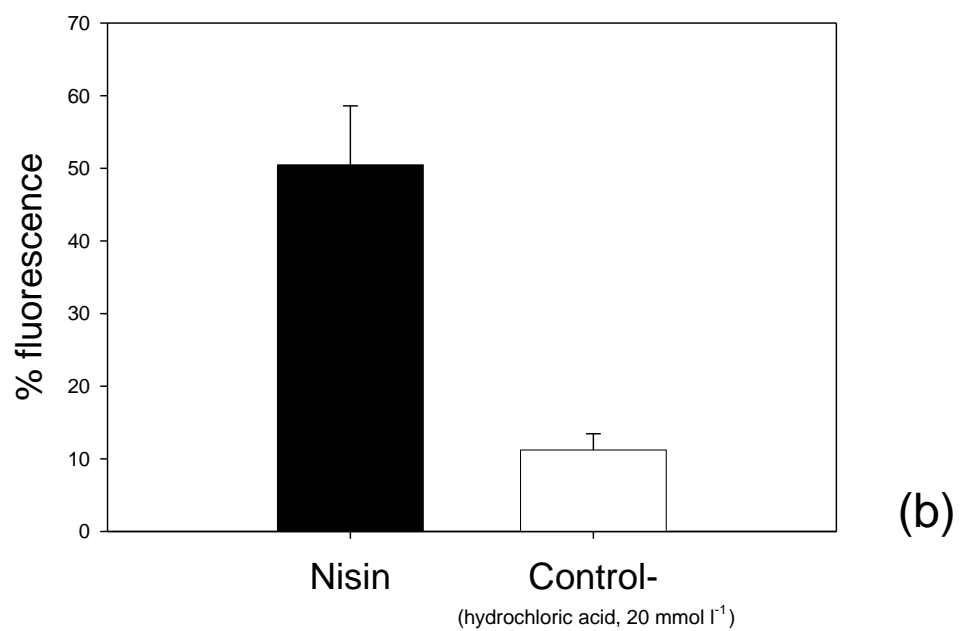
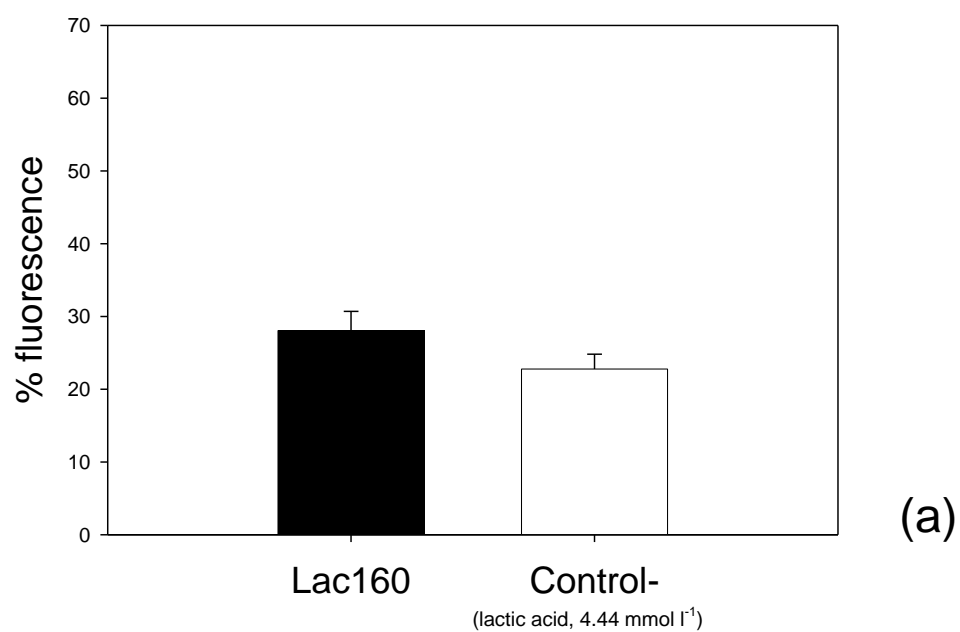
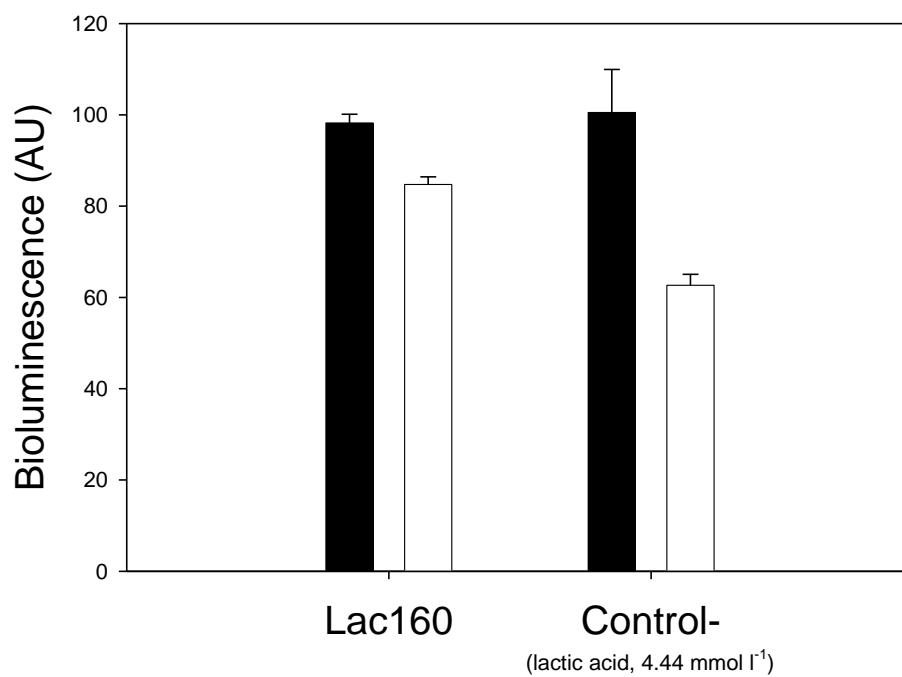
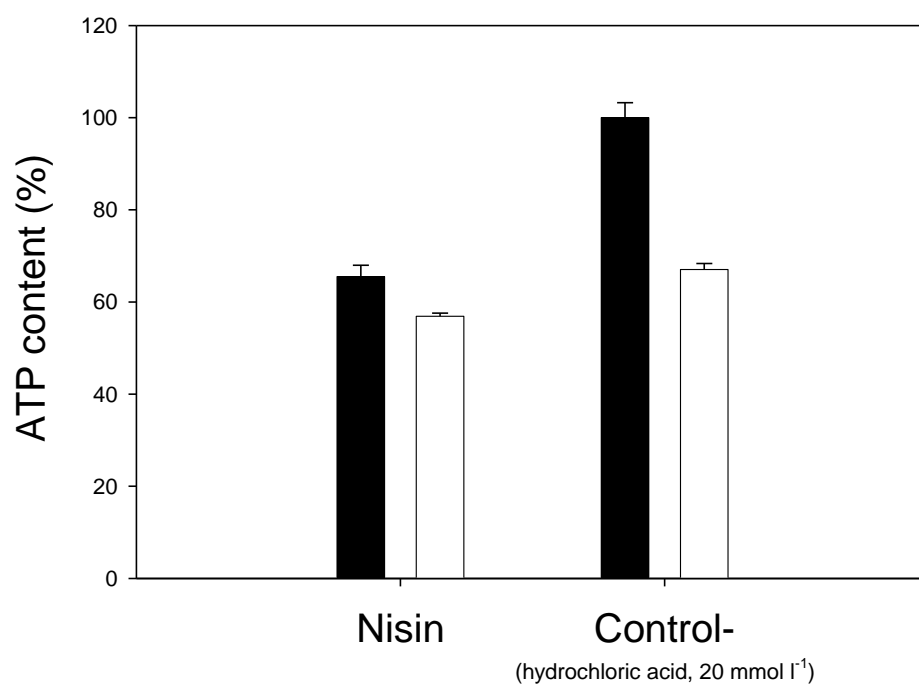
FIGURES

Fig. 1.



(a)



(b)

Fig. 2.

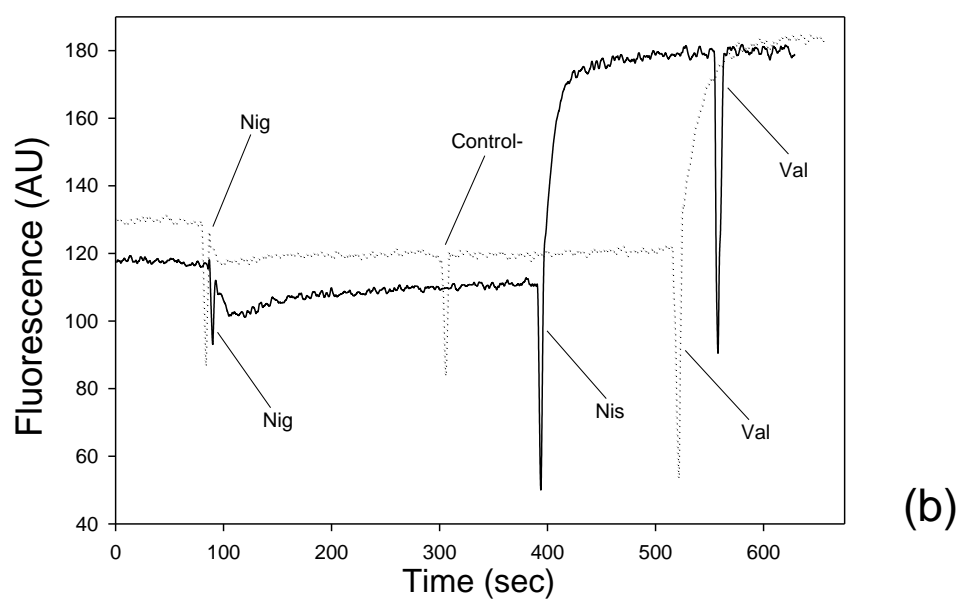
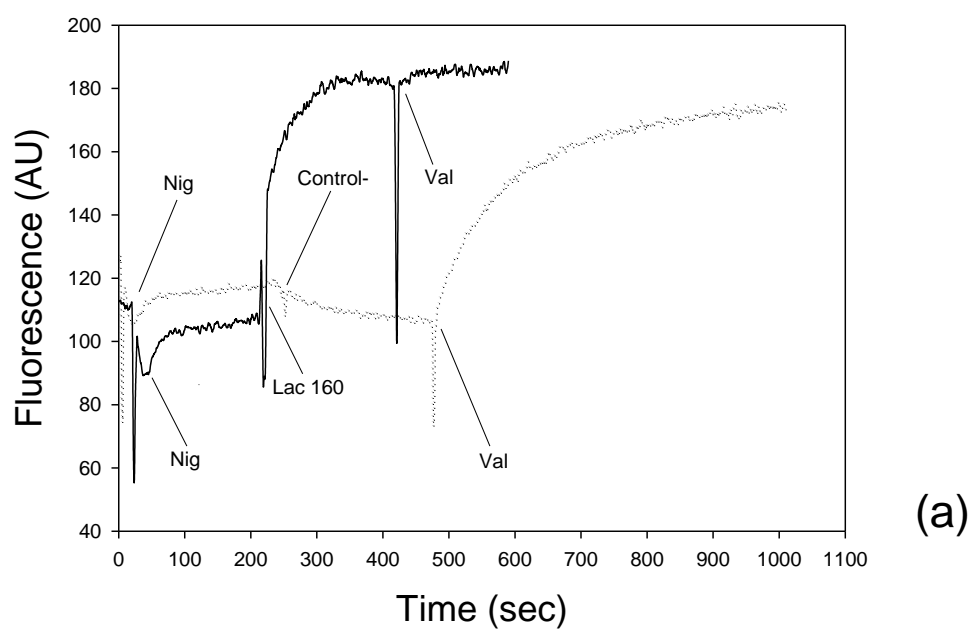


Fig. 3.

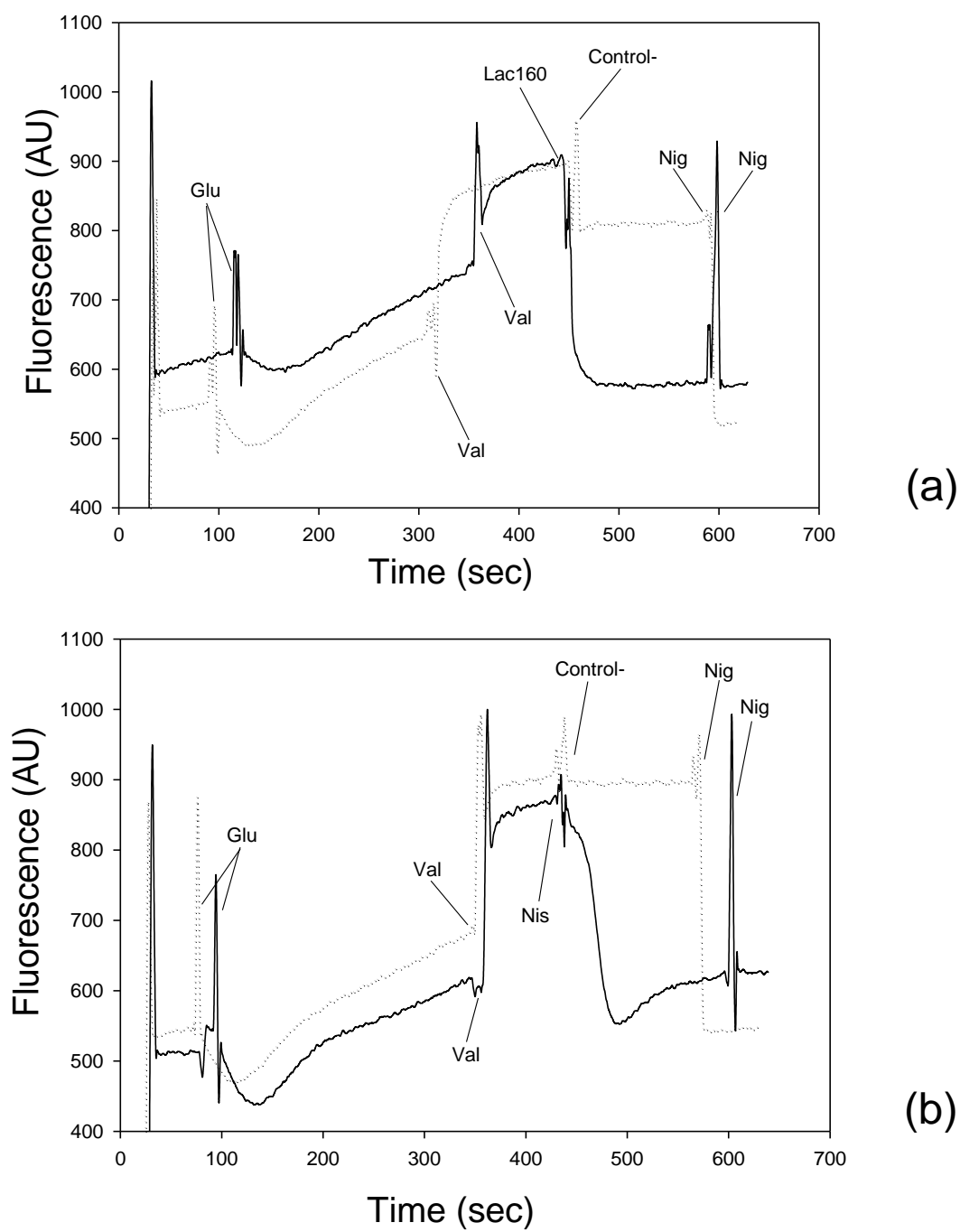


Fig. 4.

Chapter II.B.

A SHORT NOTE ON METHOD DEVELOPMENT

When the research outlined in this dissertation was initiated, very little was known about the physiology of *G. vaginalis*, aside from the fact that it is a fastidious facultative anaerobe (Chapter I.A.). Consequently, method development for the experimental procedures described in Chapter II.A. required a considerable effort, which is outlined in this section.

1. $\Delta\Psi$ DISSIPATION ASSAY

The assay monitoring changes in $\Delta\Psi$ is commonly conducted in K-HEPES buffer, with cells washed numerous times and kept on ice throughout the preparation phase. The conditions of the assay worked well for our model organism, *Micrococcus luteus*. However, *G. vaginalis* cells, after being washed with the buffer and kept on ice, did not show any ‘signs of life’. That is, neither the energization of the cells nor the addition of nigericin, nisin or valinomycin had any effect on the fluorescence of their membrane-partitioned DiSC₃ (5) (heat-killed *G. vaginalis* were used as a control). We reasoned that *G. vaginalis* is a fastidious microparasite that may poorly tolerate stresses such as starvation, being kept on ice and the oxygen-containing environment. After much trial and error, the assay was successfully designed and conducted with cells being washed and re-suspended in the fresh growth medium at ambient temperature. Surprisingly, BHI supplemented with horse serum did not interfere with the fluorescence of DiSC₃ (5). To the best of our knowledge, the $\Delta\Psi$ dissipation assay was never reported to be conducted in a bacterium’s growth medium.

2. Δ pH DISSIPATION ASSAY

The fluorescent probe commonly used for the determination of cellular cytoplasmic pH has a yellowish color (and consequently an absorbance wavelength) similar to that of BHI growth medium supplemented with horse serum. Therefore, we reasoned that the Δ pH assay had to be conducted in some kind of a buffer instead of the growth medium used for the $\Delta\Psi$ assay (this assumption was confirmed experimentally). Surprisingly, the cells of *G. vaginalis* seemed to be stable in 50 mM potassium phosphate buffer (pH=6.0) as long as they were not kept on ice. It is unclear whether it was the pH or the nature of the buffer that gave the cells their stability. This matter was not further investigated since it was not an objective of the overall study.

The original protocol by Molenaar *et al.* (1991) reports the use of acid shock for loading bacterial cells with BCECF. However, we assumed that fastidious and fragile *G. vaginalis* would not survive the shock, and therefore decided to use BCECF-AM, an acetoxymethyl ester of the probe. BCECF-AM, unlike highly charged BCECF, can passively diffuse into the cells, subsequently being converted into BCECF by the cellular esterases (providing that the bacterium possesses these enzymes (Obexer *et al.* 1995; Paradiso *et al.* 1984)). Colorless BCECF-AM was almost instantly converted into a yellowish substance (BCECF) when it was mixed into the suspension of *G. vaginalis* cells. This observation was confirmed fluorometrically, indicating esterase activity exhibited by the cells.

In the original method described by Molenaar *et al.* (1991), cells were energized with 11 mM l^{-1} glucose, which was a necessary step for the cells to build up their cytoplasmic pH. However, instead of the expected increase in fluorescence, which would

indicate a build-up of intracellular pH, the addition of glucose induced a severe drop in the fluorescence intensity of BCECF. One explanation for this observation is that under the conditions of the assay, cells could not recover from the cytoplasmic acidification caused by the intracellular fermentation of glucose (fragile *G. vaginalis* cells were sent into a 'sugar coma' from which they never recovered). A more plausible explanation for the observed effect, however, is the glucose-induced efflux of BCECF from the cytoplasm of *G. vaginalis*. The pH of the buffer is lower than the cytoplasmic pH, meaning that efflux of the probe will decrease its fluorescence intensity. The glucose-induced efflux of BCECF has been previously reported in *Lactococcus lactis* (Molenaar *et al.* 1992). To test this hypothesis, glucose-energized and non-energized *G. vaginalis* cells, both loaded with the probe, were removed from the buffer by centrifugation. There was a significantly higher amount of extracellular probe (detected by its fluorescence) in the glucose-energized cells, confirming the efflux hypothesis.

The entire experiment was repeated with the glucose-energization step being skipped. However, under these conditions the addition of 2 μM valinomycin triggered a massive decrease in detected fluorescence, instead of the increase which was expected from the valinomycin-induced conversion of $\Delta\Psi$ into ΔpH . Apparently, under the conditions of the assay, the cells did not possess sufficient resources to make this conversion without the addition of glucose. The 'middle way' was followed by energizing the cells with with 2.2 mM l^{-1} of glucose, which allowed both the retention of a sufficient quantity of the probe within the cells and the valinomycin-induced conversion of $\Delta\Psi$ into ΔpH .

Finally, we attempted to run the assay at higher pH values so that all the experiments in this study were conducted under similar conditions. Additionally, an increase in the pH of the buffer would theoretically decrease the ionophore activity of the residual lactic acid in the lactocin 160 preparation. The trial assays were carried out in 50 mM potassium phosphate buffers of pHs ranging between 6.0 and 7.8. The final addition of nigericin to the system induced a noticeable fluorescence drop even when the assay was carried at pH 7.5, suggesting that *G. vaginalis* cells have the ability to build up their intracellular pH to at least a value of 7.5. Ultimately, the effect of lactocin 160 on the transmembrane pH gradient of *G. vaginalis* was investigated using potassium phosphate buffer at pH 7.0, as described in the MATERIALS AND METHODS section of Chapter II.A.

3. THE MEMBRANE-DISRUPTION (ETHIDIUM BROMIDE) ASSAY

The cell integrity is only vital for the initial steps of this assay. Once the ethidium bromide molecules penetrate the cells through the perturbed cytoplasmic membranes, it remains bound to the cellular DNA. After the unbound ethidium bromide is washed away (MATERIALS AND METHODS of Chapter II.A.), it no longer makes a difference whether the cells are alive. Because the cells have to stay alive for just a few minutes for this method to be successful, the membrane-disruption assay worked with *G. vaginalis* in its original form, and that is why it was selected in a first place. The only problem with this method, as described by Benito *et al.* (1999), is that it requires the cytoplasmic membrane damage to be assessed against total heat-mediated cell lysis. However, the

described heating conditions (80°C for 10 minutes) failed to lyse exponential growth phase cells of *G. vaginalis*. The same conditions were effective in lysing the cells of *G. vaginalis* in their stationary growth phase, presumably because the cell wall of this microorganism becomes thinner as the culture ages (Catlin 1992; Sadhu *et al.* 1989). It is also thought that for this very same reason (thinning of the cell wall), the stationary growth phase *G. vaginalis* responds negatively to a Gram stain test. However, at mid-exponential growth phase the cell wall of this microorganism is relatively thick and its unique structure is probably responsible for increased sturdiness.

Out attempts to lyse the cells using detergents, DMSO and TRITON X-100 also did not achieve a desirable result, since the ethidium bromide assay indicated that the ‘total’ membrane damage induced by these chemical agents was still smaller than the partial damage caused by nisin. The difficulty associated with chemically-induced lysis of *G. vaginalis* has also been previously described (Catlin 1992; Greenwood and Pickett 1980; Piot *et al.* 1980; Vandamme *et al.* 1991) and is thought to be related to the unique structure of this bacterium’s cell wall. Ultimately, cell lysis was achieved by holding the cells at 100°C for ten minutes in a microcentrifuge tube with a locking cap mechanism (Fisherbrand, Atlanta, GA). The pressure created within the tube prevented boiling and the cells were successfully lysed. The final conditions used for the experimental procedure are described in the MATERIALS AND METHODS section of Chapter II.A.

4. THE ATP ASSAY

Much like the $\Delta\Psi$ and ΔpH assays, the ATP assay requires bacterial cells to be kept on ice to slow down their metabolism so they could be kept alive longer. The *G.*

vaginalis cells prepared for the assay in this manner (Li *et al.* 2005) had roughly 60% of their ATP content externalized, indicating ATP leakage; however, a subsequent treatment with bacteriocins still produced statistically significant results relative to the respective controls (Chapter II.A.). We published these original data (Turovskiy *et al.* 2009) with intention to illustrate the challenges associated with experiments involving *G. vaginalis*. Eventually, the experiment was repeated under the same conditions as described by Li *et al.* (2005) except the cells were kept at ambient temperature instead of on ice. Following these conditions, we observed the same tendencies as depicted in our published manuscript (Chapter II.A.). However, the ATP leakage was minimized, indicating that the temperature was the key limiting factor (Fig 1).

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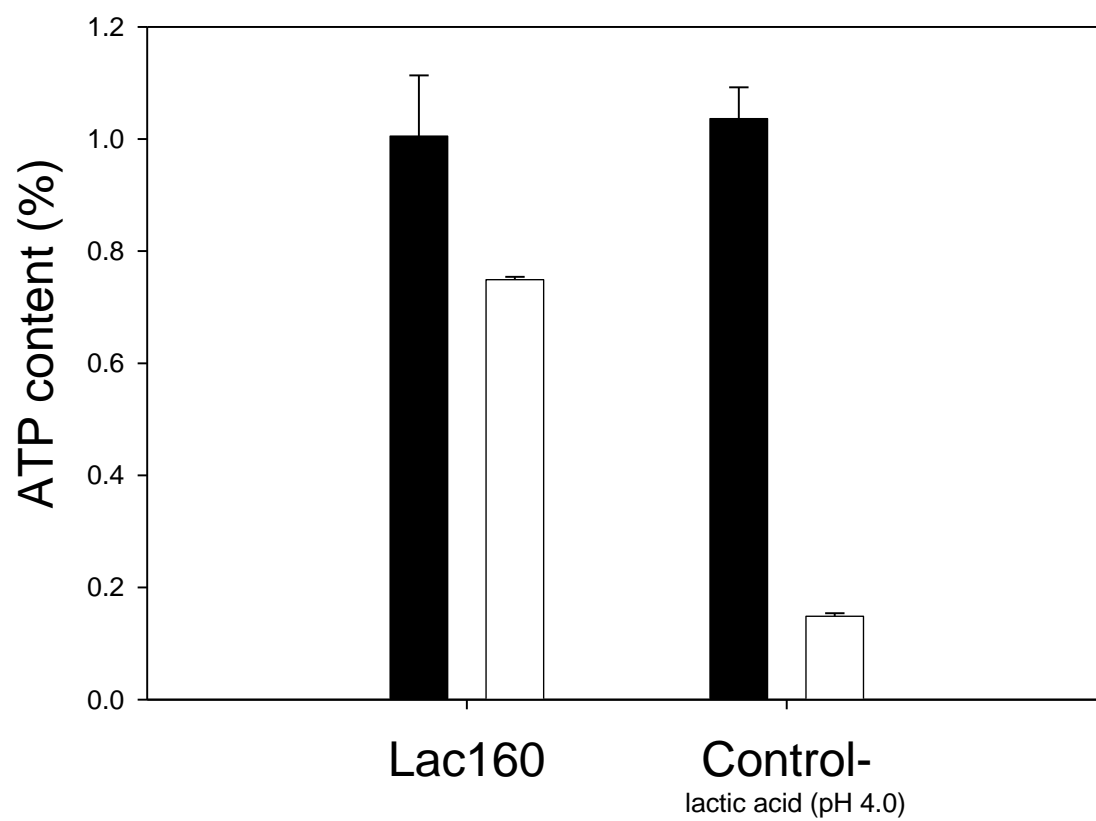
Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R. and De Ley, J. (1991) Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int. J. Syst. Bacteriol.* **41**, 88-103.

FIGURE CAPTIONS

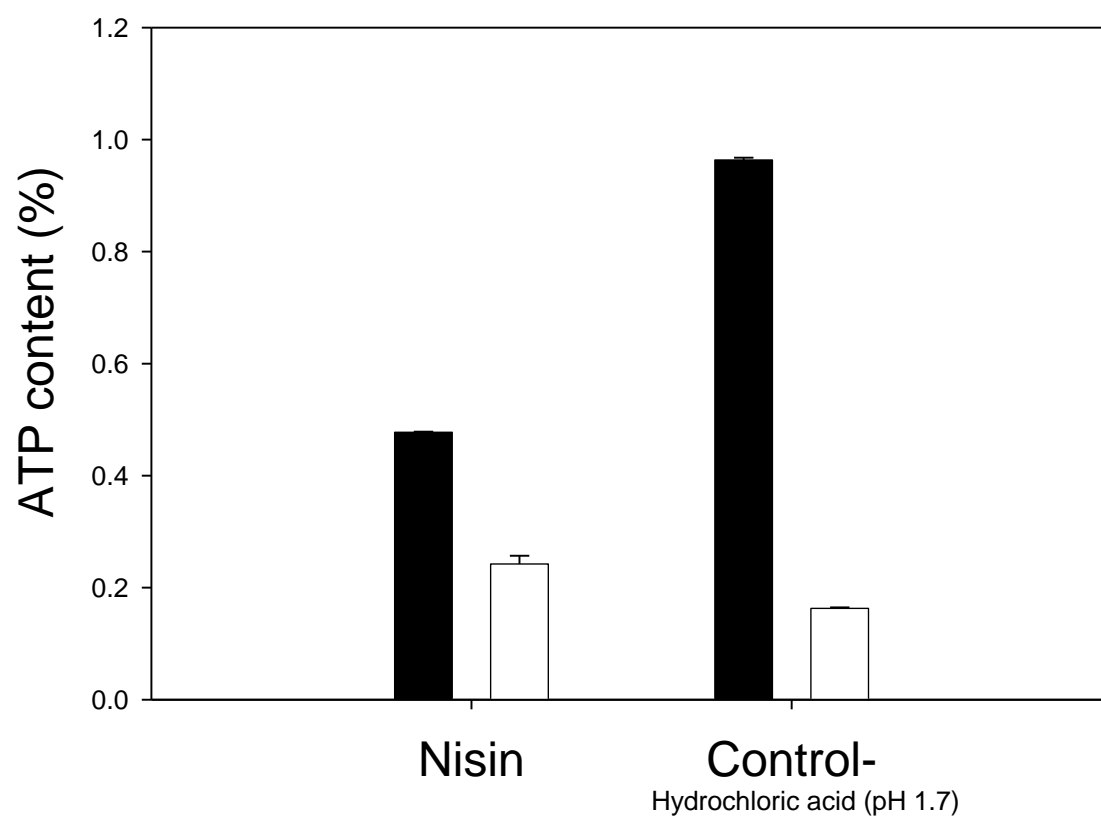
Fig. 1 The effect of lactocin160 and nisin on ATP content of *G. vaginalis* cells. This experiment was conducted with cells being kept at ambient temperature at all times instead of being kept on ice (shown in Fig. 2 of Chapter II.A.). The trend is the same, however at ambient temperature, the externalization of ATP in the controls is minimized. Open bars represent the level of extracellular ATP, while closed bars represent the total ATP content (extracellular plus the intracellular ATP). Accumulation of extracellular ATP in the *G. vaginalis* culture treated with lactocin 160 (Lac 160) indicates that the antimicrobial induced the efflux of ATP across the cytoplasmic membrane of the cells (1a). In contrast, nisin (Nisin) caused a significant decrease in the total ATP content of the culture, signifying that this bacteriocin triggered the intracellular hydrolysis of ATP in *G. vaginalis* (1b). The corresponding negative controls for Lac 160 and nisin, were the cells treated with the lactic acid solution and nisin diluent respectively (both represented as Control-).

FIGURES

Fig 1 a-b

(a)

(b)



CHAPTER III

IDENTIFICATION OF COMPOUNDS HAVING SYNERGISTIC ACTIVITY WITH LACTOCIN 160³

³The following article (III.A.) is prepared for submission to the journal of Probiotics and Antimicrobial Proteins (PAAP)

ZINC LACTATE AND SAPINDIN ACT SYNERGISTICALLY WITH LACTOCIN 160 AGAINST *GARDNERELLA VAGINALIS*

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ABSTRACT

Lactocin 160 is a vaginal probiotic-derived bacteriocin shown to selectively inhibit the growth of *Gardnerella vaginalis* and some other pathogens commonly associated with bacterial vaginosis. The natural origin of this peptide, its safety and selective antimicrobial properties make it a promising candidate for successful treatment and prophylaxis of bacterial vaginosis (BV). This study evaluated interactions between lactocin 160 and four other natural antimicrobials in the ability to inhibit *G. vaginalis*. We report that zinc lactate and soapnut extract act synergistically with lactocin 160 against this pathogen and therefore have a potential to be successfully used as the components of the multiple-hurdle antimicrobial formulation for the treatment of BV.

Key words: probiotics, lactocin 160, bacterial vaginosis, bacteriocin, synergy, synergistic interactions, *Gardnerella vaginalis*, natural antimicrobials

1. INTRODUCTION

Bacterial vaginosis is a complex multi-species infection of the lower genital tract which affects millions of women each year (Reid 2001). Aside from having a dramatic negative impact on the quality of a woman's life, this condition is notorious for causing serious gynecological and obstetric complications. Less than satisfactory results produced by the conventional antibiotic treatments for BV have prompted researchers to look for natural alternatives to antibiotics, particularly among the antimicrobial products of healthy vaginal lactobacilli. The topical application of these lactobacillus-derived antimicrobials, especially if followed by probiotic treatment, can potentially be used to restore a healthy microbial balance in BV-affected individuals (Dover *et al.* 2008). Lactocin 160, a bacteriocin-like compound produced by a vaginal isolate of *Lactobacillus rhamnosus* 160, is a promising alternative treatment for BV. This antimicrobial peptide selectively inhibits some BV-associated pathogens, including *Gardnerella vaginalis*, without affecting the commensal vaginal lactobacilli (Aroutcheva *et al.* 2001b). Moreover, the safety of lactocin 160 for topical applications has been demonstrated using both *in vivo* and *in vitro* vaginal models (Dover *et al.* 2007).

The emergence of bacterial mutants that are resistant to antimicrobials (especially multi-drug resistance) is a rapidly advancing problem in clinical microbiology (Costelloe *et al.* 2010; Kant *et al.* 2010). The widespread resistance of *G. vaginalis* to the antibiotic metronidazole, commonly used for treatment of BV, has already been reported (McLean and McGroarty 1996; Nagaraja 2008). Moreover, the ability of *G. vaginalis* to uptake DNA from other vaginal microorganisms drastically increases its chances of developing antimicrobial resistance (Harwich, Jr. *et al.* 2010; Yeoman *et al.* 2010). The most

practical way to minimize the chance of developing resistance is by using multiple antimicrobial hurdles, with each having a different mode of action (Leistner 2000).

The multiple-hurdle approach relies on the use of multiple stress factors which simultaneously deplete various resources of a target cell, making the microbial adaptation processes more challenging. Generally, stressors with different molecular targets are used as hurdles because they tend to act synergistically when used in combination (Leistner 2000). Multiple-hurdle technology has been utilized for many years to control microorganisms in clinical settings and in food preservation (Leistner 2000). A secondary advantage of this practice is its cost-effectiveness; synergistically-acting components of the antimicrobial formulation can be used in lower concentrations. In addition, the activity of an antimicrobial preparation can be modulated to a desired specificity by using a multi-component formulation (Curtis and Lee 1995; Leistner 2000). In this study we evaluated interactions between lactocin 160 and other natural antimicrobials in an effort to control the growth of *G. vaginalis* so the data can ultimately be used for the design of an effective multiple-hurdle treatment for BV.

Turovskiy *et al.* (2009) demonstrated that lactocin 160 targets the cytoplasmic membranes of *G.vaginalis* cells, ultimately dissipating both components of the proton motive force and causing depletion of the cellular ATP content. The exact molecular mechanism of action is yet to be determined, but there is evidence that lactocin 160 facilitates formation of transient pores across the cytoplasmic membranes of *G. vaginalis*, thereby triggering transmembrane traffic of ions and molecules (Turovskiy *et al.* 2009, unpublished data).

The four natural antimicrobials selected for the synergy study were zinc lactate, soapnut extract, poly-L-lysine and lauric arginate. These substances were chosen because their antimicrobial mode of action is likely to differ from the mode of action of bacteriocins, and because they have previously been approved for human use. Additionally, they have some secondary properties which could be desirable in a feminine hygiene product.

Zinc lactate is a salt of lactic acid, which is a major fermentative product of lactic acid bacteria (LAB), including vaginal lactobacilli. As a result, lactates are prevalent in the lower genital tract of healthy women (Aroutcheva *et al.* 2001a). Lactic acid is a crucial defense factor of healthy vaginal microbiota; thus, a number of feminine hygiene products, including those designed for treatment of BV, contain this compound (Andersch *et al.* 1986). Additionally, lactates are used as food preservatives. Several mechanisms are responsible for the antimicrobial properties of lactic acid and its salts. In an acidic environment, these antimicrobials act as ionophores, which drop the intracellular pH in bacteria (Cherrington *et al.* 1991; McEntire *et al.* 2003). Lactates also create a hostile environment for the proliferation of microorganisms by decreasing the water activity (McEntire *et al.* 2003). Zinc salt of lactic acid was selected for this study over other lactate species because (i) zinc ions were shown to enhance the activity of the bacteriocin nisin against *Listeria monocytogenes* (McEntire *et al.* 2003), and (ii) there are reports of ionic zinc having both antiviral and spermicidal properties (Bourne *et al.* 2005; Chvapil *et al.* 1980) - two effects desirable in a feminine hygiene product.

Epsilon-poly-L-lysine (poly-L-lysine) is a secondary metabolite secreted by various *Streptomycetaceae* bacteria. This antimicrobial is a cationic polypeptide that consists of 25-35 L-lysine residues connected by amide bonds between ϵ -amino and α -carboxyl groups (Nishikawa and Ogawa 2006). Commercially, poly-L-lysine is produced through a fermentation process involving *Streptomyces albulus* (Nishikawa and Ogawa 2006). Numerous *in vivo* studies demonstrated that poly-L-lysine is safe for consumption (Hiraki *et al.* 2003; Nishikawa and Ogawa 2006). This antimicrobial is currently on the commercial market in Japan as a food preservative (Yoshida and Nagasawa 2003). The antimicrobial activity of poly-L-lysine is related to its electrostatic adsorption to a cell's surface. The exact mechanism of its action is largely unknown, although it has been proposed that this ionic adsorption strips the outer membrane in Gram-negative cells (Shima *et al.* 1984; Yoshida and Nagasawa 2003).

Saponins are steroid or triterpenoid glycosides produced by a variety of plants and by some marine organisms. This group of natural detergents is very common in both human and animal diets (Francis *et al.* 2002). Plant extracts containing saponins are commonly used as food additives. For example, *Quillaja saponaria* extract is widely used in both the food and beverage industries without any reported toxicity (Kirk *et al.* 2004). Saponins derived from the fruit pericarp of *Sapindus mukorossi* (soapnut) are of particular interest for this study because they have only an insignificant effect on the proliferation of vaginal lactobacilli. Additionally, *Sapindus* saponins have spermicidal properties and are used as active ingredients in the contraceptive cream CONSAP (Ojha *et al.* 2003). Finally, there are some reports of saponins inhibiting the replication of the HIV-1 virus (Francis *et al.* 2002; Mengoni *et al.* 2002). Although it is clear that the

antimicrobial activity of saponins is related to their detergent-like properties, the exact mechanism of action is still unknown. Studies involving liposomes, however, suggest that saponins permanently damage cytoplasmic membranes, making them permeable to macromolecules (Francis *et al.* 2002).

Lauroamide arginine ethyl ester (LAE) is a derivative of lauric acid, L-arginine and ethanol. This antimicrobial has a generally recognized as safe (GRAS) status for use in meat, poultry and other food products (GRAS Notice No. GRN 000164). The antimicrobial activity of LAE is thought to be related to the compound's surfactant properties (Rodriguez *et al.* 2004). Studies conducted by Rodriguez *et al.* (2004) using transmission electron microscopy (TEM) indicated that LAE induced swelling of the outer membrane in a Gram-negative bacterium, *Salmonella typhimurium*. In contrast, LAE induced the formation of white spots and clear zones in the cytoplasmic membranes of *Staphylococcus aureus*, a Gram-positive bacterium. In both strains, these alterations induced the flux of potassium ions across the cytoplasmic membrane (Rodriguez *et al.* 2004).

In this manuscript, we demonstrate that zinc lactate and soapnut extract act synergistically with lactocin 160 against *G. vaginalis*. This synergistic interaction indicates a possibility of these natural antimicrobials being successfully used as the components of a multiple-hurdle approach for control of BV-related pathogens.

2. MATERIALS AND METHODS

Bacterial strains and growth conditions

Frozen stocks of *G. vaginalis* ATCC 14018 were maintained at -70°C in Brain Heart Infusion (BHI) broth (Difco, Sparks, MD) containing 3% horse serum (JRH Biosciences, KS), mixed with 15% glycerol. BHI containing 3% horse serum was also used to propagate the culture, which was passed through the medium overnight at least twice prior to being used in experiments. The cells were always inoculated into fresh medium contained in 50 ml centrifuge tubes (1% v/v) which were pre-incubated with a loosened cap, under anaerobic conditions, to minimize the stress-effect; this way, the microorganism was directly transferred into a warm, anaerobic environment. The culture was then incubated under anaerobic conditions at 37°C.

Preparation of antimicrobial solutions

The partially purified preparation of lactocin 160 was produced at the Cell Production and Recovery Facility (Waksman Institute, Rutgers University, NJ) using the method described by (Aroutcheva *et al.* 2001b; Turovskiy *et al.* 2009). The 10 AU ml⁻¹ stock solution was prepared by dissolving 300 mg of this preparation in 1 ml of double distilled water. The total protein concentration in the stock solution was quantified by using Micro BCATM Protein Assay Kit (Thermo Scientific, Rockford, IL), and it was determined to be 960 µg ml⁻¹.

The other four antimicrobials were generous gifts provided to us by their manufacturers. The sample of zinc lactate (PURAMEX ZN) was given to us by Purac

America (Lincolnshire, IL). We received soapnut extract (SAPINDIN) from Sabinsa Corp. (Piscataway, NJ). Poly-L-lysine (250 mg ml^{-1}) was sent to us by Chisso America, Inc. (Rye, NY) and lauramide arginine ethyl ester (100 mg ml^{-1} , MIRENAT-CF) was a gift from Vedeqsa Corp. (Barcelona, Spain). Prior to being used in the experiments, the aqueous solutions of all the antimicrobials were filter-sterilized through $0.2 \text{ }\mu\text{m}$ syringe filters (NALGENE, Rochester, NY).

Determination of the minimal inhibitory concentrations (MICs) of the antimicrobials

The minimal inhibitory concentrations (MICs) of all the antimicrobials were determined using the method reported by Turovskiy *et al.* (2009) with the some modifications. A separate microplate assay was conducted for each antimicrobial. Aqueous solutions covering a wide range of antimicrobial concentrations were prepared using sterile double distilled water. One hundred μl of each dilution was then placed into a 96-well plate (Corning, Inc., Corning, NY), in duplicate, immediately followed by $100 \text{ }\mu\text{l}$ of the newly inoculated *G. vaginalis* culture. Sterile, double distilled water was used as a negative control to reveal the growth patterns of *G. vaginalis* without any antimicrobial restraint. Subsequently, the surface of the wells was covered with sterilized mineral oil for prevention of condensation during the assay. The assay was conducted at 37°C under anaerobic conditions. The OD_{595} readings were taken every two hours for 48 hours using an automated microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). Some natural antimicrobials used in this study are only available in the partially purified form;

therefore we decided to express the activity of all the antimicrobials against *G. vaginalis* in arbitrary units (AU), defined as the lowest dilution of the partially purified preparation causing the full inhibition of the microorganism. The subsequent interaction analysis was conducted using arbitrary units of activity.

The checkerboard assay

The interactions between two antimicrobials were investigated using a microplate reader-based checkerboard assay described by Asok *et al.* (2004) and Badaoui *et al.* (2007). Briefly, the aqueous solutions of each pair of antimicrobials were prepared separately. The solutions were then mixed in various proportions using a 96-well plate to produce antimicrobial mixtures containing a wide range of concentrations (the highest concentration of each antimicrobial in the mixture was above the antimicrobial's MIC). In total, each well of the microplate contained 100 µl of the antimicrobial mixture (the composition of which systematically varied throughout the plate) and 100 µl of *G. vaginalis* culture. The cells used in the assay were prepared by diluting an overnight culture of *G. vaginalis* 100 times with fresh growth medium that was incubated overnight under anaerobic conditions to rid it of oxygen. The cells were then added to the wells of the microplate containing the antimicrobials (100 µl of diluted culture per well). Changes in turbidity were monitored under anaerobic conditions for 48 hours using an automated microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA). The readings at OD₅₉₅ were taken every 2 hours while the plate was incubated at 37°C. The ultimate goal of this assay is to determine the MICs of the tested antimicrobials when they are used in various combinations.

Analysis of the checkerboard assay data

The nature of interactions between lactocin 160 and other natural antimicrobials was analyzed using isobolograms. This analytical method relies on the comparison of the MICs of two antimicrobials when they are used individually to their MICs when used in combination. The isobologram method is based on a visual comparison of these values when plotted on the same coordinate axis. Initially, the individual MICs of two antimicrobials are plotted on the x - and y -axes with the coordinates $(0, x)$ and $(y, 0)$. These two points are then connected by a perforated interaction line. The fractional inhibitory concentrations (FICs), defined as the ratio of the compound's MIC when used in combination with the second antimicrobial to its MIC when used individually, of each compound are then plotted as the x and y coordinates of a single point.

3. RESULTS

The checkerboard assay allows for simultaneous evaluation of multiple concentrations of a two-component mixture and to identify antimicrobials that act synergistically with lactocin 160 (Badaoui *et al.* 2007). According to the isobologram model, the concentration combinations located below the perforated interaction line signify synergistic interactions. Conversely, the combinations located above the interaction line indicate antagonism, while the ones located along the interaction line show an additive effect between the tested antimicrobials. Finally, the lack of microbial growth inhibition by a combination of two antimicrobials at their sub-inhibitory

concentrations indicates the lack of interaction; i.e. the antimicrobials have no effect on each other's inhibitory activity (Badaoui *et al.* 2007).

Accordingly, several points representing concentration combinations of lactocin 160 and zinc lactate appeared below the interaction line on the isobologram, indicating synergy between these two antimicrobials (Fig 2a). Similarly, some synergistic effect was noticed between lactocin 160 and soapnut extract (Fig 2b). However, we did not observe any interactions between lactocin 160 and LAE; various combinations of these antimicrobials at their sub-inhibitory concentrations did not inhibit the growth of *G. vaginalis* (Fig 2c). Finally, when applied in combination lactocin 160 and poly-L-lysine had an antagonistic effect (Fig 2d).

4. DISCUSSION

Originally, we anticipated to see synergy among all four antimicrobial combinations tested in this study because of the pronounced mode of action differences between the components of each combination. Although poly-L-lysine, LAE and soapnut extract, much like lactocin 160, target bacterial cytoplasmic membranes, the damage caused by these three antimicrobials is presumably permanent, in contrast to transient pores created by bacteriocin-like substances such as lactocin 160. However, out of the four tested antimicrobials, only zinc lactate and soapnut extract synergized with lactocin 160 against *G. vaginalis*.

Lactates may act as ionophores at pH values close to and below the pK_a of lactic acid (3.86 at 25°C). However, under the conditions of the checkerboard assay (pH close to neutral), the antimicrobial activity of these salts is mainly due to a decrease in water activity within the bacterial environment. In contrast, lactocin 160 inhibits *G. vaginalis*' growth by depleting its various transmembrane gradients (Turovskiy *et al.* 2009); therefore, synergy between these antimicrobials with very different inhibition mechanisms is not surprising. Soapnut extract acts as a detergent, inducing immense, permanent membrane damage which is also significantly different from the transient channels formed by bacteriocins.

The antimicrobial activity of LAE is also thought to be related to the surfactant properties of this compound. Much like lactocin 160, LAE makes bacterial cytoplasmic membranes permeable to potassium ions, although little is known about the nature of the perturbation caused by this antimicrobial. The lack of interactions between these two antimicrobials can possibly be explained by a similar mode of action.

The reasons for antagonism between lactocin 160 and poly-L-lysine are unclear. It is possible that the electrostatic interactions between these two peptides reduce their activity against a target cell. The second possibility is that lactocin 160 adsorbed to the cell surface may hinder interactions between poly-L-lysine and its cellular targets. Finally, it is possible that a sub-inhibitory concentration of the one antimicrobial triggers some adaptive response in the target cell, making it resistant to the second antimicrobial. Interestingly, the antagonism effect was only observed at sub-inhibitory concentrations of lactocin 160; the effect was not evident at the inhibitory concentrations. For that reason,

poly-L-lysine can, theoretically, still be included in an antimicrobial formulation involving lactocin 160, as long as both these antimicrobials are used at concentrations at or above their MICs. However, due to their synergy with lactocin 160, zinc lactate and soapnut extract are the most promising candidates for a lactocin 160-based multiple-hurdle preparation for control of *G. vaginalis*.

The pH of the system used in this study was around neutral, resembling the elevated vaginal pH characteristic of BV. This system is reflective for the use of lactocin 160-based formulations for treatment of BV. However, these antimicrobials can also be potentially used to prevent recurrence of BV by suppressing the growth of *G. vaginalis* in successfully treated patients. The healthy vaginal environment has an acidic pH (<4.5). Therefore, if the formulations were used for prophylaxis of recurrent BV, the interactions between antimicrobials would take place in acidic conditions. We were unable to grow *G. vaginalis in vitro* under acidic conditions; thus, we can only speculate about the antimicrobial interactions in these conditions. Theoretically, the acidic environment should enhance the bactericidal properties of the antimicrobials, because this environment provides an additional stress for the microorganism. This is especially true for antimicrobials such as lactocin 160 and zinc lactate, which act as ionophores, making the bacterial cytoplasmic membranes permeable to traffic of hydrogen ions. However, it is also possible that the acid tolerance response (ATR) of *G. vaginalis* would induce resistance to other antimicrobials. For instance, induction of ATR in *Listeria monocytogenes* through exposure to lactic acid increased this bacterium's tolerance to the bacteriocin nisin (Bonnet *et al.* 2006; Bonnet and Montville 2005). Therefore, the experimental approach is ultimately unavoidable. Accordingly, future studies can

evaluate the effectiveness of the selected antimicrobial combinations against biofilms of *G. vaginalis*, which are known to have an inherent tolerance of lactic acid (Patterson *et al.* 2007). The biofilms can be grown to maturity at neutral pH and then can be re-suspended in acidic media to test the antimicrobial interactions under acidic conditions resembling a healthy vaginal environment.

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FIGURE CAPTIONS

Fig. 1 Growth kinetics of *G. vaginalis* in the presence of poly-L-lysine. Typically, at sub-inhibitory concentrations of an antimicrobial ($<1 \text{ AU ml}^{-1}$) the lag phase is prolonged because the cells are stressed and/or because some portion of the bacterial population is killed. Sub-inhibitory concentrations may also decrease the growth rate during the exponential growth phase (slope of the curve) or decrease the cell density reached by the culture at the onset of the stationary phase. The antimicrobial poly-L-lysine is used as an example to demonstrate these tendencies. All the concentrations were tested in duplicates; however the replicates of the OD values were averaged to simplify the figure.

Fig. 2 Isobolograms showing interactions between lactocin 160 and four natural antimicrobials against the vaginal pathogen *G. vaginalis*. Lactocin 160 synergizes with zinc lactate (a) and soapnut extract (b). For the most part, there are no interactions between lactocin 160 and the LAE preparation (c). Finally, there is a marked antagonism between lactocin 160 and poly-L-lysine (d).

FIGURES

Fig. 1

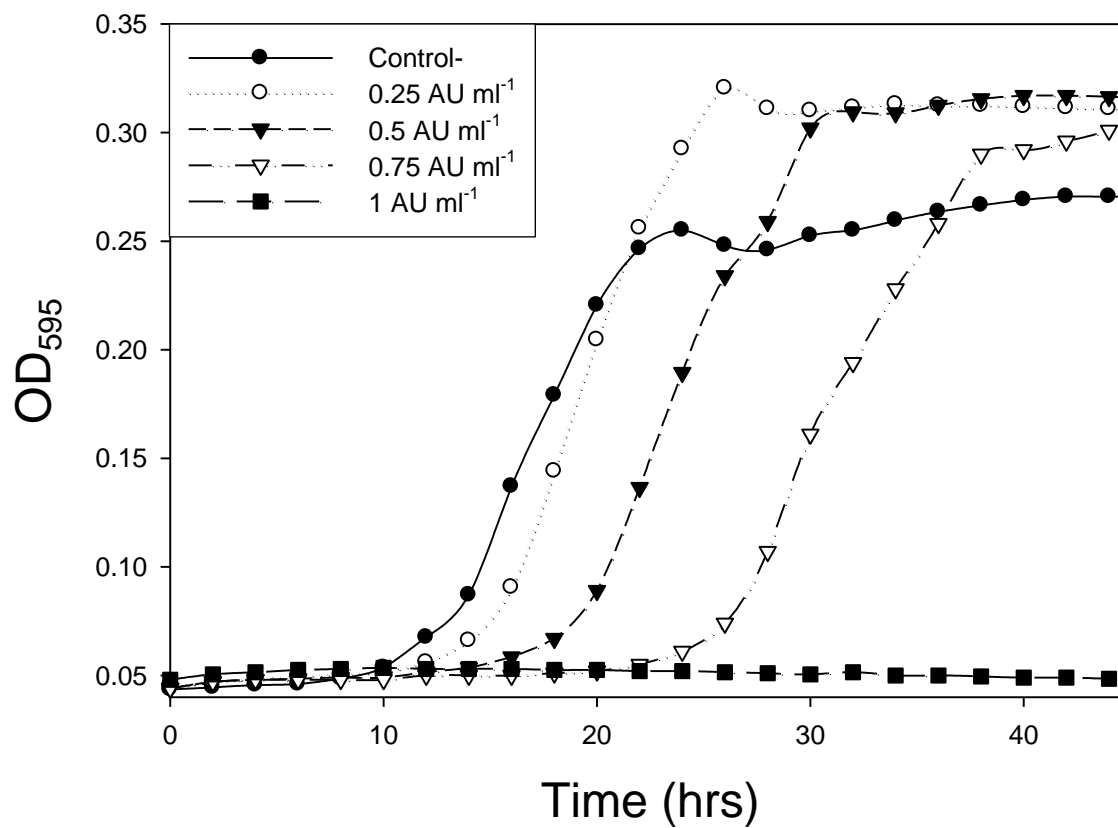


Fig. 2a

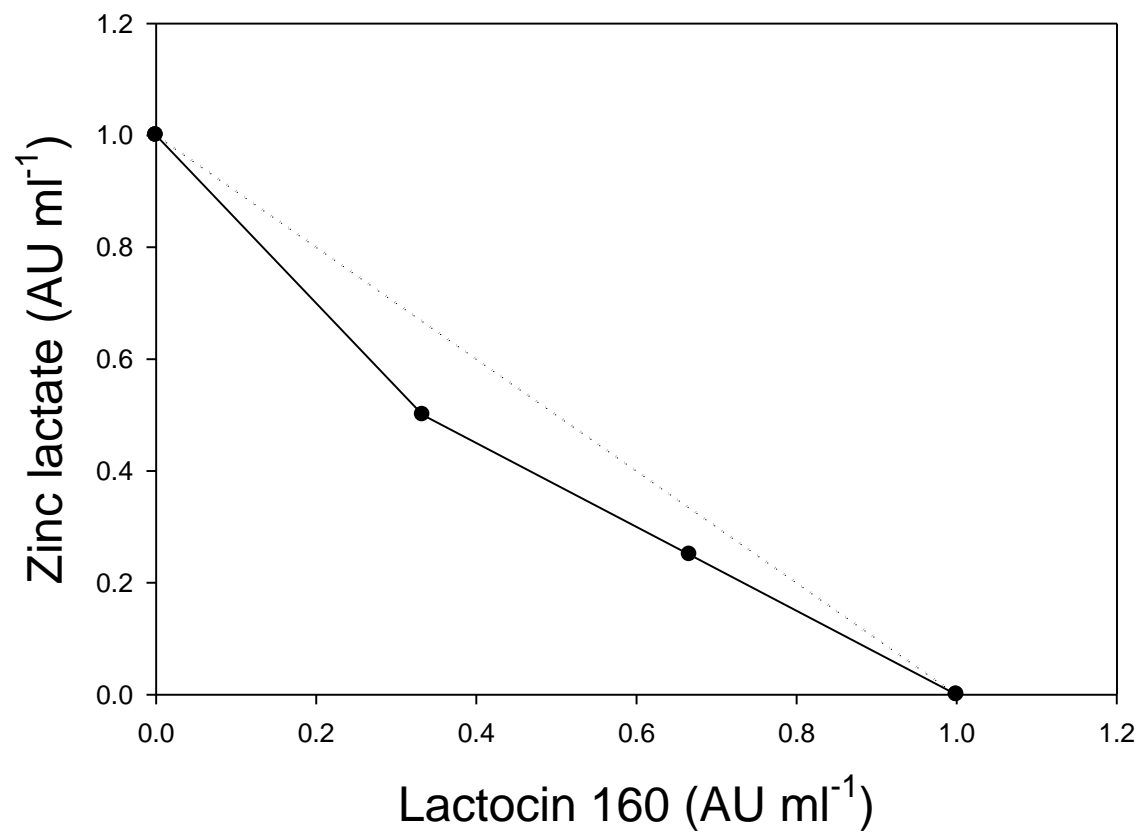


Fig. 2b

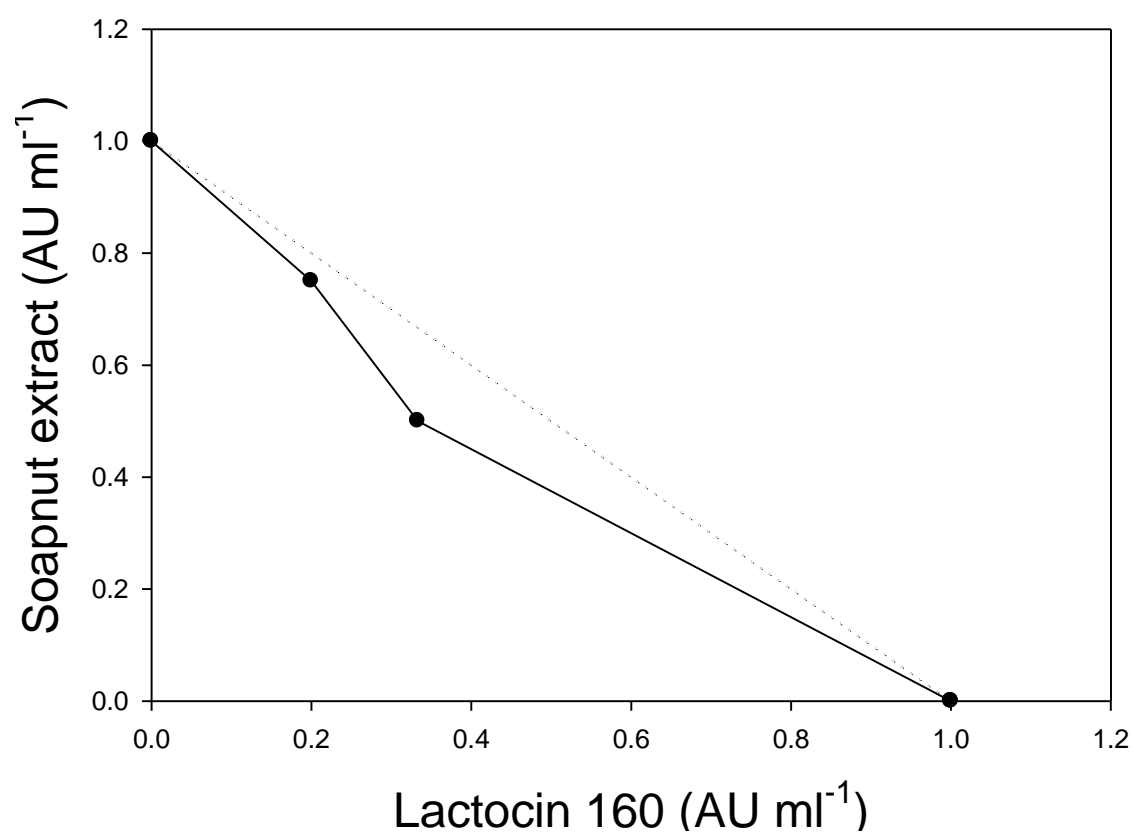


Fig. 2c

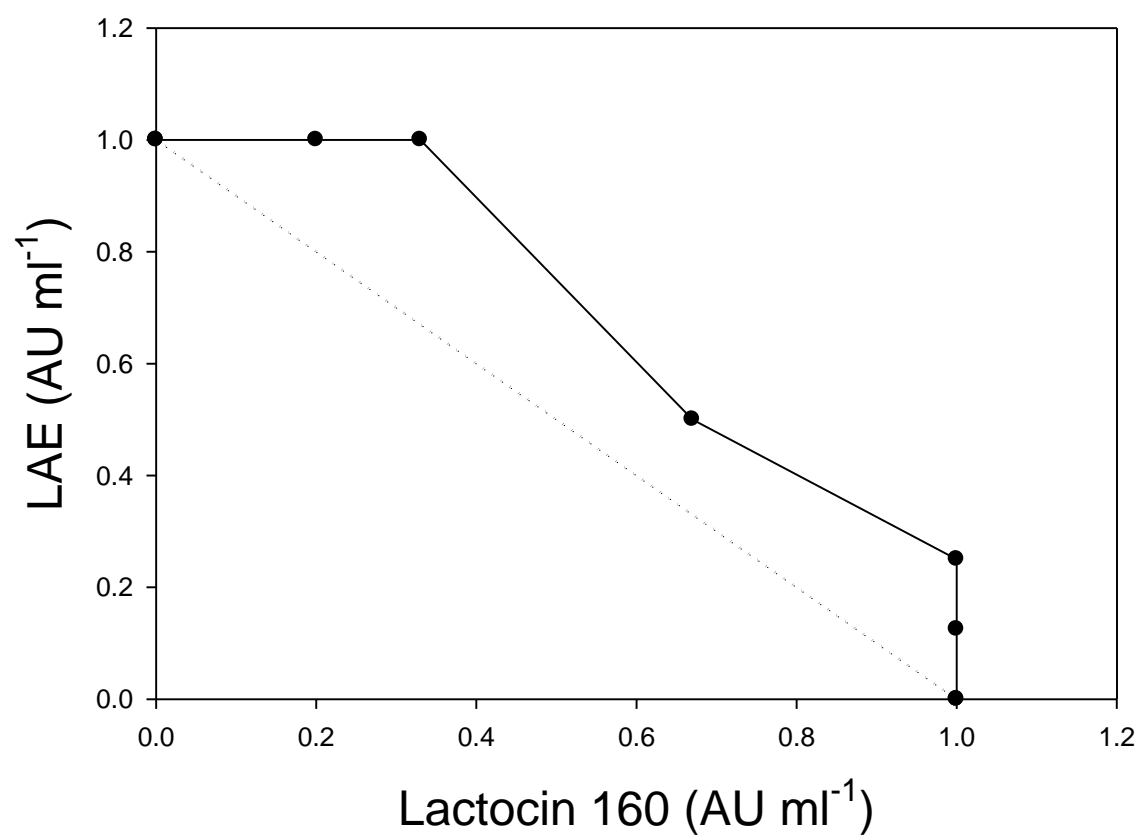
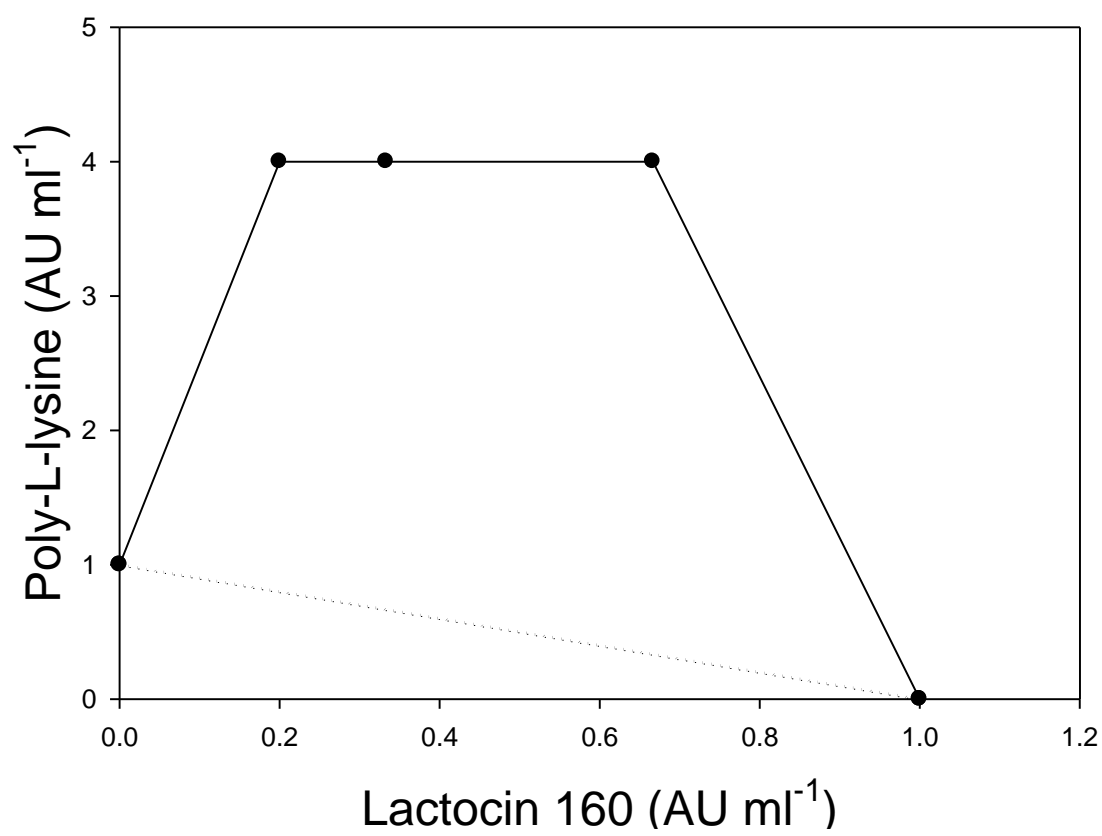


Fig. 2d



CHAPTER IV

SUGGESTIONS FOR THE FUTURE RESEARCH

Although *G. vaginalis* is thought to play the leading role in the etiology of BV, the fully developed disease is characterized by drastic increases in the numbers of multiple pathogenic species. Thus, in order to successfully restore the vaginal microbial balance, pathogens such as *Prevotella* and *Bacteroides* spp. must be inhibited along with *G. vaginalis*. In the future, we are planning to investigate the mode of action of lactocin 160 against *Prevotella bivia* and *Bacteroides* spp. Additionally, interactions between lactocin 160 and other natural antimicrobials will be tested against these pathogens. In order to create more options for the formulation that will effectively control multiple BV-associated pathogens, the panel of antimicrobials tested for interactions with lactocin 160 will also be extended to include H₂O₂, glycerol monolaurate, and other natural safe antimicrobials.

Model studies described in the previous chapters were conducted using planktonic *G. vaginalis* cells; however *in vivo*, BV-associated pathogens mostly exist in a biofilm form. Biofilm-imbedded bacteria are generally more resistant to antimicrobials than planktonic cells, and this tendency has been demonstrated *in vitro* for *G. vaginalis* (Patterson *et al.* 2007). Our future studies will focus on testing lactocin 160 and the selected antimicrobial combinations against the biofilms formed by BV-related pathogens on inanimate surfaces. The abilities of multiple-hurdle formulations involving lactocin 160 to inhibit the formation of these biofilms are going to be tested using the scheme described below.

Furthermore, we will test the bactericidal properties of these formulations against the fully matured biofilms of *G. vaginalis* and the other BV-associated pathogens. Accordingly, plate counting methods as well as a variety of fluorescent and luminescent procedures (Borucki et al. 2003; Patterson et al. 2007) can be used to assess the viability of biofilm-imbedded cells after a fully grown biofilm has been treated with the selected antimicrobial combinations. We also anticipate that mature biofilms resuspended in acidified media with pH values resembling those of normal vaginal secretions (<4.5) would stay viable (Patterson et al. 2007), allowing them to be used for testing of the antimicrobials at acidic conditions. Studies conducted by Swidsinski *et al.* (2008) indicate that biofilms of *G. vaginalis* persist even after a successful metronidazole treatment, eventually inducing a recurrence of BV. Therefore, the antimicrobial assays conducted against biofilms under acidic conditions would model the use of the selected antimicrobials for prophylaxis of BV.

Finally, our lab is currently working on developing a model for growing *G. vaginalis* on the surface of vaginal epithelia using the commercially-available EpiVaginal™ Tissue Model (MatTek Corporation, Ashland, MA). In the future, we are planning to test the effectiveness of selected lactocin 160-based formulations utilizing this model prior to testing these formulations in clinical trials.

We conducted a preliminary study to test the ability of lactocin 160 to inhibit formation of *G. vaginalis* biofilms *in vitro*. Future studies will use similar methods to test other natural antimicrobials in their ability to prevent adhesion of *G. vaginalis* to a surface. The result of this preliminary experiment is presented bellow (in a brief form).

The adhesion of bacterial cells to surfaces is an initial step in biofilm formation which is thought to be essential for the pathogenesis of *G. vaginalis* (Harwich, Jr. *et al.* 2010). Some antimicrobials at their sub-inhibitory concentrations can prevent the adhesion of bacterial cells to various surfaces (Allison *et al.* 2000; Peeters and Piot 1985; Reid *et al.* 2001; Yamanaka *et al.* 2004). It was reasonable to assume that lactocin 160 produced by vaginal *Lactobacillus rhamnosus* to eliminate ‘the competitors’ would have these properties against the vaginal pathogen, *G. vaginalis*.

Initially, we tested several media, eventually deciding to use BHI supplemented with 1% glucose for this preliminary biofilm study. *G. vaginalis* formed considerably denser biofilms ($P < 0.01$) in this medium compared to the BHI supplemented with 3% horse serum that was used in previous experiments (Chapter II and III). This observation is consistent with the results published by (Patterson *et al.* 2007).

Lactocin 160 was prepared by the method previously described by Aroutcheva *et al.* (2001) and Turovskiy *et al.* (2009). One AU ml⁻¹ of activity was defined as the lowest dilution of lactocin 160 preparation that would completely inhibit the growth of *G. vaginalis*, and it corresponded to 96 µg of protein. The aqueous solution containing 15 AU ml⁻¹ of lactocin 160 was then filter-sterilized using 0.2 µm syringe filters (NALGENE, Rochester, NY). *Gardnerella vaginalis* biofilms were grown in a 96-well tissue culture plate (BD, Franklin Lakes, NJ) using BHI medium supplemented with 1 % glucose. Each well of the plate contained 200 µl of culture with lactocin 160 concentrations ranging between 0-1 AU ml⁻¹. The plate was incubated anaerobically for 24 hours at 37°C, and the total cell growth was quantified by measuring the absorbance at OD₆₅₀ with a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

The biofilms were quantified using the method described by Borucki *et al.* (2003). Briefly, the unattached cells in each well were washed three times with 200 μ l of PBS. The attached cells were then stained for 40 minutes at ambient temperature with 100 μ l of 0.1% aqueous crystal violet solution. Each well was washed three further times with 200 μ l of PBS to remove the unbound dye. Subsequently, crystal violet dye bound to cells was solubilized by pipetting 200 μ l of 95% ethanol into each well and incubating the plate for 30 minutes at 6°C. This solution was then gently transferred into a new 96-well plate in order to quantify the crystal violet dye by its absorbance at 595 nm using a microplate reader (BioRad Model 550, Hercules, CA). When necessary, the statistical significance of the results was evaluated by using the Student's *t*-test. The image of the biofilm grown on the glass cover slip was taken under 600X magnification using an Eclipse 80i digital imaging system operated with NIS-Elements 3.0 (NIKON INSTRUMENTS INC., Melville, NY). The image of the magnified biofilm formed by *G. vaginalis* on a glass cover slip is shown in Fig 1.

Treatment of *G. vaginalis* cells with lactocin 160 did not influence their ability to bind crystal violet (data not shown). Data represented in Figure 2b indicate a significant, concentration-dependent decrease in the quantity of surface-attached *G. vaginalis* cells that is due to the activity of lactocin 160. Antimicrobials may inhibit formation of bacterial biofilms through several different mechanisms. Firstly, antimicrobials can interfere with bacterial metabolism (i.e. exopolysaccharide synthesis) or with quorum sensing, thereby preventing biofilm formation. Alternatively, the antimicrobial's adhesion to the cell envelope may hinder bacterial attachment to a surface. Finally, a

decrease in biofilm quantity can simply be caused by a partial inhibition of bacterial growth.

Lactocin 160, even at sub-inhibitory concentrations, noticeably interfered with the growth of *G. vaginalis* as evident by the absorbance of the culture taken at OD₆₅₀ following 24 hours of incubation (Fig 2a). We determined the ratios of surface-attached cells to the total cell growth (surface attached cells along with the planktonic cells) for *G. vaginalis* grown at various subinhibitory concentrations of lactocin 160 (0.2-0.8 AU ml⁻¹) and for the negative control (Fig. 2c). The differences between these ratios did not have a statistical significance ($P \geq 0.1$) indicating that the decrease in number of the surface-attached cells caused by lactocin 160 is simply due to growth inhibition. To summarize, we did not find any evidence for the interference of lactocin 160 with surface adhesion of *G. vaginalis* cells. Prevention of biofilms due to growth inhibition is an obvious and not particularly intriguing effect, and for that reason the concept was not investigated any further.

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FIGURE CAPTIONS

Fig. 1 Biofilm of *G. vaginalis* grown on a glass cover slip.

Fig. 2 Lactocin 160, does not prevent formation of *G. vaginalis* biofilms. At sub-inhibitory concentrations, lactocin 160 partially inhibits the growth of *G. vaginalis* as measured by absorbance at OD₆₅₀ (a), also causing a noticeable decrease in biofilm quantity (measured by a crystal violet staining method) (b). The lack of statistically significant differences ($P \geq 0.1$) between the ratios of biofilm-attached cells to the averaged total cellular growth (planktonic cells and biofilm-attached cells) indicates that the biofilm prevention induced by lactocin 160 is solely due to growth inhibition.

FIGURES

Fig. 1

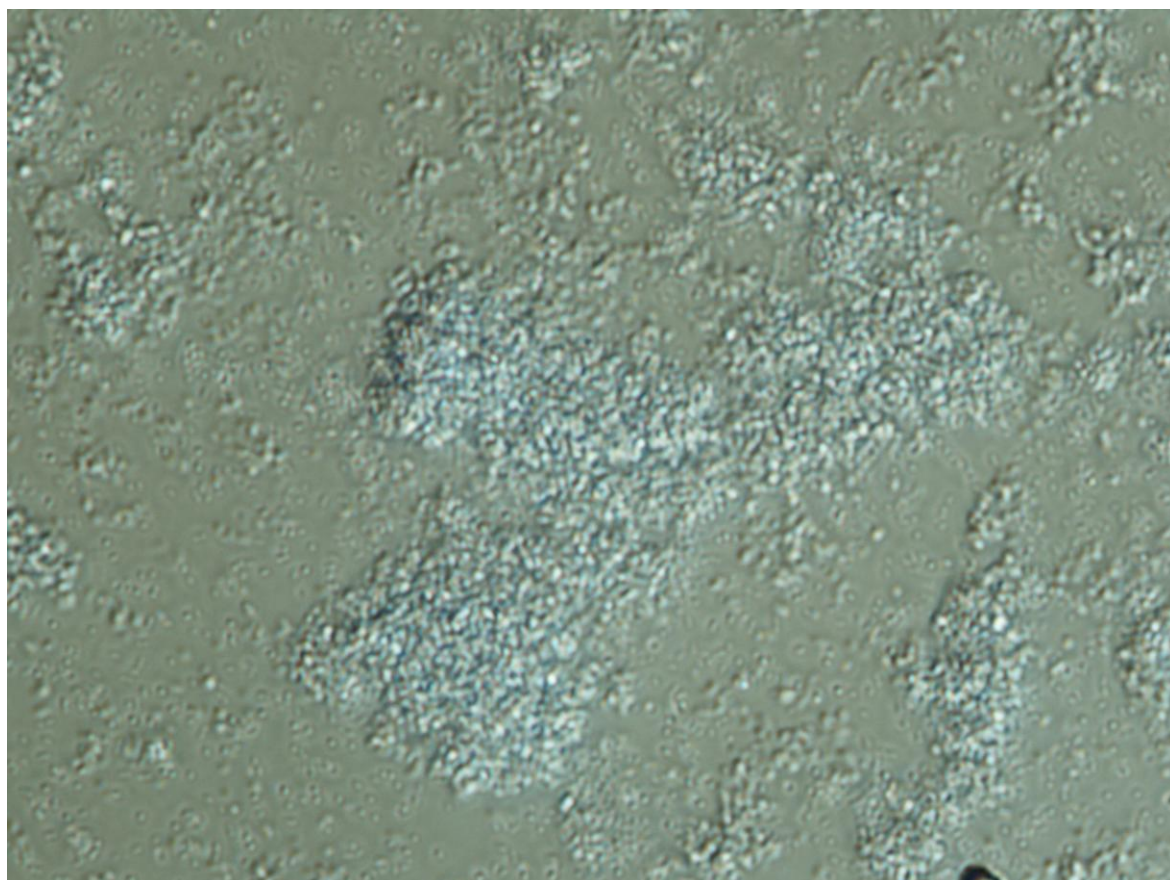


Fig. 2a

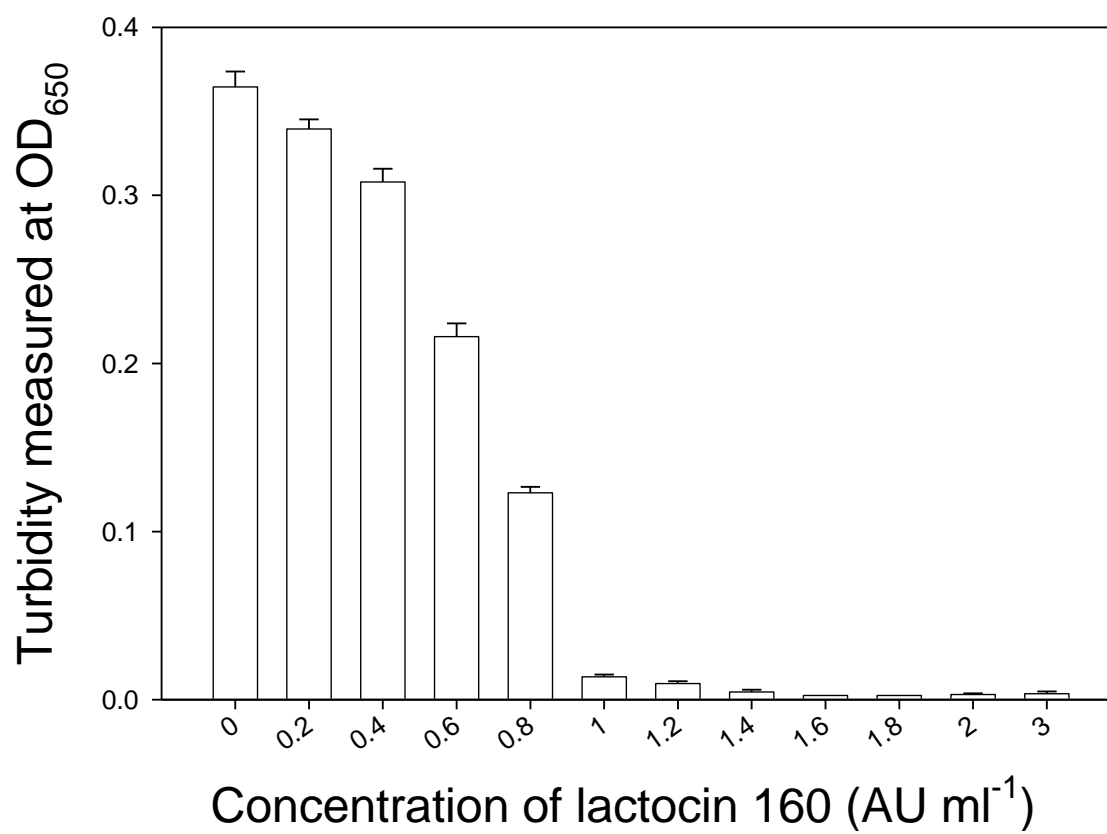


Fig. 2b

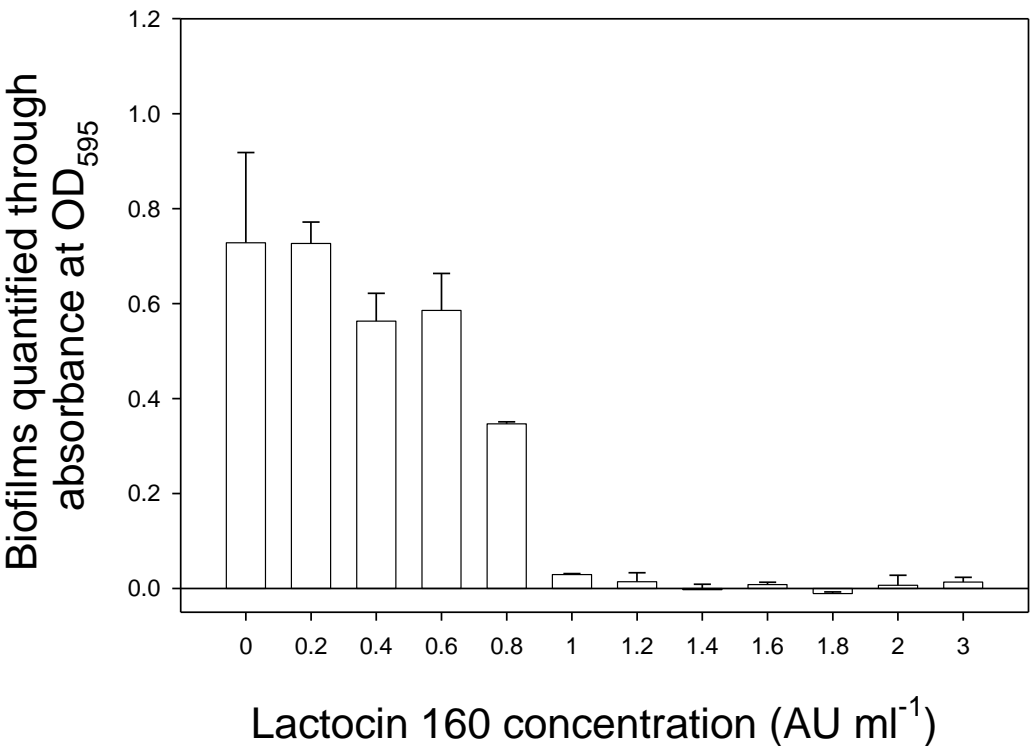
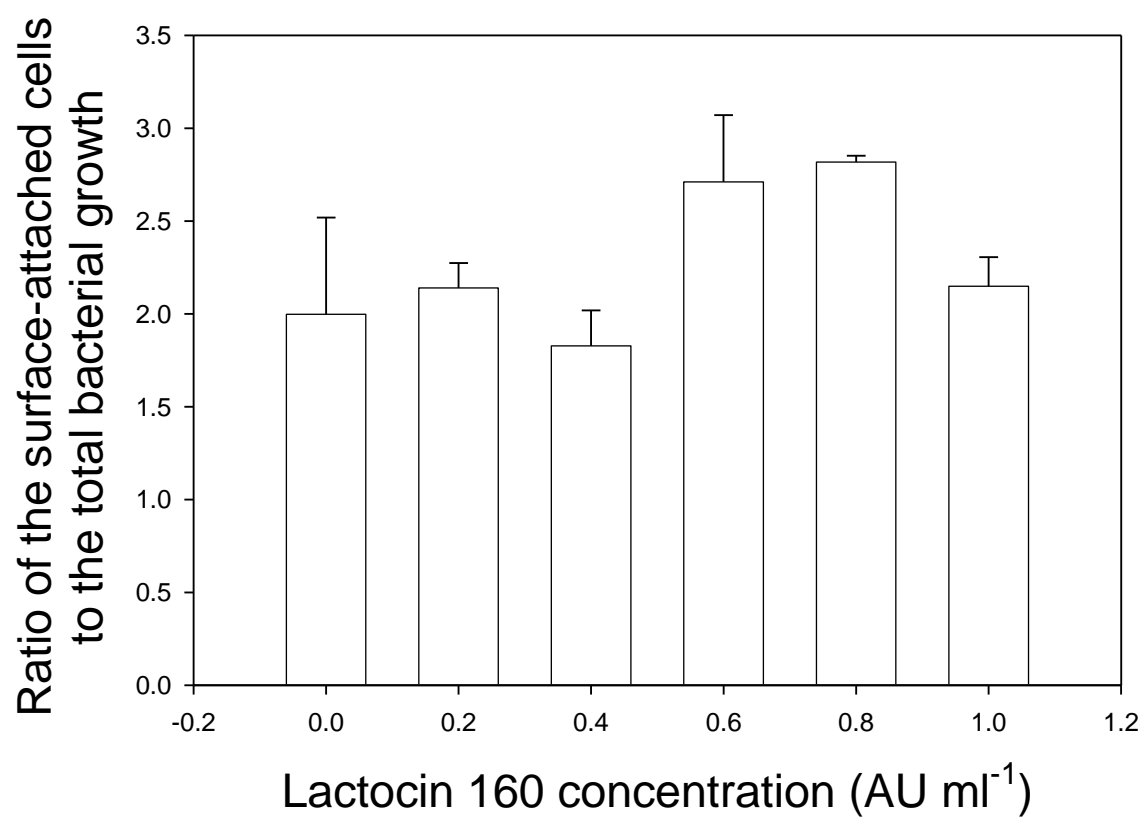


Fig. 2c



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